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Evaluation of a Bivalent (CVD 103-HgR/CVD 111) Live Oral Cholera Vaccine in Adult Volunteers from the United States and Peru

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To provide optimum protection against classical and El Tor biotypes of *Vibrio cholerae* O1, a single-dose, oral cholera vaccine was developed by combining two live, attenuated vaccine strains, CVD 103-HgR (classical, Inaba) and CVD 111 (El Tor, Ogawa). The vaccines were formulated in a double-chamber sachet; one chamber contained lyophilized bacteria, and the other contained buffer. In the first study, 23 U.S. adult volunteers received CVD 103-HgR at 10^8 CFU plus CVD 111 at 10^8, 10^7, or 10^6 CFU, CVD 111 alone at 10^7 CFU, or placebo. In the second study, 275 Peruvian adults were randomized to receive CVD 103-HgR at 10^9 CFU plus CVD 111 at 10^9 or 10^8 CFU, CVD 111 alone at 10^9 CFU, CVD 103-HgR alone at 10^9 CFU, or placebo. Three of 15 U.S. volunteers who received CVD 111 at 10^7 or 10^8 CFU developed mild diarrhea, compared to none of 4 who received CVD 111 at 10^6 CFU and 1 of 4 who received placebo. Twelve (63%) of 19 vaccine recipients shed the El Tor vaccine strain. All but one volunteer developed significant Ogawa and Inaba vibriocidal antibody titers. Volunteers who received CVD 111 at 10^7 CFU had geometric mean Ogawa titers four to five times higher than those of volunteers who received the lower dose. In the second study, all dosage regimens were well tolerated in Peruvians. About 20% of volunteers who received CVD 111 at the high dose excreted the El Tor organism, compared to 7% in the low-dose group. CVD 111 was detected in the stools of two placebo recipients, neither of whom had symptoms or seroconverted. In all vaccine groups, 69 to 76% developed fourfold rises in Inaba vibriocidal antibodies. Among those who received the bivalent vaccine, 53 to 75% also developed significant rises in Ogawa vibriocidal antibodies. We conclude that it is feasible to produce a single-dose, oral bivalent vaccine that is safe and immunogenic against both biotypes (El Tor and classical) and both serotypes (Inaba and Ogawa) of cholera for populations in both developed and developing parts of the world.

With the introduction of epidemic cholera into Latin America in 1991, cholera is now capable of causing explosive epidemics in all regions of the developing world (5, 6). Vaccines may be a valuable method of protection for travelers and aid workers who are traveling or working in areas where cholera is endemic (1). Because single-dose, oral vaccines are easy to administer and rapidly induce immunity, they may also have considerable public health impact as a method to control epidemics (19).

An ideal oral cholera vaccine would provide protection against classical and El Tor biotypes and Inaba and Ogawa serotypes of *Vibrio cholerae* O1. CVD 103-HgR is a vaccine derived from a *V. cholerae* O1 classical Inaba (12). This vaccine strain has proven exceptionally safe and in U.S. volunteer studies provided 80 to 100% protection overall against classical cholera challenge, approximately 50 to 60% protection against El Tor challenge, and 100% protection against severe or moderate diarrhea after challenge with either biotype (11, 12, 19). CVD 103-HgR has also been shown to be safe and immunogenic in Latin American and Asian children and adults (7, 10, 15–17). The epidemic cholera strain in Latin America and most other regions of the world is biotype El Tor, predominantly serotype Ogawa. The addition of an El Tor Ogawa strain to CVD 103-HgR might provide broader protection against all strains of *V. cholerae* O1.

It has been difficult to construct a safe El Tor vaccine strain. CVD 110 was constructed from wild-type strain El Tor Ogawa E7948. Although attenuated in much the same manner as CVD 103-HgR, CVD 110 caused mild to moderate diarrhea in 7 of 10 adult volunteers (mean stool volume, 861 ml) (20). Analysis of a series of other El Tor strains that were attenuated showed that there is a wide variability in the virulence of the strains (21); however, an El Tor Inaba strain (C6709) was safe and protective against an El Tor Inaba challenge strain (9, 21). CVD 111 was constructed by using a less virulent parent strain, El Tor Ogawa N16117 (11). N16117 was modified by deleting *ctx*, *zot*, *cep*, and *ace* and reintroducing *ctxB* to create CVD 111.

