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Volunteer Studies of Deletion Mutants of *Vibrio cholerae* O1 Prepared by Recombinant Techniques

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***Vibrio cholerae* O1 A^-B^- vaccine strain JBK 70 and A^-B^+ CVD 101 prepared by recombinant DNA techniques from pathogenic El Tor Inaba N16961 and classical Ogawa 395, respectively, were fed to 38 volunteers in single doses of 10^4 to 10^{10}. Although severe diarrhea did not occur in any vaccinee, more than one-half developed mild diarrhea. These attenuated strains colonized well and elicited prominent vibriocidal and antitoxic (CVD 101) antibody responses. Recipients of a single dose of JBK 70 were significantly protected when challenged with 10^6 wild-type N16961. Diarrhea occurred in 7 of 8 controls but in only 1 of 10 vaccinees ($P < 0.003$, 89% vaccine efficacy), demonstrating the potency of immune mechanisms that do not involve cholera antitoxin. Further derivatives were prepared to explore the pathogenesis of the residual diarrhea, considering that either intestinal colonization by the vaccine itself or accessory toxins might be responsible. CVD 102, an auxotrophic mutant of CVD 101, did not cause diarrhea but colonized poorly and elicited feeble immune responses. Derivatives of JBK 70 and CVD 101 (CVD 104 and 105) deleted of genes encoding the El Tor hemolysin still caused mild diarrhea. Genetically engineered strains can be colonizing, highly immunogenic, and protective single-dose oral vaccines, but they must be further attenuated before they can be considered for use as public health tools.**

Cholera, the diarrheal disease due to enterotoxigenic *Vibrio cholerae* O1, in its severe form can lead to fatal diarrheal dehydration and typically occurs in explosive epidemics. The severe purging of cholera is due to the secretogenic effects of cholera enterotoxin on the gut mucosa, and purified cholera toxin in quantities as small as 5 μg can cause copious purging when fed to volunteers (20). Studies of volunteers (3, 14, 15, 18, 21), as well as epidemiologic studies in a cholera-endemic area (7), have shown that an initial clinical episode of cholera provides highly potent, long-lived protective immunity. Both antibacterial and antitoxic immunity apparently play roles in protection against cholera, although there has been considerable debate regarding the relative importance of each component (9, 11, 20, 25). While the neutralizing effect of antitoxic antibodies is known to be directed overwhelmingly against the B (G_{M1} ganglioside-binding) subunit of cholera toxin (8), there is no agreement on the precise nature of the protective antigens involved in antibacterial immunity (9, 11, 20).

The studies described herein represent steps in a long-term program towards development of attenuated strains of *V. cholerae* O1 to use as live oral vaccines. By starting with strains of *V. cholerae* O1 known to cause cholera and to give rise to protection in volunteers (14, 18, 21), it has been possible by recombinant DNA techniques to prepare a series of derivatives from which the genes encoding both the A (ADP-ribosylating) and B subunits of cholera toxin or just the A subunit have been deleted (13). The evaluation of these strains as live oral vaccine candidates, as well as further derivatives deleted of genes encoding the El Tor hemolysin/cytotoxin, has led to important insights into the pathogenesis of and immunity to cholera.

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MATERIALS AND METHODS

Bacterial strains. A summary of the origin, biotype, serotype, and relevant genotypic and phenotypic characteristics of the strains used in volunteer studies is shown in Table 1. Pathogenic parent strains El Tor Inaba N16961, isolated from a patient in Bangladesh with cholera, and classical Ogawa 395 from India have previously been shown to cause cholera in experimental challenge studies in volunteers and to lead to protective immunity (3, 14, 15, 18, 21). Candidate vaccine strain JBK 70 was derived from N16961 by deletion of the genes encoding both the A and B subunits of cholera toxin by site-directed mutagenesis; the preparation of this A^-B^- strain has been described in detail (13). A^-B^+ vaccine strain CVD 101 was derived from Ogawa 395 by deletion of the genes encoding the A subunit, as previously described (12). Vaccine strain CVD 102 is a spontaneously derived, thymine-dependent, auxotrophic mutant of CVD 101. CVD 104 is a further derivative of JBK 70 from which the genes encoding the El Tor hemolysin were deleted by site-directed mutagenesis, whereas CVD 105 is a further derivative of CVD 101 from which the El Tor hemolysin genes were similarly deleted (J. B. Kaper, H. L. T. Mobley, J. M. Michalski, D. A. Herrington, and M. M. Levine, *in* Y. Takeda and R. B. Sack, ed., *Advances in Research on Cholera and Related Diarrheas*, vol. 6, in press).

