INFECTION AND IMMUNITY, May 2005, p. 3018–3024 Vol. 73, No. 5
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.5.3018–3024.2005
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The Vaccine Candidate *Vibrio cholerae* 638 Is Protective against Cholera in Healthy Volunteers

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Received 2 September 2004/Returned for modification 19 November 2004/Accepted 17 January 2005

***Vibrio cholerae* 638 is a living candidate cholera vaccine strain attenuated by deletion of the CTXΦ prophage from C7258 (O1, El Tor Ogawa) and by insertion of the *Clostridium thermocellum* endoglucanase A gene into the hemagglutinin/protease coding sequence. This vaccine candidate was previously found to be well tolerated and immunogenic in volunteers. This article reports a randomized, double-blind, placebo-controlled trial conducted to test short-term protection conferred by 638 against subsequent *V. cholerae* infection and disease in volunteers in Cuba. A total of 45 subjects were enrolled and assigned to receive vaccine or placebo. The vaccine contained 10^9 CFU of freshly harvested 638 buffered with 1.3% $NaHCO_3$, while the placebo was buffer alone. After vaccine but not after placebo intake, 96% of volunteers had at least a fourfold increase in vibriocidal antibody titers, and 50% showed a doubling of at least the lipopolysaccharide-specific immunoglobulin A titers in serum. At 1 month after vaccination, five volunteers from the vaccine group and five from the placebo group underwent an exploratory challenge study with 10^9 CFU of ΔCTXΦ attenuated mutant strain *V. cholerae* 81. Only two volunteers from the vaccine group shed strain 81 in their feces, but none of them experienced diarrhea; in the placebo group, all volunteers excreted the challenge strain, and three had reactogenic diarrhea. An additional 12 vaccinees and 9 placebo recipients underwent challenge with 7×10^5 CFU of virulent strain *V. cholerae* 3008 freshly harvested from a brain heart infusion agar plate and buffered with 1.3% $NaHCO_3$. Three volunteers (25%) from the vaccine group and all from the placebo group shed the challenge agent in their feces. None of the 12 vaccinees but 7 volunteers from the placebo group had diarrhea, and 2 of the latter exhibited severe cholera (>5,000 g of diarrheal stool). These results indicate that at 1 month after ingestion of a single oral dose (10^9 CFU) of strain 638, volunteers remained protected against cholera infection and disease provoked by the wild-type challenge agent *V. cholerae* 3008. We recommend that additional vaccine lots of 638 be prepared under good manufacturing practices for further evaluation.**

Cholera continues to be a serious health problem in many developing countries, including the western hemisphere (20). Cholera is an epidemic illness caused by *Vibrio cholerae* of the O1 and O139 serogroups through the elaboration and intestinal release of a potent enterotoxin termed cholera toxin (9). Serogroup O1 is subdivided into two biotypes, classical and El Tor, both of which are further distinguished into two main serotypes, Ogawa and Inaba, which have different lipopolysaccharide (LPS) structures (8). The El Tor biotype is the most frequent worldwide as part of the seventh cholera pandemic that began in 1961 and has not receded yet (20). This biotype is now endemic in many areas in southern Asia and Africa (20).

Convalescence from cholera leads to strong and long-lasting protective immunity directed mainly against the O-antigen portion of LPS (14, 16). Thus, vaccination seems to be a powerful and feasible prevention strategy against this disease. The recognition that protection relies on efficient stimulation of the mucosal immune system has favored the concept of single-dose live attenuated oral cholera vaccines (14, 18). CVD103HgR (classical biotype) and Peru-15 (El Tor biotype) are two genetically engineered O1 vaccine strains of the Inaba serotype that have been claimed to be well tolerated, immunogenic, and protective in the human cholera challenge model (6, 12, 24, 26). However, their efficacy remains to be confirmed by field trials and demonstration experiments in areas of endemicity.