In a phase 1 study of CVD 111, 25 volunteers received 3×10^8 CFU of freshly harvested CVD 111 with buffer. A mild diarrhea that was not accompanied by malaise, nausea, vomiting or other notable adverse effects was observed in 12% of volunteers. The immune response was strong: 92% of volunteers developed vibriocidal antibodies, with a mean reciprocal antibody titer of 2,291. When challenged with wild-type *V. cholerae* O1 El Tor Ogawa, 7 (88%) of 8 unimmunized control volunteers, compared with 3 (17%) of 18 immunized volunteers, developed diarrhea (vaccine efficacy, 81%) (18).

The Swiss Serum and Vaccine Institute prepared a lyophi-

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TABLE 1. Dosage regimens used in U.S. and Peru studies to evaluate safety and immunogenicity

Group	Vaccine dose (CFU)			
	U.S. study (n = 23)		Peru study (n = 275)	
	CVD 103-HgR[a]	CVD 111	CVD 103-HgR	CVD 111
1	10^8	$^7 10^8$	10^9	10^9
2	10^8	10^7	None	10^9
3	10^8	10^6	10^9	10^8
4	None	10^7	10^9	None
5 (*E. coli* K-12 placebo)				

[a] The licensed dose of CVD 103-HgR (Orochol) is 10^8 cells.

lized formulation of CVD 111. The purpose of these studies was to determine the safety and immunogenicity of several dosages of the lyophilized formulation of CVD 111 given alone or in combination with CVD 103-HgR in healthy U.S. and Peruvian adult volunteers. We tested the bivalent vaccine in populations from developed and developing parts of the world because it has been shown previously that dosing, safety, and immunogenicity profiles may differ considerably between populations in developing and developed parts of the world (7, 15, 16).

MATERIALS AND METHODS

Vaccine and placebo preparations. The vaccine formulation consisted of two aluminum foil sachets. One sachet contained buffer which was added to 100 ml of distilled water in a cup. The other sachet contained the lyophilized mono- or bivalent vaccine preparation. The lot release specification was 2×10^6 to 8×10^6, 10^7, 10^8, or 10^9 cells. In previous studies, the counts have averaged $\sim 4 \times 10^6$, 10^7, 10^8, or 10^9 cells. The vaccine was stirred into the buffered water solution consisting of 2.65 g of $NaHCO_3$ and 1.65 g of ascorbic acid. Volunteers were asked not to eat or drink for 30 min before and after dosing.

Subjects. U.S. volunteers were community members seen as outpatients recruited at the Center for Vaccine Development, Baltimore, Md. The Peruvian volunteers were military recruits stationed around Lima, Peru. Both groups consisted of young adults 18 to 40 years old, who gave informed signed consent. Persons were excluded if they had a history of acute or chronic intestinal infection, were taking antidiarrheal or antacid medications, had a history of cholera infection or vaccination in the last 2 years, were taking antibiotics within 7 days before dosing, had multiple drug or doxycycline allergies, had a history of immunosuppression or use of immunosuppressive drugs, were pregnant, or had tested positive for human immunodeficiency virus antibody.

Study design. Both studies were outpatient studies. In the first study, 23 U.S. adult volunteers were randomized into five groups, in blocks of five. Three groups received CVD 103-HgR at 10^8 CFU plus CVD 111 at 10^8, 10^7, or 10^6 CFU. The fourth group received CVD 111 at 10^7 CFU alone, and the fifth group received inactivated *Escherichia coli* K-12 placebo (Table 1). U.S. volunteers kept a diary of symptoms for 3 days. Stools were cultured on days 1, 3, 5, 7, 10, and 14 after vaccination, and blood for immune responses was collected before and on days 7 and 10 after vaccination.