Volunteers. Participants in vaccination and efficacy challenge studies were college students and other healthy young adults from the metropolitan Baltimore community. The methods of medical screening, care of the volunteers, and informed consent have been previously described (17). To ensure the informed nature of consent, volunteers had to pass a written examination containing questions on all aspects of the study, including risks, benefits, procedures, bacteriology, and immunology. Both vaccination and chal-

TABLE 1. Origin and characteristics of *V. cholerae* O1 strains used in these studies

Vaccine strain	Pathogenic parent strain	Cholera toxin genotype	El Tor hemolysin genotype	Shiga-like toxin	Other notable characteristic
JBK 70	El Tor Inaba N16961	A^-B^-	+	+	
CVD 101	Classical Ogawa 395	A^-B^+	+	+	
CVD 102	Classical Ogawa 395	A^-B^+	+	+	Thymine-dependent auxotroph of CVD 101
CVD 104	El Tor Inaba N16961	A^-B^-	−	+	Derived from JBK 70
CVD 105	Classical Ogawa 395	A^-B^+	−	+	Derived from CVD 101

lenge studies were carried out under legal quarantine in the 22-bed isolation ward maintained by the Center for Vaccine Development in the University of Maryland Hospital.

Cohorts of approximately 5 to 10 volunteers were given a single dose of one of the vaccine strains and observed for 5 days, after which they received 5 days of tetracycline therapy before being discharged.

Oral vaccination or challenge. The vaccine or challenge strains were stored at −70°C. Inocula were prepared as previously described and were given to volunteers with 2.0 g of $NaHCO_3$ to neutralize gastric acid (17); vaccination or challenge was carried out at 3 p.m., with volunteers fasting for 90 min before and after.

Clinical surveillance and definition of diarrhea. After ingesting vibrios, the volunteers were kept under surveillance for 96 (challenge studies) or 120 (vaccination studies) h before receiving a 5-day course of tetracycline (500 mg every 6 h). All stools were collected in sterilizable plastic bedpans that fit on the commode, and their volumes were measured. The consistency of stools was graded on a five-point scale: grade 1, formed stool; grade 2, soft but formed stool; grade 3, thick liquid; grade 4, opaque watery liquid; grade 5, rice water. Diarrhea was defined as passage of two or more loose stools (grades 3 to 5) within 48 h and at least 200 ml in volume or a single loose stool of 300 ml or greater.

Volunteers were examined at least once daily. During challenge studies, volunteers with diarrhea were given oral glucose-electrolyte solution to maintain hydration (27).

Bacteriology. Specimens of all stools were cultured. If no stool specimen was available in a 24-h period, a rectal swab was obtained. Stool and swabs were inoculated directly onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (BBL Microbiology Systems, Cockeysville, Md.) and inoculated into alkaline peptone water and sodium-gelatin-phosphate enrichment broths (32). After overnight incubation, the enrichment broths were subcultured onto TCBS agar. Quantitative culture of stools was performed as previously described (17). Suspicious colonies from TCBS agar were confirmed as *V. cholerae* and serotyped as previously described.

Gelatin string capsule devices were used to obtain duodenal fluid for culture 20 and 44 h after ingestion of the vaccine (17). Duodenal fluid was tweezed from the bile-stained, distal 15 cm of the string into a sterile petri dish; direct cultures on TCBS agar and enrichment in alkaline peptone water were performed as described above. For quantitative cultures, 0.1 ml of duodenal fluid was diluted in 9.9 ml of 0.85% saline; two further 100-fold dilutions were also prepared. The diluted duodenal fluids were inoculated directly onto TCBS agar and cultured 24 h at 37°C, and the colonies were enumerated.