We have also genetically modified several strains of *V. cholerae* for vaccine development. Deletion of the entire CTXΦ prophage from *V. cholerae* C7258 (10), a wild-type El Tor Ogawa strain isolated in Peru in 1991, produced atoxigenic strain *V. cholerae* 81 (2). In a subsequent step, this strain was

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further manipulated to introduce the *Clostridium thermocellum* endoglucanase A gene (*celA*), disrupting the hemagglutinin/protease gene *hapA*, thus creating *V. cholerae* 638 (19). *Vibrio cholerae* 81 and 638 colonize well in mice and are immunogenic in rabbits when given by the intraduodenal route (4, 11). Strain 638 has also proven to be safe, immunogenic, and excreted by a high percentage of volunteers (3). However, no data on the safety and immunogenicity of *V. cholerae* 81 have been published, and no data on the protective capacity conferred by vaccine candidate 638 are available at present. The experimental volunteer challenge model (21) allows evaluation of the efficacy of cholera vaccine candidates before extensive field trials are conducted in areas of endemicity (6, 24, 25) and has been accepted as evidence of protection by the U.S. Food and Drug Administration (5). In challenge studies, a group of volunteers immunized with a single oral dose of the vaccine strain and a control group inoculated with placebo are challenged with a fully virulent strain under quarantine conditions in a hospital setting (6, 24, 25).

To determine whether to manufacture clinical trial lots of 638 for phase I, II, and III vaccine evaluations, we undertook volunteer studies of *V. cholerae* 81 to check the level of attenuation attained by this progenitor strain during the construction of 638. Volunteer studies were also conducted to explore whether vaccination with a single oral dose of 10^9 CFU of *V. cholerae* 638 conferred protection against colonization and symptoms induced by the subsequent ingestion of 10^9 CFU of *V. cholerae* 81. Finally, we examined whether a single oral dose of 10^9 CFU of 638 conferred protection against colonization and diarrhea caused by challenge with fully virulent *V. cholerae* 3008. The results obtained and presented in this article make strain 638 an attractive vaccine candidate for preparation of lots under good manufacturing practices for phase I, II, and III vaccine evaluations.

MATERIALS AND METHODS

***V. cholerae* strains.** Attenuated strains used in volunteer studies were *V. cholerae* 81 and 638. Procedures used to obtain *V. cholerae* 81 (ΔCTXΦ) and 638 (ΔCTXΦ *hapA*::*celA*) were reported elsewhere (2, 3, 19). The wild-type El Tor Ogawa strain used for challenge, *V. cholerae* 3008 (25), was kindly provided by James B. Kaper. *V. cholerae* VC12 (classical Ogawa) was used as an indicator strain in vibriocidal tests.

Subjects, inclusion criteria, and informed consent. Volunteers were recruited from among 18- to 40-year-old male workers in the western scientific community of Havana City. All subjects were in good health, did not have a recent history of diarrheal disease or cholera vaccination, and were not taking any medication at the time of recruitment. Before admission to these studies, volunteers were medically and psychologically screened and passed a written examination to ensure their understanding of the study and elemental knowledge of cholera. Screening examination included a complete blood count, chemistry panel, human immunodeficiency virus and hepatitis C virus antibodies, hepatitis B virus antigen, enteropathogenic bacteria in feces, and a psychological test. Any abnormality in clinical laboratory tests, stool cultures positive for an enteric pathogen, recent antibiotic use, positive serological test results for human immunodeficiency virus antibody, hepatitis B antigen, or hepatitis C antibody, failure to pass the written examination, or psychological incompatibility with accepting quarantine conditions impaired admission of a volunteer to these studies. Finally, each volunteer signed a witnessed informed consent for each study.

Allocation of trials. All studies were conducted as inpatient trials at the Unit for Isolation of Biological Risks at the Institute for Tropical Medicine Pedro Kouri. The ethics committee of this institution revised and approved the clinical trial protocols. Studies were authorized and audited by the State Center for Drug Control and conducted under a license from the National Center for Biological Safety, which revised the protocol and inspected the facilities.

Preparation of freshly grown inocula and administration. Bacterial strains were streaked to confluence on brain heart infusion agar, grown for 5 h at 37°C, and harvested in saline. The suspension was adjusted spectrophotometrically to the desired concentration. The suspension in 30 ml of 1.3% bicarbonate buffer was administered to volunteers, and an aliquot was diluted appropriately and plate counted. Thirty minutes before inoculation, volunteers ingested 120 ml of bicarbonate buffer. The placebo consisted of bicarbonate buffer alone and was indistinguishable in appearance from the vaccine preparation. Food and drinks were withheld from volunteers for 90 min before and 90 min after dosing.

