Expanded Safety and Immunogenicity of a Bivalent, Oral, Attenuated Cholera Vaccine, CVD 103-HgR Plus CVD 111, in United States Military Personnel Stationed in Panama

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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-chambered sachet; one chamber contained lyophilized bacteria, and the other contained buffer. A total of 170 partially-immune American soldiers stationed in Panama received one of the following five formulations: (a) CVD 103-HgR at 10^8 CFU plus CVD 111 at 10^7 CFU, (b) CVD 103-HgR at 10^8 CFU plus CVD 111 at 10^6 CFU, (c) CVD 103-HgR alone at 10^8 CFU, (d) CVD 111 alone at 10^7 CFU, or (e) inactivated Escherichia coli placebo. Among those who received CVD 111 at the high or low dose either alone or in combination with CVD 103-HgR, 8 of 103 had diarrhea, defined as three or more liquid stools. None of the 32 volunteers who received CVD 103-HgR alone or the 35 placebo recipients had diarrhea. CVD 111 was detected in the stools of 46% of the 103 volunteers who received it. About 65% of all persons who received CVD 103-HgR either alone or in combination had a fourfold rise in Inaba vibriocidal titers. The postvaccination geometric mean titers were comparable among groups, ranging from 450 to 550. Ogawa vibriocidal titers were about twice as high in persons who received CVD 111 as in those who received CVD 103-HgR alone (600 versus 300). The addition of CVD 111 improved the overall seroconversion rate and doubled the serum Ogawa vibriocidal titers, suggesting that the combination of an El Tor and a classical cholera strain is desirable. While CVD 111 was previously found to be well tolerated in semiimmune Peruvians, the adverse effects observed in this study indicate that this strain requires further attenuation before it can be safely used in nonimmune populations.

The worldwide increase in cholera in the last decade in all areas of the developing world has renewed interest in control methods (1, 13, 22). Until recently, vaccination was not recommended for the control of cholera because of the poor efficacy of the parenteral whole-cell vaccine (2, 23). This vaccine, which is the only licensed vaccine in the United States, lacks efficacy, causes local side effects, and does not interrupt transmission. The new live, attenuated and inactivated oral cholera vaccines are safer and more effective than the parenteral killed vaccine. They have the potential for widespread use in areas where cholera is endemic, where they could have considerable impact on public health (12). These types of vaccines would also be valuable to persons traveling or working in such areas (1, 20). Protective immunity has been demonstrated in less than a week after a single dose of a live, attenuated vaccine (18). The induction of rapid immunity suggests that this type of vaccine could aid in the control of cholera outbreaks or ongoing epidemics.

The epidemic cholera strain in most regions of the world, including Latin America, is biotype El Tor, predominantly serotype Ogawa (22). However, cholera caused by both classical and El Tor biotypes and Inaba and Ogawa serotypes of V. cholerae O1 regularly occur (3). CVD 103-HgR is a vaccine strain derived from a Vibrio cholerae O1 classical Inaba strain (9). This vaccine strain was safe and immunogenic in populations in developed and lesser-developed countries and has been licensed in Canada and in some countries in Europe, Latin America, and Asia (6, 8, 14–16). In studies with U.S. volunteers, CVD 103-HgR protected against severe cholera caused by all strains; however, protection against any diarrhea was more protective against challenge with the classical biotype than the El Tor biotype (9), suggesting that a vaccine prepared from an El Tor Ogawa strain might provide better protection against the currently circulating strains of V. cholerae O1 (7, 19). In a field study of natural cholera infection, recurrent episodes of cholera were documented after an initial El Tor biotype infection but not after a classical biotype infection, suggesting a longer-lasting protection after infection with the classical biotype (4). Thus, it appears that the optimum vaccine would be a combination of strains representing El Tor and classical biotypes and Ogawa and Inaba serotypes.