Serology. Sera were collected from all volunteers before and 10, 21, and 28 days after ingestion of vaccine or pathogenic challenge strains of *V. cholerae*. Immunoglobulin G (IgG) cholera antitoxin (by enzyme-linked immunosorbent assay [ELISA]) and vibriocidal antibody were measured as previously described (4, 23). Serum IgG and IgA antibodies were measured by ELISA against purified TCP pili prepared from classical Inaba strain 569B, lipopolysaccharide (LPS) (2), outer membrane protein preparations (35), inactivated homologous whole vibrios (5), and a lysate of *V. cholerae* (19).

Before and 9 days after ingestion of vaccine or pathogenic vibrios, many volunteers ingested polyvinyl chloride intestinal tubes to collect jejunal fluid for measurement of specific secretary IgA (s IgA) ELISA antibody to various antigens, including cholera toxin (14), homologous LPS (2), outer membrane protein preparations (34), purified TCP pili, inactivated whole vibrios, and a lysate of the homologous vibrio strain. For logistical reasons, it was not possible to have all the volunteers return for an additional collection of intestinal fluid at a time point (14 to 18 days postvaccination) optimal for detection of sIgA immune responses. Fourfold rises in titer were considered significant.

RESULTS

JBK 70. A^-B^- strain JBK 70 was fed to volunteers in doses of 10^6, 10^8, or 10^{10} organisms (Table 2). Compared

TABLE 2. Clinical and bacteriological response of volunteers after ingestion of A^-B^- El Tor Inaba vaccine strain JBK 70 prepared by recombinant DNA techniques or its virulent parent *V. cholerae* El Tor Inaba N16961

Strain and dose ingested	No. of volunteers	No. (%) with diarrhea	Mean diarrheal stool vol/ill volunteer (ml)	No. (%) with stool vol ≥5.0 liters	Mean no. of loose stools/ill volunteer	Coprocultures		Duodenal fluid cultures	
						No. (%) positive	Geometric mean excretion[a]	Proportion (%) positive[b]	Geometric mean titer[a]
JBK70									
10^6	4	1 (25)	543	0	4	3 (75)	1.5×10^6	0/4	
10^8	5	2 (40)	1,180	0	8.5	5 (100)	2.4×10^7	1/2 (50)	2×10^3
10^{10}	5	4 (80)	802	0	6	5 (100)	4.9×10^7	5/5 (100)	6×10^2
N16961 (10^6)	38	35 (92)	4,227	10 (26)	15.2	38 (100)	3.0×10^7		

[a] Number of *V. cholerae* organisms per gram of stool or per milliliter of duodenal fluid among positive samples.
[b] Number positive/number with duodenal fluid cultures.

TABLE 3. Clinical spectrum of vaccinees who developed diarrhea after ingestion of genetically engineered nonenterotoxinogenic *V. cholerae* El Tor Inaba strain JBK 70

Volunteer	Dose	Total diarrheal stool vol (ml)	No. of loose stools/day of observation:					Symptom			
			1	2	3	4	5	Abdominal cramps	Anorexia	Vomiting	Fever (°F)
5012-2	10^6	543	1	2	1	0	0	+	−[a]	−	−
5012-5	10^8	710	0	5	0	0	0	+	+	+	100 (37.8°C)
5012-21	10^8	1,649	0	4	6	2	0	+	−	−	−
5012-16	10^{10}	1,878	0	4	7	1	0	+	+	−	−
5012-15	10^{10}	499	0	3	1	0	0	+	−	−	−
5012-7	10^{10}	596	2	3	1	0	0	+	+	−	100 (37.8°C)
5012-9	10^{10}	235	0	1	0	1	0	+	+	−	−

[a] −, No symptoms.

with the clinical responses of 38 volunteers to ingestion of the toxigenic A^+B^+ parent strain N16961, 35 of whom developed diarrhea with a mean diarrheal stool volume of 4.23 liters and 10 (29%) of whom each purged at least 5.0 liters, JBK 70 was greatly attenuated. Nevertheless, although no severe purges occurred, at each dose 25 to 80% of the volunteers passed one or more loose stools and met the definition of diarrhea. Table 3 shows the number of loose stools passed by each volunteer with diarrhea, the days with loose stools, and the total diarrheal stool volume; in several instances, loose stools were accompanied by abdominal cramps, anorexia, and low-grade fever. Stool volumes were all below 2.0 liters and in five of the seven individuals did not exceed 1.0 liter.