In the second study, 275 Peruvian adult volunteers were divided into five groups in a double-blind, randomized fashion and received a single oral dose of CVD 103-HgR at 10^9 CFU plus CVD 111 at 10^8 CFU or 10^9 CFU, CVD 103-HgR at 10^9 CFU alone, CVD 111 at 10^9 CFU alone, or inactivated *E. coli* K-12 placebo (Table 1). Vaccine randomization was done in blocks of 20. Side effect information was collected during daily interviews for 3 days after vaccination, stool specimens were obtained every other day for 5 days to assess transmissibility and duration of excretion, and a blood specimen was obtained before and 10 to 14 days after vaccination to measure the immune response. The Peru study was performed in two parts, with approximately 75 volunteers in the first part and 200 in the second part. The safety results of the first part were analyzed by an outside data monitoring board before proceeding to the second part.

Definitions of adverse reactions and treatment. In the U.S. study, diarrhea was defined as the passage of three or more unformed stools over a 24-h period, and fever was defined as an oral temperature of 38°C (100.4°F). In the Peru study, stools were described as normal, soft, or liquid, and diarrhea was defined as the passage of liquid stool.

Bacteriology. In the U.S. study, whole stools were collected in a clean container and refrigerated. Rectal swabs were collected if whole stool was not available and stored in Cary-Blair transport medium at room temperature. In Peru, rectal swabs were placed into Cary-Blair transport medium and transported to the laboratory on the same day. For isolation and identification of *V. cholerae* O1, the swab was removed from the transport medium, inoculated directly onto TCBS, and after enrichment for 6 to 18 h at 37°C, placed in alkaline peptone water, pH 8.6. Presumptive identification was based on a positive oxidase test and Gram stain. *V. cholerae* colonies from each plate were serotyped by using Inaba and Ogawa antisera. All *V. cholerae* O1 strains were stored at −70°C and lyophilized.

A sample of 32 strains from Peruvian volunteers that were identified as El Tor Ogawa was sent to the University of Maryland for verification. All were streaked on TCBS for isolation and to verify the presence of *V. cholerae*. All strains were then inoculated onto L agar and transferred to Whatman paper filters as described previously (4). Filters were then hybridized with a 540-bp *Xba*I-*Cla*I fragment probe derived from the *ctxA* gene (14).

Immunology. Serum vibriocidal antibodies against Ogawa and Inaba and immunoglobulin G (IgG) cholera antitoxin were measured in pre- and postvaccination specimens by previously described methods (3, 13). In the U.S. study, the highest of the two postvaccination specimens was defined as the peak. In both studies, a fourfold or greater rise in vibriocidal titer was considered significant (seroconversion). IgG cholera antitoxin in serum diluted 1:50 was measured by enzyme-linked immunosorbent assay. A ≥0.20 rise in the net optical density of the postvaccination specimen over that of the prevaccination specimen was considered significant (seroconversion).

Statistical methods. The diarrheal rate was compared for all five groups by a 5×2 Fisher's exact test at the $P = 0.05$ level. Seroconversion was defined as a ≥4-fold rise in reciprocal serum vibriocidal titer from pre- to postvaccination. Separate analyses were run for Inaba and Ogawa serum vibriocidal titers. For vibriocidal titers, all five groups were compared by 5×2 χ^2 test at the $P = 0.05$ level.

Geometric mean reciprocal Inaba and Ogawa antibody titers (GMTs) were compared initially among the four vaccine groups by separate single-classification analyses of covariance (independent variable = vaccine group; dependent variable = log-transformed reciprocal day 10 titers; covariate = log-transformed reciprocal day 0 titers). If the null hypothesis (no heterogeneity among the four geometric means) was rejected, then pairwise comparisons were performed with Bonferroni corrections applied.

RESULTS

Study 1 (United States). (i) Safety. One of five U.S. volunteers in each group who received CVD 111 at a dose of 10^7 or 10^8 CFU alone or in combination developed diarrhea (5 to 12 loose stools), usually accompanied by abdominal cramps. None of four volunteers who received CVD 111 at 10^6 CFU in combination with CVD 103-HgR developed diarrhea. One of the four U.S. volunteers who received placebo reported diarrhea consisting of three loose stools.