Clinical surveillance. Volunteers were monitored to detect the occurrence of nausea, vomiting, abdominal cramps, malaise, gurgling, headache, fever, and diarrhea for 5 days after inoculation. The intensity of each symptom was classified as mild if it did not interfere with daily activities, moderate if it interfered with but did not impair daily activities, or severe if it impaired daily activities.

All stools were collected in disposable plastic bedpans and weighed, and their consistency was graded on a 5-point scale (grade 1, formed; grade 2, soft but formed; grade 3, thick liquid; grade 4, opaque watery; and grade 5, rice water). The total diarrheal stool volume was determined, including loose stools after doxycycline administration.

Treatment. Any volunteer who developed diarrhea after challenge received an oral glucose-electrolyte solution in a volume of 1.5 ml for each ml of diarrheal stool to prevent dehydration. When clinically indicated, intravenous Ringer's lactate was given, in a quantity matching the diarrheal stool volume. In vaccination as well as in challenge studies, volunteers received 300 mg of doxycycline at day 5 after inoculation and were released from quarantine after the third negative coproculture.

Definitions. Diarrhea was defined as the passage of two or more loose stools of at least 200 g (grades 3 to 5) within 48 h or a single loose stool of 300 g or greater. The standard definition of a cholera case was used, i.e., diarrheal losses produced by infection with a virulent strain producing a positive stool culture for O1 *V. cholerae*. Reactogenic diarrhea was defined as the occurrence of diarrhea after inoculation of a *V. cholerae* CTXΦ deletion mutant.

Study design to evaluate the safety of *V. cholerae* 81. A randomized, double-blind, placebo-controlled study was planned. Thirteen subjects were assigned to the vaccine group, and four were assigned to the placebo group. The vaccine consisted of 10^9 CFU of *V. cholerae* 81 buffered with 30 ml of 1.3% $NaHCO_3$; the placebo was 1.3% $NaHCO_3$ alone. Preparation of the inoculum, administration, clinical surveillance, and treatment were performed as indicated above.

Immunization phase to test the protective capacity of *V. cholerae* 638. A total of 45 volunteers were assigned at random and in a double-blind manner to receive, with 1.3% $NaHCO_3$, a single oral dose of strain 638 (10^9 CFU) ($n = 24$) or placebo ($n = 21$). Preparation of the inoculum, administration, clinical surveillance, and treatment were performed as indicated above. Specimens for excretion and immune response evaluations were obtained and analyzed as described below.

Attenuated challenge. At 1 month after inoculation, five subjects from the vaccine group and five from the placebo group were readmitted to the isolation ward for challenge with 10^9 CFU of *V. cholerae* 81. Inoculum administration and clinical surveillance and treatment were done as described above. Specimens for bacteriological and serological analyses were taken and analyzed as described below to evaluate protection against reinfection afforded by vaccination with *V. cholerae* 638.

Virulent challenge. At 1 month after vaccination, 12 of the subjects vaccinated with 638 and 9 of the placebo recipients were admitted again to the isolation ward to be orally challenged with 7×10^5 CFU of fully virulent El Tor Ogawa strain 3008. Preparation of the inoculum, administration, clinical surveillance, and treatment were performed as indicated above. Specimens to detect the excretion of *V. cholerae* and for immune response evaluations were obtained and analyzed as described below.

Specimens. The first stool excreted on each day was collected and weighed, and 1-g aliquots were brought to the microbiology laboratory to establish bacteriological counts of shed vibrios. Sera were collected on days 0, 7, 14, 28, 35, and 42 relative to vaccine dosing to determine the titers of vibriocidal as well as immunoglobulin A (IgA) antibodies directed against Ogawa LPS. Venous blood samples were obtained on the day of each inoculation (day 0) and 7 days later to enumerate LPS-specific IgA antibody-secreting cells (IgA-ASC). Saliva samples were collected by stimulation of mastication on days 0 and 9 in each protocol phase, incubated at 56°C for 15 min, clarified by centrifugation at $9{,}000 \times g$ for 10 min, and kept frozen at −20°C until used.