JBK 70 was recovered from coprocultures of all vaccinees and from duodenal cultures of 55%. As expected, no recipients of this A^-B^- strain manifested rises in serum IgG antitoxin. However, the vibriocidal antibody response was impressive, even in comparison with individuals who ingested the fully toxigenic parent strain N16961 (Table 4).

Although JBK 70 was associated with adverse reactions, it apparently colonized the intestine and stimulated a prominent antibacterial immune response. Therefore, we took advantage of a unique opportunity to assess the relative potency of immunity to cholera in the absence of cholera antitoxin; 1 month after vaccination, recipients of JBK 70 were challenged with wild-type toxigenic strain N16961. Results of this challenge study are summarized in Table 5. Diarrhea occurred in 7 of 8 controls but in only 1 of 10 JBK vaccinees (89% vaccine efficacy, $P < 0.003$, Fisher's exact test). That antibacterial mechanisms were involved in the protection is demonstrated in Table 5, in which it is shown that vaccinees had a significantly lower recovery of *V. cholerae* by direct coproculture than controls, as well as a 1,000-fold lower excretion per gram of stool.

CVD 101. The reason for the residual, albeit mild, diarrhea encountered in recipients of JBK 70 was not clear. It was important to determine whether such adverse reactions were related to the El Tor biotype origin of JBK 70 and whether genetically engineered nontoxigenic vaccine strains derived from the classical biotype of *V. cholerae* might perhaps be better tolerated. Accordingly, a rather comprehensive dose-response evaluation of CVD 101, the A^-B^+ derivative of classical Ogawa 395, was carried out. Some diarrhea occurred in 40 to 67% of recipients at every dose (Table 6); in no instance did severe diarrhea occur. The total stool volumes, similar to those seen after ingestion of JBK 70, represent a reduction by 84% of the copious stool volumes (mean, 5.5 liters) that occurred in volunteers after ingestion of the wild-type parent strain Ogawa 395 (Table 6). CVD 101 was recovered in coprocultures of 22 of 24 vaccinees (92%).

The serological response of volunteers after ingestion of a single dose of CVD 101 is shown in Table 6. Both the vibriocidal and IgG ELISA antitoxin responses approached but did not quite match those that occur after infection with the pathogenic parent strain, classical Ogawa 395; a single dose of CVD 101 induced a geometric mean vibriocidal titer and a mean antitoxin response that were approximately three-fourths of those seen after infection with Ogawa 395. Because of the adverse reactions that occurred with CVD 101, it was not deemed appropriate to carry out challenge studies to assess the efficacy of this vaccine.

Rationale for further clinical studies. At this point it appeared that, irrespective of biotype or serotype, deleting the genes that encode the ADP-ribosylating A subunit from pathogenic *V. cholerae* O1 was insufficient to render the strains completely nonreactogenic. However, we did show that single doses of as few as 10^4 organisms of genetically engineered nontoxigenic live oral *V. cholerae* vaccines readily colonized the intestine and elicited potent antibody responses (Table 6). Two hypotheses were invoked to explain the residual mild diarrhea observed in a proportion of vaccinees. One hypothesis suggested that the act of colonization of the proximal small intestine by adherent vibrios by

TABLE 4. Comparison of vibriocidal antibody responses of volunteers after ingestion of pathogenic or attenuated vaccine strains of *V. cholerae* O1

Strain	Dose ingested	*n*[a]	% Volunteers with fourfold or greater antibody rises	Peak geometric mean titer	% Volunteers with titers:	
					>2,560	≥10,240
JBK 70, A^-B^- El Tor Inaba vaccine	10^6–10^{10}	14	100	5,940	86	79
	10^6	4	100	1,522	50	25
	10^8	5	100	11,763	100	100
	10^{10}	5	100	8,914	100	100
N16961, pathogenic El Tor Inaba	10^6	21	90	4,793	81	33
E7946, pathogenic El Tor Ogawa	10^6	21	95	3,674	81	43

[a] *n*, Number of volunteers who ingested strain.