(ii) Immunogenicity. Immune responses measured by serum Ogawa and Inaba vibriocidal antibodies developed in all but one U.S. volunteer in the vaccine groups (Table 2). The peak

TABLE 2. Vibriocidal antibody titers after vaccination with a single-dose, bivalent, live oral cholera vaccine in healthy U.S. volunteers

Group	Dose (CFU)		Seroconversion rate (Inaba)	Inaba vibriocidal peak GMT	Seroconversion rate (Ogawa)	Ogawa vibriocidal peak GMT	IgG antitoxin seroconversion rate
	CVD 103-HgR	CVD 111					
CVD 103-HgR/CVD 111	10^8	10^8	5/5	1,470	4/5	1,689	4/5
	10^8	10^7	5/5	13,512	5/5	4,457	2/5
	10^8	10^6	4/4	3,044	4/4	1,076	2/4
	None	10^7	5/5	320	5/5	6,756	4/5
E. coli K-12 placebo			0/4	48	0/4	48	0/4

TABLE 3. Postvaccination side effects in 275 healthy Peruvian volunteers, Lima, Peru, 1995 to 1996

Group	Dose (CFU)		No. with symptoms/55 vaccinated			
	CVD 103-HgR	CVD 111	Liquid diarrhea	Abdominal pain	Vomiting	Fever
CVD 103-HgR/CVD 111	10^9	10^9	1	7	2	0
	None	10^9	1	7	0	1
	10^9	10^8	3	8	0	1
	10^9	None	0	8	0	0
E. coli K-12 placebo			1	9	0	0

GMT against the Ogawa serotype was several times higher among the volunteers who received CVD 111 at the 10^7 CFU dose because one volunteer in the 10^8 CFU group did not respond as well as the others. The difference between groups was not statistically significant.

(iii) Fecal excretion. Twelve (80%) of 15 volunteers who received CVD 111 at 10^7 or 10^8 CFU shed the vaccine on one or more occasion in the 10 days following vaccination. None of the four volunteers who received 10^6 CFU of CVD111 shed vaccine organisms.

Study 2 (Peru). The first part took place from November to December 1995 among Peruvian Marine recruits stationed at Ancon Marine Base. The second part was performed February to March 1996 among Peruvian naval recruits at the CITEN naval training academy, Callao, Peru. All volunteers were male. The mean age at Ancon was 18 years, and the mean age at CITEN was 20 years. Because there were no differences for anti-cholera toxin fold rise or pre- or postvaccination Inaba or Ogawa titers at the two locations, the two parts were combined.

(i) Safety. The vaccines were very well tolerated. Few postdosing side effects were elicited among the volunteers, and there were no differences between the vaccine groups and the placebo recipients (Table 3). Among 165 Peruvian volunteers who received CVD 111 at a dose of 10^9 or 10^8 CFU, either alone or in combination, 5 (3%) developed diarrhea, compared to 1 (1.8%) of 55 placebo recipients and none in the group that received CVD 103-HgR alone ($P = 0.53$). The number of liquid stools ranged from one to five; diarrhea was reported to begin 1 to 3 days after vaccination and lasted 1 day. Two volunteers who reported vomiting received the highest dose of vaccine ($P = 0.20$). Abdominal cramps were reported equally among vaccine and placebo recipients.

(ii) Immunogenicity. As evidence of prior exposure to *V. cholerae* O1, 35% of volunteers had Inaba vibriocidal titers of ≥1:40 and 56% had Ogawa vibriocidal titers of ≥1:40 before vaccination. Between 69 and 76% of Peruvian volunteers who received CVD 103-HgR developed fourfold rises in Inaba vibriocidal antibodies, compared to 62% of the volunteers who received CVD 111 alone (Table 4). A range of 53 to 75% of Peruvian volunteers who received CVD 111 developed fourfold rises in Ogawa vibriocidal antibodies, compared to 45% of the volunteers who received CVD 103-HgR alone ($P < 0.02$). None of the placebo recipients developed a fourfold antibody titer rise in response to either Inaba or Ogawa organisms.