Bacteriological analysis. The CFU of *V. cholerae* 638, 81, and 3008 excreted per g of stool were determined by dispersion of 1 g of each fecal specimen in 1 ml of 0.9% NaCl, serial dilution, and plating on thiosulfate-citrate-bile salts-sucrose (Merck) agar. The identity of the colonies was checked by agglutination

TABLE 1. Comparison of reactions of 24 subjects to a single oral dose of 10^9 CFU of *Vibrio cholerae* 638 and 21 volunteers receiving placebo

Parameter	No. (%) of volunteers experiencing reaction with the following grade in response to the indicated treatment:						P value[c]
	No reaction		Mild[a]		Moderate[b]		
	638 (n = 24)	Placebo (n = 21)	638 (n = 24)	Placebo (n = 21)	638 (n = 24)	Placebo (n = 21)	
Diarrhea	20 (84)	19 (90)	4 (16)	2 (10)			0.670
Gurgling	11 (46)	11 (53)	13 (54)	10 (47)			0.768
Abdominal cramps	17 (71)	15 (72)	7 (29)	6 (28)			1.000
Headache	18 (76)	18 (86)	5 (20)	3 (14)	1 (4)		0.701
Vomiting	24 (100)	21 (100)					
Fever	24 (100)	21 (100)					

[a] Short-duration reaction that did not interfere with the daily activities of the volunteers.
[b] Reaction that interfered with but did not impair the daily activities of the volunteers.
[c] For vaccine versus placebo recipients.

with specific antisera, resistance to polymyxin B, and expression of the *celA* marker gene (19).

Serological analysis. Vibriocidal antibody titers in sera were determined by using a microassay as previously reported with *V. cholerae* VC12 (classical Ogawa) as the target strain (1, 3). The titer was defined as the highest serum dilution that inhibited bacterial growth, as determined by visual color examination.

Anti-Ogawa LPS IgA antibodies in sera were determined by an enzyme-linked immunosorbent assay (ELISA) with *V. cholerae* C7258 Ogawa LPS (3) as the solid-phase antigen. The highest serum dilution, determined by interpolation, giving 0.4 unit of absorbance above the background was reported as the titer for each serum (2, 23).

Specific IgA-ASC were enumerated by a modification of the enzyme-linked immunospot assay (7). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation on Histopaque 1077 (Sigma Chemical Co., St. Louis, MO). Individual wells of MultiScreen-HA plates (Millipore, MA) were coated overnight at 4°C with *V. cholerae* Ogawa LPS (25 μg ml^{-1}), 0.5% bovine serum albumin (negative control), or anti-human IgA (positive control) diluted in phosphate-buffered saline (PBS). The plates were washed and filled with RPMI 1640 complete medium supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO) and gentamicin (50 μg ml^{-1}). This step was followed by incubation for 30 min at 37°C in a 7.5% CO_2 atmosphere at 37°C. A volume of 90 μl was withdrawn from each well and replaced with 50 μl of PBMC suspension (10^5 to 10^6 cells), and the plates were further incubated for 4 h. The imprints of specific ASC were developed with the addition of peroxidase-conjugated anti-human IgA and incubation overnight at 4°C. Spots were counted under low magnification, and the results were expressed as ASC per 10^6 PBMC. Any increase in the ASC count at day 7 postdosing was considered a positive response.

Salivary IgA to Owaga LPS was determined by an ELISA with 96-well plates (MaxiSorp F96; A/S Nunc, Roskilde, Denmark) (13). Polystyrene plates were coated at 4°C for 16 h with Ogawa LPS at 25 μg ml^{-1} in PBS (pH 7.2). Nonspecific binding sites were blocked with a solution containing 3.0% nonfat dry milk dissolved in PBS. Saliva from volunteers and reference samples were added and incubated for 2 h. Peroxidase-conjugated goat anti-human IgA (Sigma Chemical Co., St. Louis, MO) was added, and the plates were further incubated for 1 h and developed with *o*-phenylenediamine (Sigma Chemical Co., St. Louis, MO). Absorbance readings were obtained with a microplate reader at 492 nm. A twofold or greater increase in optical density was considered positive.