TABLE 5. Study to assess the efficacy of a single dose of JBK 70 oral attenuated *V. cholerae* El Tor Inaba vaccine strain prepared by recombinant DNA techniques[a]

Group	Diarrhea attack rate[b]	Mean diarrheal stool vol (range)/ill volunteer	No. of volunteers with positive coprocultures		Geometric mean excretion (vibrios/g of stool)
			Direct[c]	After enrichment	
Controls	7/8	4.5 (1.1–7.9)	8	8	3.5×10^6
Vaccinees	1/10	1.6	2	6	3.8×10^{3}[d]

[a] Volunteers were challenged with 10^6 pathogenic *V. cholerae* El Tor Inaba N16961 with $NaHCO_3$.
[b] Number positive/number challenged; $P < 0.003$ by Fisher's exact test.
[c] $P = 0.001$ by Fisher's exact test.
[d] Geometric mean of positives only.

itself results in net intestinal secretion manifested clinically as mild diarrhea. The alternative hypothesis argues that the *V. cholerae* vaccine strains possess additional secretogens, distinct from cholera toxin, such as the hemolysins/cytotoxins, cytotoxins/proteases, or Shiga-like toxins (10, 24, 28, 29, 34, 38, 39), and that these are responsible for the mild diarrhea seen in the vaccinees. We proceeded to develop additional vaccine strains to test these hypotheses.

CVD 102. A thymine-dependent auxotrophic mutant of CVD 101, CVD 102, was derived to determine whether reactogenicity would diminish if a live but nonproliferating *V. cholerae* strain was fed to volunteers. CVD 102, in a dose of 10^7 organisms (Table 7), was given to five volunteers, none of whom manifested adverse reactions. However, the absence of reactogenicity occurred at a great cost in immunogenicity; vaccine organisms could be recovered from only two of five individuals, only two of five had vibriocidal seroconversions (low titers), and none had antitoxin seroconversions.

CVD 104 and 105. In addition to cholera toxin, *V. cholerae* can elaborate other products that might have a secretogenic effect on intestinal mucosa (10, 24, 28, 29, 34). For example, *V. cholerae* El Tor elaborates a hemolysin that is active on sheep and rabbit erythrocytes; a related hemolysin occurs in some non-O1 *V. cholerae* (10, 38, 39). The genes for the El Tor hemolysin are also found in classical biotype vibrios, although in vitro classical organisms elaborate little if any hemolysin. Nevertheless, hemolysin genes cloned from classical Ogawa 395 are expressed in *Escherichia coli* as biologically active hemolysin (33). Madden et al. (24) identified a cytotoxin in *V. cholerae* of both O1 and non-O1 serogroups that stimulates secretion in rabbit ileal loops. This cytotoxin appears to be identical to the El Tor hemolysin (24). The genes encoding the El Tor hemolysin were deleted from JBK 70 and from CVD 101, resulting in the further derivatives CVD 104 and CVD 105, respectively (Table 1). These were fed to volunteers in doses of 10^5 to 10^7 (Table 7). Deletion of the genes encoding the El Tor hemolysin did not notably diminish the rate of adverse mild diarrheal reactions in the recipients. All vaccinees shed the vaccine, and immune responses were quite satisfactory.

Adverse reactions and blood group. In view of the relationship between blood group O and severity of cholera diarrhea (7, 22), the occurrence of diarrheal adverse reactions was investigated in relation to blood group. Diarrhea after ingestion of JBK 70 and CVD 101 was not seen more frequently in persons of blood group O.

Further measurements of serum and intestinal immune response. Having noted the impressive vibriocidal antibody responses after immunization with JBK 70 and CVD 101 and the high level of protection conferred by JBK 70, we attempted to measure with greater precision the immune response stimulated by these vaccines and the identity of the antigens to which the responses were directed. In Table 8 are shown the serum and intestinal immune responses to various *V. cholerae* antigens, including cholera toxin, LPS, outer membrane proteins, purified TCP pili from classical Inaba strain 569B (the recently described putative intestinal colonization factor of pathogenic *V. cholerae* O1 [37]), killed whole *V. cholerae* (homologous strain), and a lysate of the homologous *V. cholerae* strain measured by ELISA in JBK 70 and CVD 101 vaccinees. Measurement of serum antibody