The GMTs were approximately twofold higher in the bivalent group that received the high dose (10^9 CFU) of CVD 111. The Inaba reciprocal vibriocidal titers of the four vaccine groups were homogeneous (analysis of covariance, $P = 0.18$); however, the vaccine groups differed with respect to postvaccination Ogawa reciprocal vibriocidal titers, adjusted for prevaccination reciprocal titers ($P = 0.002$). Pairwise analyses of covariance were run on the four vaccine groups, with Bonferroni adjustment of the resultant probabilities. These analyses indicated that the high-dose bivalent vaccine (CVD 103-HgR and CVD 111 at 10^9 CFU) generated significantly higher Ogawa vibriocidal antibody titers than either the low-dose bivalent vaccine (CVD 103-HgR at 10^9 CFU and CVD 111 at 10^8 CFU, adjusted $P = 0.015$) or CVD 103-HgR alone (adjusted $P = 0.007$). The other vaccine groups were statistically homogeneous.

Persons with low prevaccination vibriocidal titers had mean fold increases ranging from 30 to 80 times the baseline, while persons with high prevaccination titers had 7- to 10-fold increases (data not shown). Vaccinees with prevaccination titers of ≥1:160 had lower vibriocidal seroconversion rates; however, over 70% of those with a prevaccination titer of 1:40 or 1:80 seroconverted.

From 62 to 78% of vaccinees had a significant antibody titer rise in response to cholera antitoxin (Table 4). Seroconversion rates were higher (78 to 82%) among those who received the bivalent vaccine than among those who received either monovalent vaccine (62 to 69%). Twenty-five percent of the placebo recipients also seroconverted.

TABLE 4. Serum Inaba and Ogawa vibriocidal antibody and IgG antitoxin responses following ingestion of a single dose of CVD 103-HgR and CVD 111 at various dosages or placebo by Peruvian military recruits, November 1995 to March 1996[a]

Group	Dose (CFU)		Vibriocidal antibody								IgG antitoxin antibody			
			Seroconversion rate				Reciprocal GMT				Seroconversion		Optical density	
			Inaba		Ogawa		Inaba		Ogawa					
	CVD 103-HgR	CVD 111	No.	%	No.	%	Pre	Post	Pre	Post	No.	%	Pre	Post
CVD 103-HgR/CVD 111	10^9	10^9	42	76	41	75	41	557	68	699	43	78	0.57	1.13
	None	10^9	34	62	37	67	47	337	77	564	34	62	0.59	0.93
	10^9	10^8	38	69	29	53	57	391	95	387	45	82	0.55	0.95
	10^9	None	42	76	25	45	30	363	56	279	38	69	0.56	0.90
E. coli K-12 placebo			0	0	0	0	29	31	58	59	14	25	0.56	0.57

[a] Each group contained 55 subjects. Pre, prevaccination; post, postvaccination.

TABLE 5. Postvaccination cholera vaccine excretion rates in 275 Peruvian volunteers, Lima, Peru, 1995 to 1996

Group[a]	Dose (CFU)		Excretion[b]			
			V. cholerae O1 Ogawa		*V. cholerae* O1 Inaba	
	CVD 103-HgR	CVD 111	No.	%	No.	%
CVD 103-HgR/CVD 111	10^9	10^9	16	29	0	0
	None	10^9	12	21	0	0
	10^9	10^8	4	7	0	0
	10^9	None	1	2	1	2
E. coli K-12 placebo			2	4	0	0

[a] Each group contained 55 subjects.
[b] The excretion rate was significantly higher among those subjects who received CVD 111 at the high dose (16 of 55) than in those who received the low dose (4 of 55) (two-tailed Fisher's exact test, $P = 0.006$).

(iii) Excretion and transmission. CVD 111 was isolated from 25% (28 of 110) of vaccinees who received CVD 111 at the high dose (9 logs), compared to 7% of those who received it at the low dose (8 logs) (Table 5). There was evidence of transmission of CVD 111 to two persons who received placebo and one person who received CVD 103-HgR alone. Neither of the placebo recipients had symptoms or seroconverted. Excretion peaked on day 3 or 5. About 10% of those who received 9 logs of CVD 111 were still excreting the vaccine on day 9, the last culture. All 32 strains were negative for *ctxA* hybridization.