Statistical processing of the data. Statistical analysis was done by using Statistic software (22). The rates of occurrence of adverse reactions among vaccine and placebo groups were compared by using the Fisher exact test (FET). The Mann-Whitney U test was used to compare the numbers and grades of diarrheal episodes in vaccine and placebo recipients as well as the fecal excretion of *V. cholerae* 3008 in vaccine and placebo recipients who underwent challenge. Prechallenge and postchallenge antibody titers in the vaccine group were compared by the Wilcoxon matched-pairs test. The seroconversion rates among placebo and vaccine groups after challenge with 3008 were compared by using the FET. Challenge studies with 3008 were designed to have 80% power to detect an 80% protective efficacy against cholera by using a two-sided analysis with a P value of 0.05.

RESULTS

Bacteriological response to *V. cholerae* 81. *Vibrio cholerae* 81 is a CTXΦ deletion mutant of *V. cholerae* C7258 (El Tor Ogawa, Peru, 1991) (3). Thirteen volunteers ingested a single $NaHCO_3$-buffered dose of 10^9 CFU of 81 to assess strain safety. Another group of 4 control volunteers ingested $NaHCO_3$ as placebo. All subjects ingesting 81, but none of the 4 controls ingesting placebo, got infected and tested positive for *V. cholerae* in all fecal cultures done on thiosulfate-citrate-bile salts-sucrose in the 5 days that followed administration. The CFU counts excreted per g of feces had a geometric mean of 1.3×10^5 and lower quartile (LQ) and upper quartile (UQ) at 1×10^4 and 1×10^6 CFU g^{-1}, respectively. We conclude that strain 81 can colonize the intestinal tract.

Clinical response to *V. cholerae* 81. No volunteer had severe symptoms. Reactogenic diarrhea (grade 3) was observed in six (46%) out of the 13 volunteers ingesting 81; 3 were mild and 3 were moderate episodes. They were distributed as follows: One volunteer had 13 episodes (total volume [V], 1.6 liters), one had 8 events (V, 2.0 liters), one had 6 (V, 1.3 liters), one had 3 (V, 0.5 liter), one had 2 (V, 0.5 liter) and the other had a single episode of 0.3 liter. In addition, two moderate and one mild cases of vomiting were recorded. Also some volunteers had reactions like mild gurgling (23%), abdominal cramps (23%) and headache (30%). In the placebo group, only one among four subjects experienced mild diarrhea (two grade 3 events, V, 0.4 liter) and abdominal cramps. We conclude that strain 81 induces a high rate of undesired symptoms and would require more attenuation for use in humans. Thus, its inclusion in a cholera vaccine preparation is not recommended. However, the strong colonizing ability of 81, as well as the profile of side effects it induces, could make it an attractive and more convenient yet-to-be-tested surrogate for fully virulent wild-type agents in the volunteer challenge model for studies of protective efficacy against infection by *V. cholerae*.

Volunteer studies with *V. cholerae* 638. The studies were designed to test the capacity of a single oral dose (10^9 CFU) of freshly harvested 638 to confer short term protection against infection and disease provoked by a subsequent challenge with the virulent strain *V. cholerae* 3008. Assignment to receive vaccine or placebo was done with independence to blood group; however, subjects of the O group distributed evenly, 46% to vaccine and 41% to placebo.

Clinical response to *V. cholerae* 638. No subject had severe effects capable of impairing their daily activities. With the exception of one moderate episode of headache, the rest of the reactions seen were mild and distributed equally between the vaccine and the placebo groups (Table 1). The most important

TABLE 2. Clinical and bacteriological responses of volunteers in vaccine and placebo groups to challenge with *Vibrio cholerae* 81 or 3008

Parameter[a]	Value for the indicated subjects challenged with:			
	V. cholerae 81[b]		*V. cholerae* 3008[c]	
	Vaccine (n = 5)	Placebo (n = 5)	Vaccine (n = 12)	Placebo (n = 9)
Subjects shedding the challenge strain	2 (40)	5 (100)	3 (25)	9 (100)
Median CFU per gram of feces[(d)]	6×10^4	3×10^5	2.1×10^4	2.3×10^6
Lower quartile (LQ)	1×10^2	1×10^4	6.6×10^3	3.3×10^5
Upper quartile (UQ)	2×10^5	1×10^7	3.4×10^5	1.6×10^7
Median duration (days) of shedding (LQ − UQ[d])	2 (3–5)	5 (5–5)	0 (0–1)	4 (4–5)
Subjects with any diarrhea	0	3 (60)	0	7 (78)
Subjects with severe or moderate diarrhea (≥3 kg)	0	0	0	2 (22)