TABLE 6. Clinical, bacteriological, and serological responses after ingestion of A^-B^+ *V. cholerae* vaccine strain CVD 101 prepared by recombinant DNA techniques or its virulent parent, classical Ogawa 395

Strain and vaccine dose	Rate of diarrhea	Mean diarrheal stool vol (liters) (range)	Mean no. (range) of loose stools	No. (%) with stool vol >5.0 liters	No. of volunteers seroconverting	Vibriocidal antibody		Antitoxin	
						Seroconversion rate[a]	Peak GMT[b]	Seroconversion rate[c]	Peak GMT
CVD 101									
10^8	2/3[d]	1.3 (1.2–1.3)	10 (9–11)	0	2	2/3	806	2/3	0.80[e]
10^7	3/5	1.2 (0.5–2.1)	8 (3–13)	0	5	5/5	3,880	4/5	0.80
10^6	2/5	0.4 (0.38–0.42)	3	0	5	5/5	2,560	4/5	0.89
10^5	2/5	1.1 (0.7–1.5)	9 (4–13)	0	5	5/5	3,880	5/5	0.83
10^4	4/6	0.7 (0.3–1.1)	5 (2–7)	0	6	6/6	2,032	4/6	0.73
Ogawa 395 (10^6)	33/36	5.5 (0.3–17.5)	19.5 (1–60)	15 (45)	35	33/36	4,223	32/36	1.17

[a] Significant seroconversion refers to a fourfold or greater rise in titer after vaccination or challenge.
[b] Geometric mean titer.
[c] Significant seroconversion is defined as a rise of 0.15 or greater in IgG-ELISA net optical density units in the postvaccination or postchallenge sera over the prevaccination or prechallenge specimen tested at a single 1:50 dilution.
[d] Number positive/number vaccinated.
[e] ELISA net optical density units.

TABLE 7. Clinical and bacteriologic responses of volunteers to various doses of three A^-B^+ classical Ogawa *V. cholerae* oral attenuated vaccine strains prepared by recombinant DNA techniques

Vaccine	Dose	Diarrhea attack rate	Mean diarrheal stool vol (ml) (range)	Bacteriology		No. of significant seroconversions	
				No. of volunteers with positive coprocultures	No. of vibrios/g of stool	Vibriocidal[a]	Antitoxin[b]
CVD 102	10^7	0/5[c]	0	2	3×10^2[d]	2	0
CVD 104	10^7	2/6	729 (209–1249)	6	3×10^5	6	0
CVD 105	10^5–10^6	3/9	415 (231–509)	9	2×10^6	8	8

[a] A fourfold or greater rise in vibriocidal antibody was considered significant.
[b] A rise in IgG-ELISA net optical density units of 0.15 or greater between the pre- and postvaccination sera tested at 1:50 dilution was considered significant.
[c] Number positive/number vaccinated.
[d] Geometric mean excretion.

against the *V. cholerae* lysate proved to be the most sensitive assay. However, considering the prominent vibriocidal titers, the responses against specific vibrio antigens were relatively modest.

Most of the postvaccination specimens of jejunal fluid were collected only on the eighth day postvaccination, too early to detect responses in many of these unprimed individuals. Nevertheless, significant rises in sIgA antibody to various vibrio antigens were detected in some of the vaccinees (Table 8).

DISCUSSION

Early in our program to develop a live oral vaccine to prevent cholera, we reported the high level and long duration of protective immunity elicited by a clinical infection due to pathogenic *V. cholerae* (14, 15, 18, 20, 21). The inability to culture (classical rechallenges) (15, 21) or difficulty in culturing (El Tor rechallenges) (18, 20) vibrios from direct coprocultures of rechallenged volunteers suggested to us that antibacterial mechanisms were playing an important role in protection. The striking inverse correlation between prevalence and level of vibriocidal antibody in populations living in a cholera-endemic area and the incidence of cholera supports this contention; for example, as the prevalence and geometric mean vibriocidal titer rise with age in Bangladesh, the incidence of cholera falls (26). The identity of the antigens against which the antibacterial component of the cholera immune response is directed has been a point of disagreement among investigators. For this reason, in our approach to cholera vaccine development, we have attempted to mimic, with live oral vaccines that do not elaborate cholera holotoxin, the broad immunity conferred by natural infection. In years past, the attenuated strains that were evaluated included environmental isolates (16) and a chemically mutagenized strain, Texas Star (17). The advent of recombinant DNA technology has permitted the preparation of a series of *V. cholerae* mutants having precise deletions of genes encoding putative virulence properties, while leaving intact all other surface antigens and gene products. The clinical studies with these strains, reported herein, have provided some unexpected insights into the pathogenesis of diarrheal infection due to *V. cholerae* and of the immune response to the organism.