DISCUSSION

No serological method exists to measure an immune response that is made solely to El Tor; however, data from challenge studies indicate that classical strains are highly protective against classical strains of either the homologous or heterologous serotype but are less protective against El Tor strains of either serotype (12). Since El Tor is the major circulating strain of the seventh pandemic, it is important to build a vaccine that is as protective as possible against this serotype. This is the first report on the use of a multivalent live oral O1 cholera vaccine in populations in developed and developing parts of the world. The two major obstacles in the development of live attenuated vaccines for enteric diseases are safety in developed country populations and immunogenicity in developing country populations. One of the ways to balance these competing problems is to adjust the vaccine dose for each population. In the case of CVD 103-HgR and CVD 111, the dose is increased 10-fold in order to immunize developing country populations (15, 16). CVD 103-HgR has an excellent safety profile in all populations tested, including infants, at doses of up to 10^9 CFU. CVD 111 colonizes very well and elicits strong immune responses, but it also appears to cause symptoms in North Americans. Therefore, the dose must be reduced in nonimmune populations to between 10^6 and 10^7 CFU. Additional safety studies in North American adults are under way to determine the optimal dose.

In contrast to U.S. volunteers, Peruvian adult volunteers tolerated CVD 111 very well at 10^8 and 10^9 CFU; however, the vibriocidal titers were also considerably lower. Lower titers in developing country populations has been a consistent finding which has been attributed to preexisting antibody titers and/or to bacterial competition in the intestine (16). The bivalent vaccine induced high-titer vibriocidal immunity to both the Ogawa and Inaba strains and appeared to induce higher titers and higher rates of seroconversion in persons with preexisting antibodies than CVD 103-HgR alone. In a previous study, only 3 (38%) of 8 persons with a vibriocidal titer of 1:40 or 1:80 seroconverted, while 32 (84%) of 38 persons receiving the bivalent vaccine seroconverted (7). Although there is no way to measure an antibody response specific to the El Tor biotype, we assume that a good Ogawa antibody response may also reflect a good response to the El Tor biotype since the Ogawa serotype resides on the El Tor strains. Since El Tor Ogawa is the predominant strain in Latin America, the addition of CVD 111 may be advantageous in providing protection against El Tor cholera. The addition of CVD 103-HgR may also play an important role because it provides immunity against the Inaba serotype and because there is some evidence that classical strains provide a more complete protection against subsequent infection than El Tor strains (2).

In this study, as in the previous study with freshly harvested organisms, CVD 111 vaccine organisms were shed for several weeks after vaccination. We found several instances among Peruvian military recruits where El Tor Ogawa organisms were transmitted to placebo recipients or to a person who was vaccinated with only CVD 103-HgR. These recruits lived closely together in barracks. While some evidence of transmission of CVD 103-HgR was found in Indonesian sibling children (15), CVD 111 appears to colonize much more effectively than CVD 103-HgR. CVD 111 vaccine organisms transmitted from other subjects did not appear to cause a biological response leading to either symptoms or seroconversion. Similar to the recommendations for oral poliovirus vaccine, vaccine might not be recommended in households where immunocompromised persons might inadvertently become infected. Hygienic measures such as hand washing and disinfection of toilets would make transmission unlikely under most circumstances.

The vaccine strain was prepared by recombinant techniques resulting in a precise deletion of the toxin cassette containing genes encoding the A (biologically active) subunit of cholera toxin, *zot*, *ace*, and *cep*. Since this is a deletion, it is not possible for these vaccine strains to revert to toxigenicity. In theory, the A subunit genes could be regained by a recombinational event with wild-type toxigenic *V. cholerae*. Recent observations describing the phage transmission of toxin genes may increase the possibility of this occurring (22), but at present a cholera toxin containing phage has not been isolated in nature. In the laboratory, the chance of a recombination occurring without selective pressure is very small (10^{-6}) (8). Polymyxin B susceptibility may prove to be a simple microbiological test to identify CVD 111 El Tor vaccine organisms. None of the El Tor organisms that were susceptible to polymyxin B had cholera toxin genes, confirming that they were vaccine organisms.

The main advantage of live cholera vaccines is that they are capable of inducing rapid immunity after a single dose. We demonstrated that it is feasible to vaccinate simultaneously with two live strains of cholera without apparent loss of activity of either strain. Simultaneous administration did not increase postdosing side effects and led to an immune response to both strains. Since the two strains are complementary in both biotype and serotype and induce a strong immune response to both Inaba and Ogawa organisms, it is hoped that this vaccine will induce an immunity that is broadly protective against all strains of O1 cholera.

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