[a] Reported as number (percentage) unless otherwise indicated.
[b] Attenuated challenge. P values were 0.166 for subjects shedding the challenge strain and subjects with any diarrhea.
[c] Fully virulent challenge. P values were 0.001, 0.000, and 0.171 for subjects shedding the challenge strain subjects with any diarrhea, and subjects with severe or moderate diarrhea, respectively.
[d] Values were determined only for subjects who shed the challenge agents.

effect observed was mild diarrhea. It was seen in 4 of the 24 vaccinees and included one subject with 5 episodes (total purge, 780 g), two subjects with 3 episodes (total output, 520 g) and another subject with one episode (total, 300 g). The numbers of episodes per volunteer (P = 0.487) or their intensity rates (P = 0.643) did not differ significantly between vaccine and placebo recipients when compared by the Mann-Whitney U test. These results suggest that 638 may be included in a candidate cholera vaccine to undergo further phases of evaluation.

Fecal shedding of *V. cholerae* 638. Vaccine strain was detected in the feces of 21 of the 24 subjects (87.5%) that ingested the vaccine suspension. The incubation period to excretion had a median of 3 days (LQ, 2; UQ, 4 days) and only 3 volunteers excreted during all 5 days of surveillance. Among those who shed 638, the geometric mean was 7.3×10^4 CFU g^{-1} (LQ, 9.6×10^3; UQ, 4.3×10^5). The numbers of volunteers shedding 638 peaked on days 4 and 5. None of the placebo recipients excreted 638. These results concur with a previous report (3) that indicated that 638 effectively colonizes the intestinal tract of humans.

Immune response to *V. cholerae* 638. 96% of vaccinees developed a fourfold increase in the titers of vibriocidal antibodies against the indicator strain VC12 (serotype Ogawa). The geometric mean of reciprocal titers was 1,837 (range, 320 to 20,480) 14 days after inoculation. The LPS-specific IgA seroconversion rate was lower (50%) as measured in LPS-specific IgA ELISAs. The geometric mean of reciprocal titers at day 14 among subjects who seroconverted was 387 (range, 115 to 1,585). These results indicate that strain 638 primes the immune system and induce IgA-producing cells with specificity for the Ogawa LPS of *V. cholerae* as well as a systemic response of bactericidal antibodies. This immune status might be expected to afford protection against infection and disease in a subsequent challenge with wild-type *V. cholerae*.

Clinical and bacteriological responses to challenge. All 45 subjects who participated in the immunization phase (21 placebo and 24 vaccine recipients) were available for the challenge phase one month later. Among them, 5 subjects from the vaccine and 5 from the placebo group were randomly selected for an exploratory challenge with 10^9 CFU of the attenuated strain *V. cholerae* 81. Additionally, 12 vaccine and 9 placebo recipients were randomly selected and underwent challenge with 7×10^5 CFU of the fully virulent El Tor Ogawa strain 3008.

In the exploratory study, all five subjects from the placebo group got infected and excreted strain 81 in their feces during the 5 days that followed oral ingestion (Table 2). The median CFU counts per g of feces was 3×10^5, with LQ and UQ at 1×10^4 and 1×10^7 CFU g^{-1}, respectively. In contrast, only two subjects from the vaccine group shed *V. cholerae* 81 in their feces (Table 2). One of them had a single episode of fecal shedding, while the other excreted during all 5 days. The median CFU counts per g of their feces was 6×10^4, with LQ and UQ at 1×10^2 and 2×10^5 CFU g^{-1}, respectively. Additionally, none of the vaccinees but 3 out of five placebo recipients experienced reactogenic diarrhea after ingestion of *V. cholerae* 81. The mean diarrheal volume was 373 ml (range, 300 to 480 ml). These results suggested that vaccination with 638 protected subjects against a subsequent challenge dose of $\sim 10^9$ CFU of *V. cholerae* 81. Finally, ingestion of *V. cholerae* 81 did not boost the preexistent immune status of vaccinees as detected with tests for vibriocidal antibodies or anti-LPS IgA (not shown). However, this strain elicited seroconversion in all subjects from the placebo group.