The most important observations include the following: (i) the demonstration that deletion of the structural genes necessary for production of cholera holotoxin from a *V. cholerae* O1 strain renders the organism unable to cause the severe purging of cholera gravis but does not completely attenuate it (even in doses as low as 10^4 organisms, such mutants can cause mild diarrhea in approximately one-half of the recipients); (ii) the discovery that a single oral dose of the attenuated *V. cholerae* strains readily colonizes the human intestine and elicits potent vibriocidal and (depending on the strain) antitoxic antibody responses; (iii) a clear-cut demonstration that a high level of protection can be elicited by a genetically engineered A^-B^- strain (JBK 70) that does not stimulate cholera antitoxin, conclusively showing the potency of immune mechanisms that do not involve cholera antitoxin.

A mild diarrhea that occurred in a proportion of recipients of our A^-B^- and A^-B^+ oral vaccine candidates was a bothersome observation. We believe that this residual mild diarrhea is a consequence of the production of other secretogenic substances by the engineered strains. *V. cholerae* O1 and non-O1 strains are known to produce a variety of

TABLE 8. Serum IgG and IgA and intestinal fluid sIgA antibody responses to various *V. cholerae* antigens in recipients of a single oral dose of JBK 70 and CVD 101 vaccines

Vaccine	No. of volunteers with significant rises[a] in serum IgG or IgA antibody to:						No. of volunteers with fourfold or greater intestinal sIgA antibody response to:					
	Whole vibrio	Vibrio lysate	Outer membrane protein	LPS	Cholera toxin	TCP pili	Outer membrane protein	LPS	Cholera toxin	TCP pili	Whole vibrio	Vibrio lysate
JBK 70[b]	10[c]	12	7	11	0	1	1	2	0	0[d]	0[d]	2[d]
CVD 101[b]	8[d]	6[d]	9	8[d]	10	3[d]	4	4	4	0[d]	2[d]	2[c]

[a] For all assays except serum cholera antitoxin, a fourfold or greater rise in ELISA titer was considered significant. For cholera antitoxin, sera were assayed at a single dilution of 1:50 and a rise in net optical density units of 0.15 or greater was considered significant.
[b] Recipients of 10^6, 10^8, or 10^{10} organisms. n = 14 and 13 for JBK 70 and CVD 101, respectively.
[c] Number with significant rises/number tested.
[d] Sera or jejunal fluids were not available from one vaccinee for these assays.

cytotoxins (24, 28, 29, 34, 39), some of which have the ability to stimulate net secretion by intestinal mucosa (24, 28). It should be possible to prepare additional mutants deleted of the genes encoding the accessory toxins, perhaps resulting in fully attenuated vaccine strains. Two possible candidate toxins to explain the residual diarrhea are the El Tor hemolysin/cytotoxin (10, 24, 38, 39) and the Shiga-like toxin (29) elaborated by *V. cholerae* O1. Deletion of the genes encoding the El Tor hemolysin from strains JBK 70 and CVD 101, resulting in CVD 104 and CVD 105, respectively, did not notably diminish the reactogenicity of the vaccine strains. Pathogenic *V. cholerae* O1 have been found to elaborate a HeLa cell cytotoxin that is partially neutralized by Shiga antitoxin (29), thereby demonstrating an immunological relationship to Shiga toxin. Work is under way to prepare analogous mutants deleted of the genes encoding the Shiga-like toxin. While such strains have not yet been created, an A^-B^+ cholera toxin mutant, CVD 103, has been engineered from classical Inaba 569B, a pathogenic strain that phenotypically does not elaborate the Shiga-like toxin (29). In volunteers, CVD 103 causes significantly fewer diarrheal reactions than CVD 101 or JBK 70 (M. M. Levine, J. B. Kaper, J. G. Morris, D. Herrington, G. Losonsky, B. Tall, and R. Hall, *in* S. Kuwahara and N. Pierce, ed., *Advances in Research on Cholera and Related Diarrheas*, vol. 5, in press). Only 11% of 46 CVD 103 vaccinees who have ingested 10^8 organisms have manifested diarrheal reactions; in no individual has the stool volume exceeded 1.0 liter, and in only one has it surpassed 400 ml. These observations suggest that Shiga-like toxin may play an important causative role in the mild diarrheal reactions that occur in recipients of live oral *V. cholerae* vaccine candidates.