CVD 111 was constructed from V. cholerae O1 El Tor Ogawa parent strain, N16117 (11). N16117 was modified by deleting the virulence cassette containing the toxin genes, ctx, zot, cep, and ace. The gene for the binding portion of cholera toxin (ctxB) and that for mercury resistance (mer) were inserted into the hemolysin locus (hlyA) to produce CVD 111 (11, 17). Twenty-five U.S. adults were given a single oral dose
of $3 \times 10^8$ CFU of freshly harvested CVD 111 with buffer (17). Three (12%) volunteers developed mild diarrhea (mean stool volume = 813 ml) but no systemic symptoms. CVD 111 was highly immunogenic, with 23 (92%) volunteers developing high-titer serum vibriocidal antibodies (geometric mean titer = 12,291). When challenged with wild-type V. cholerae O1 El Tor Ogawa, 7 (88%) of 8 unimmunized control volunteers compared to 3 (17%) of 18 immunized volunteers developed diarrhea (vaccine efficacy, 81% (17)). This level of vaccine efficacy against El Tor Ogawa was 15 to 20% higher than what had been achieved with CVD 103-HgR (10).

Therefore, a lyophilized formulation of CVD 111 was prepared. In a randomized trial, 275 Peruvian adults received efficacy against El Tor Ogawa was 15 to 20% higher than what had been achieved with CVD 103-HgR (10).

Therefore, a lyophilized formulation of CVD 111 was prepared. In a randomized trial, 275 Peruvian adults received CVD 103-HgR at $10^8$ CFU plus CVD 111 at $10^7$ or $10^8$ CFU, CVD 111 alone at $10^8$ CFU, CVD 103-HgR at $10^8$ alone, or placebo, in lyophilized formulations (21). All dosage regimens were well tolerated. In all vaccine groups, 69 to 76% of the subjects developed fourfold increases in Inaba vibriocidal antibodies. Among those who received the bivalent vaccine, 53 of 75% also developed significant increases in Ogawa vibriocidal antibodies.

In summary, CVD 111 is immunogenic and protective in U.S. volunteers but induced diarrhea in 12% of these volunteers. CVD 111 was well tolerated by Peruvian adults and enhanced the vibriocidal response compared to that of CVD 103-HgR alone. We undertook the present study to further study CVD 111 in combination with CVD 103-HgR, attempting to adjust the dose to a safe and immunogenic level.

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The vaccine formulation consisted of two aluminum foil sachets. One sachet contained buffer, consisting of 2.65 g of NaHCO₃ and 1.65 g of ascorbic acid, 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 ($2 \times 10^8, 2 \times 10^7, 2 \times 10^8, 2 \times 10^9$ cells. In this study the counts averaged $4 \times 10^8, 10^7, 10^8, 10^9$ cells. The vaccine was stirred into the buffered-water solution. Volunteers were asked not to eat or drink for 30 min before and after immunization.

One hundred seventy U.S. military volunteers (mean age, 28 years) stationed in Panama were divided into five groups in a double-blind, randomized fashion. Group 1 received CVD 103-HgR at $10^8$ CFU plus CVD 111 at $10^7$ CFU. Group 2 received CVD 103-HgR alone at $10^8$ CFU plus CVD 111 at $10^6$ CFU. Group 3 received CVD 111 alone at $10^7$ CFU. Group 4 received CVD 103-HgR alone at $10^8$ CFU, and group 5 received an inactivated Escherichia coli placebo (Table 1). Vaccine randomization was done in blocks of 20. Information about side effects 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.

Stools were described as normal, soft, or liquid, and diarrhea was defined as the passage of three or more liquid stools in a 24-h period.

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 and inoculated directly onto thiosulfate-citrate-bile salts-sucrose and after enrichment for 6 to 18 h at 37°C 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 with V. cholerae O1 Inaba and Ogawa antisera. Since cholera was not circulating in Panama at the time of this study, we defined all isolates of V. cholerae O1 Inaba as CVD 103-HgR and all isolated of V. cholerae O1 Ogawa as CVD 111.