The data obtained heretofore do not allow us to disprove the hypothesis that the act of colonization of the small intestine by adherent vibrios by itself results in mild diarrhea in some vaccinees. If this is true, it suggests that a single-dose, nonreactogenic live oral cholera vaccine might be very difficult, if not impossible, to achieve. Smith and Linggood (36) showed that *E. coli* strains that possess the plasmid encoding K-88 fimbriae could cause mild diarrhea in piglets even in the absence of a plasmid encoding enterotoxin; in contrast, the same strain possessing the enterotoxin plasmid but lacking the K-88 plasmid did not cause diarrhea. These results have been used to argue that colonization of the small intestine by adherent bacteria can lead to mild diarrhea. The data reported by Smith and Linggood clearly demonstrate that colonization and adherence to the mucosa are prerequisites for diarrhea by such *E. coli*. However, it is not known whether the *E. coli* strain used by Smith and Linggood elaborated other toxins, distinct from heat-labile or heat-stable enterotoxin, that might also have played a role in causing diarrhea in the piglets.

Auxotrophic strain CVD 102, the thymine-dependent derivative of CVD 101, did not cause diarrhea in five volunteers given a single dose of 10^7 organisms. However, neither did it colonize well or elicit prominent immune responses (Table 7). Even if larger doses of similar auxotrophs prove to be well tolerated, it is unlikely that elicitation of a protective immune response could be achieved without multiple spaced doses. Nevertheless, among oral cholera vaccines that require multiple doses, it may be worthy to explore further the potential of auxotrophic strains. The reason is that live strains interact with the gut mucosal immune system in a qualitatively different manner than killed vibrios (30), suggesting that even nonproliferating live *V. cholerae* vaccines might offer advantages over nonliving antigen vaccines in stimulating antibacterial immunity.

Strain JBK 70 gave us a unique opportunity to investigate the level of protection that can be achieved in the absence of an immune response to cholera toxin. The challenge of JBK 70 vaccinees showed that a very high level of protection (89%) can occur in the absence of cholera antitoxin (Table 5). This level of protection (89%) was equal to that conferred by pathogenic El Tor vibrios (90%) (18, 20) and is higher than that seen following immunization of Maryland volunteers with combination B subunit-killed whole vibrio oral vaccine (64% protection) or after ingestion of killed whole vibrios alone (56% protection) (2).

The volunteer studies with CVD 101 parallel observations made in rabbits wherein CVD 101 readily colonized the intestine (albeit at a slightly lower level than the pathogenic parent strain Ogawa 395) and elicited antitoxin responses that approached but did not quite equal those elicited by the pathogenic parent strain (31).

The identity of the antigens involved in the antibacterial component of immunity is under investigation. On the basis of the high vibriocidal titers elicited by JBK 70 and the importance of vibrio LPS in the vibriocidal process (9), some of the response must be directed against the LPS antigens. The identity of the other antigens involved in antibacterial immunity has been more controversial. Recently, Taylor et al. (37) have identified a fimbrial colonization factor elaborated by classical *V. cholerae* O1. Preliminary studies show that only a minority of volunteers immunized with the engineered strains or challenged with pathogenic vibrios manifest serological responses to a fimbrial antigen prepared from classical Inaba 569B. More studies must be carried out to determine what role the fimbrial antigens of *V. cholerae* play in immunity.

On the basis of the observations in this report and other emerging data, we remain optimistic that it will be possible to create an attenuated *V. cholerae* vaccine strain that stimulates high levels of antitoxic and anticolonization antibodies and confers potent, long-lived protection after a single oral dose, without causing significant adverse reactions.

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