Serum specimens were prepared and stored at ~70°C in the Department of Clinical Pathology at the Gorgas Army Hospital in Panama. At the end of the study, specimens were shipped on dry ice to the Center for Vaccine Development for immunological testing. Serum vibriocidal antibodies against Ogawa and Inaba and immunoglobulin G (IgG) cholera antitoxin were measured in pre- and postvaccination specimens by previously described methods (5, 10). A fourfold or greater rise in vibriocidal titer was considered significant (seroconversion). IgG cholera antitoxin in serum diluted 1:50 was measured by a semiquantitative enzyme-linked immunosorbent assay. A $\geq 0.20$ rise in the net optical density of the postvaccination specimen over that of the prevaccination specimen was considered significant (seroconversion).

The diarrheal rate was compared for all five groups by a $5 \times 2$ Fisher’s exact test at the $P = 0.05$ level. Separate analyses were run for Inaba and Ogawa seroconversion; all four nonplacebo groups were compared by $4 \times 2$ Fisher’s exact test at the $P = 0.05$ level.

Geometric mean reciprocal Inaba and Ogawa antibody titers 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. All other analyses were carried out by using Fisher’s exact test. Two-tailed hypotheses were evaluated at the $5\%$ level, except where noted. The volunteers were predominantly males in their late 20s. Among those who received CVD 111 either alone or in combination with CVD 103-HgR, 8 of 103 had diarrhea, defined as three or more liquid stools in 24 h (Table 1). Excretion of CVD 111 was observed in all 8 persons with diarrhea and in 39

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result for CVD 103-HgR/CVD 111 vaccine dose (CFU) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^8/10^7$</td>
</tr>
<tr>
<td>No. with other symptoms (%)</td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Fever</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* 0/0, E. coli placebo.

** Diarrhea was defined as three or more liquid stools in 24 h.
of 95 persons without diarrhea, \( P < 0.01 \). None of the 32 volunteers who received CVD 103-HgR alone or of the 35 who received placebo had diarrhea as defined above. The diarrhea rate (8 of 103) in subjects who received CVD 111 was significantly higher than the rate (0 of 67) subjects who received placebo or CVD 103-HgR alone \( (P = 0.023; \text{Fisher’s exact test, two-tailed}) \). Loose stools and other gastrointestinal or systemic symptoms were equally common in the five groups. Among the eight volunteers who reported diarrhea, six reported diarrhea on day 2 after vaccination, one reported diarrhea on day 3 after vaccination, and one reported diarrhea on day 4 after vaccination. Five volunteers reported 3 to 6 liquid bowel movements, and three reported 10 or greater. The three volunteers with the most-pronounced diarrhea received the lower dose \( (10^6 \text{ CFU}) \) of CVD 111.

CVD 111 was isolated from half \( (35 of 69) \) of those who received the bivalent vaccine (Table 2). There was no difference in excretion rate among those who received the higher dose and those who received the lower dose, but the percentage of subjects excreting CVD 111 was about 15% lower among those who received CVD 111 alone (Table 2). CVD 103-HgR was isolated from only 2 of 101 persons who received the vaccine. CVD 103-HgR and CVD 111 were both isolated once from persons who had not been vaccinated with these strains (Table 2). CVD 111 was isolated from a placebo recipient 5 days after vaccination. This volunteer had no symptoms and exhibited no rise in antibody titers, despite having a prevaccination vibriocidal titer of <1:20. CVD 103-HgR was detected 7 days after immunization in one person who was immunized with CVD 111 alone. This volunteer had no symptoms and no other positive cultures for either vaccine strain. He exhibited a 64-fold increase in serum vibriocidal titers for the Ogawa strain and a twofold increase for the Inaba strain.

The prevaccination vibriocidal titers were higher for Ogawa than for Inaba (Table 3). About 40% of volunteers had elevated titers (\( \geq 1:40 \)) for the Inaba strain, and about 60% had elevated titers for the Ogawa strain (Table 3). There were no significant differences in prevaccination titers among groups. Two-thirds of all persons who received CVD 103-HgR either alone or in combination with CVD 111 had a fourfold rise in Ogawa vibriocidal titers (Table 3). The postvaccination reciprocal geometric mean titers were very comparable, ranging from 450 to 550. Only 38% of persons who received CVD 111 alone developed significant Inaba vibriocidal titers.

CVD 111 enhanced the Ogawa vibriocidal titers (analysis of covariance, \( P = 0.002 \)). The Ogawa vibriocidal titer was twice as high among persons who received CVD 111 as among those who received CVD 103-HgR alone (reciprocal geometric mean titers, 602 versus 300). Over half (53 to 65%) of volunteers who received CVD 111 developed serum IgG anti-toxin antibodies compared to 38% of those received CVD 103-HgR alone.

Among the 101 persons who received CVD 111, 47 persons...
excreted CVD 111. Of these, 43 (91%) seroconverted to the Inaba strain and 44 (94%) seroconverted to the Ogawa strain. In contrast, among 54 persons with consistently negative cultures, only 16 (30%) and 18 (33%) seroconverted to Inaba and Ogawa, respectively (P < 0.001). All eight of the subjects with diarrhea seroconverted to Inaba and Ogawa. This group also had the highest titers; reciprocal geometric mean Inaba titers rose from 26 to 8,611, and reciprocal geometric mean Ogawa titers rose from 37 to 7,896.

As expected, no one in the placebo group seroconverted to either Inaba or Ogawa. Over half of the nonresponders had vibriocidal titers that were below 1:40, so the lack of response did not appear to be related to elevated prevaccination titers. In the bivalent groups 20 (29%) of 69 persons failed to seroconvert. None of the nonresponders excreted the CVD 111, and none had symptoms. The most likely explanation for lack of seroconversion in these groups is lack of colonization.

There was no difference in vibriocidal response between the bivalent groups (n = 69) and the monovalent groups (n = 66). The difference between the bivalent groups (29% failure rate) compared to CVD 103-HgR alone (47%) was greater but still not significant (P = 0.07). The difference between the high-dose bivalent group (26%) and CVD 103-HgR (47%) was also not significant (P = 0.065).

The difference in the number that converted to both Ogawa and Inaba was significant between groups. In the high-dose bivalent group, 24 (49%) of 50 seroconverted to both strains, compared to 21 (62%) of 34 in the low-dose bivalent group, 13 (38%) in the monovalent CVD 103-HgR group, and 17 (53%) of 32 in the monovalent CVD 111 group. Combining the bivalent groups, 45 (65%) of 69 seroconverted to both strains compared to 30 (45%) of 66 in the two monovalent groups, P = 0.02. Each bivalent group had significantly higher seroconversion rates compared to CVD 103-HgR alone (P < 0.025) but not to CVD 111 alone.

Among U.S. volunteers stationed in Panama, El Tor Ogawa cholera vaccine CVD 111 improved the serum immune response, especially in regard to the Ogawa vibriocidal titers. However, 8% of subjects who received CVD 111 also had diarrhea. Diarrhea was observed only in subjects who excreted the CVD 111 vaccine strain. There was little difference between the 10^6 and 10^7 dose of CVD 111 in terms of colonization or symptoms or in the time of onset of symptoms. All vaccinees who reported postvaccination diarrhea developed high vibriocidal titers. Thus, colonization and symptoms were overt signs of a good immune response to CVD 111, in contrast to CVD 103-HgR, for which colonization and postvaccination symptoms are rarely observed. The Ogawa vibriocidal titer was higher in subjects who received CVD 111 at the high dose compared to the low-dose group (reciprocal mean titer, 879 versus 533), but the mean fold increases were nearly identical. CVD 103-HgR induces an immune response in the majority of vaccinees without cultureable excretion of the vaccine strain. CVD 111 also induces an immune response in the majority of vaccinees, but seroconversion rates are associated with colonization in the gut and excretion of the vaccine strain, both when administered alone and when administered in combination with CVD 103-HgR.

CVD 111 is a good colonizer, excreted by half of the persons who received it. CVD 103-HgR is a poor colonizer, excreted by fewer than 5%. Vaccine strain transmission may have occurred in two instances. CVD 103-HgR was detected in one person who was given only the CVD 111 vaccine, and CVD 103-HgR was detected in a placebo recipient. In these two instances, excretion was documented to occur on only one occasion; neither volunteer had clinical symptoms or developed an immune response. It appears that transmission can occur even in circumstances of relatively high sanitation, leading to transient colonization that has no clinical or immunological consequences.

It is useful to compare the results of the three trials with CVD 111 in U.S. inpatient volunteers, U.S. military personnel stationed in Panama, and Peruvian military personnel (Table 4). As expected, prevaccination vibriocidal titers were higher in Peruvians and lowest in the U.S. volunteers from Maryland. Elevated vibriocidal titers were observed in over 40% of the U.S. military volunteers based in Panama. Postvaccination diarrheal rates were highest in the Maryland subjects, slightly lower in the U.S. military group, and lowest in Peruvians, who ingested the highest dosages. Similarly, the reciprocal geometric mean titer in the Maryland subjects was nearly 2,300, compared to 657 in the U.S. military group in Panama. The difference in vibriocidal titers may be due to the preparation of the vaccine, which was freshly harvested in the Maryland study and lyophilized in the Panama study.

CVD 111 was well tolerated by Peruvians, presumably because they receive greater exposure to cholera and enterotoxigenic *E. coli* in their environment. The diarrhea rate in U.S. volunteers in Panama was modest (8%) but well above the rate observed in persons immunized with CVD 103-HgR or placebo. These symptoms are undesirable but were associated with the best immune response. With one exception, all the volunteers described these symptoms as tolerable because of the lack of other symptoms such as fever or malaise. It is not known if the symptoms were caused by robust colonization or by some other unidentified pathogenic factor. However, the symptoms associated with Peru-14, an attenuated El Tor Inaba vaccine strain, could be eliminated by decreasing the strain’s motility (7, 19), which is important in colonization and in the close adherence of the strain to the gut.

<table>
<thead>
<tr>
<th>Subject nationality</th>
<th>American</th>
<th>American</th>
<th>Peruvian</th>
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<tbody>
<tr>
<td>Study location</td>
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<td>Panama</td>
<td>Peru</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>25</td>
<td>103</td>
<td>165</td>
</tr>
<tr>
<td>Dose (CFU)</td>
<td>10^6</td>
<td>10^6 or 10^7</td>
<td>10^6 or 10^9</td>
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<td>% Subjects with titer ≥40</td>
<td>40</td>
<td>44</td>
<td>61</td>
</tr>
<tr>
<td>Pre-Inaba</td>
<td>60</td>
<td>64</td>
<td>72</td>
</tr>
<tr>
<td>% Subjects with diarrhea postvaccine</td>
<td>12</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>% Subjects with ≥fourfold increase in Ogawa</td>
<td>92</td>
<td>62</td>
<td>69</td>
</tr>
<tr>
<td>Geometric mean titer</td>
<td>Pre-Ogawa</td>
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<td>53</td>
</tr>
<tr>
<td>Post-Ogawa</td>
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<td>657</td>
<td>534</td>
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<tr>
<td>Fold increase</td>
<td>51.3</td>
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<td>6.7</td>
</tr>
</tbody>
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*In the study based in the United States, the vaccine inoculum was freshly harvested, while in the other two studies it was lyophilized and stored in aluminum foil sachets.*

*TABLE 4. Summary of data from three studies on safety and immunogenicity of CVD 111 El Tor Ogawa.*

\[ Geometric mean titer = \frac{\text{Minimum titer} \times \text{Number of subjects}}{\text{Maximum titer} \times \text{Number of subjects}} \]

\[ Fold increase = \frac{\text{Post-vaccination titer}}{\text{Pre-vaccination titer}} \]
In summary, the addition of CVD 111 improved the overall seroconversion rate and doubled serum Ogawa vibriocidal titers, suggesting that the combination of an El Tor and a classical cholera strain is desirable. The adverse effects observed in this study indicate that CVD 111 requires further attenuation before it can be safely used in nonimmune populations.

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REFERENCES


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