In the fully virulent challenge, the dose of *V. cholerae* 3008 used has been reported to infect 100% of volunteers, causing diarrhea in 80% of them (25). As indicated in Table 2, among the placebo group 100% of the 9 volunteers excreted the challenge agent (median CFU per gram of feces, 2.3×10^6 CFU g^{-1}; LQ, 3.3×10^5; UQ, 1.6×10^7), while only 3 of the 12 vaccinees did (P value determined by FET, 0.001). The counts of vibrios excreted (median CFU per gram of feces, 2.1×10^4 CFU g^{-1}; LQ, 6.6×10^3; UQ, 3.4×10^5) in the vaccine group differed significantly ($P < 0.05$) when compared with the placebo recipients by the Mann-Whitney U test. Also, challengees of the placebo group shed *V. cholerae* 3008 during a mode of 4 days (LQ, 4; UQ, 5), while those of the vaccine group did during a modal value of 0 days (LQ, 0; UQ, 1). Seven members of the placebo group (77.7%) that shed strain 3008 had diarrhea and were consequently counted as cholera cases, while none of the 12 vaccinees had any diarrhea. Two out of seven subjects with diarrhea were severe cases having total outputs greater than 5 liters and required intravenous rehydration. The

TABLE 3. Systemic immune response to challenge with *V. cholerae* 3008 for subjects in vaccine and placebo groups

Group	Response rate and geometric mean reciprocal titer (range) for:							
	No. responding[a]/ total no. tested	Vibriocidal antibodies			No. responding[c]/ total no. tested	LPS-specific IgA in serum		
		Day 0[b]	Day 7	Day 14		Day 0[b]	Day 7	Day 14
Vaccine	0/12	381 (80–2,560)	349 (160–1,280)	226 (80–640)	7/12	59 (<50–228)	148 (<50–489)	104 (<50–234)
Placebo	9/9	23 (20–80)	806 (160–5,120)	1,382 (160–5,120)	7/9	<50 (<50–<50)	195 (<50–2,089)	395 (<50–4,265)

[a] With increases of fourfold or more.
[b] Challenge day before ingestion of the inoculum of 3008, 1 month after vaccine (638) or placebo intake.
[c] With increases of twofold or more.

mean diarrheal stool volume excreted by challengees of the placebo group was 2,231 ml (range, 330 to 5,731 ml), with a mean number of diarrheal episodes of 14 (range, 3 to 34) and a mean peak stool excretion of vibrios of 2.3×10^6 CFU ml^{-1}. These results indicate that Cuban volunteers are susceptible to infection, symptoms, and disease provoked by strain 3008 and that the vaccine recipient group was protected by immunization with 638.

Immune response to virulent challenge. Serum samples taken at the challenge day and one and two weeks postchallenge were analyzed for vibriocidal antibodies and for LPS-specific IgA. All nine challengees of the placebo group but none of the 12 challengees of the vaccine group responded with fourfold increases in titers of vibriocidal antibodies (*P* value determined by FET, 0.000) (Table 3). However, serum IgA anti-LPS levels increased by twofold or more after challenge in 7 of 12 subjects (58%) from the vaccine group and in 7 of 9 (78%) from the placebo group (*P* value determined by FET, 0.3235). At day 7 postchallenge, titers did not differ significantly among groups (GMT of 148 versus GMT of 195; *P* value determined by the Mann-Whitney U test, 0.7543) but peak titers at day 14 postchallenge differed significantly when compared by the same test (GMT of 104 versus GMT of 395; *P*, 0.0491). At day 21 postchallenge, titers were returning to baseline levels and were not statistically different in both groups (not shown).

IgA-ASC against the Ogawa LPS were evaluated at days 0 and 7 (Table 4). All challengees of the vaccine group had slight increases in the numbers of LPS-specific IgA-ASC (15; range, 4 to 35), while all those of the placebo group had prominent increases (1,063; range, 35 to 2,600) after the challenge day. Also, in the vaccine group, postchallenge titers did not reach those attained in the vaccination phase in any of the subjects, resulting in statistically significant differences in the primary responses induced by strain 638 (236; range, 10 to 1,500) and secondary responses evoked by strain 3008 in the same group (15; range, 4 to 35) (*P* value determined by the Wilcoxon matched-pairs test, 0.0009). Taken together, these results indicate that there was a modest secondary mucosal response to ingestion of strain 3008 that could be detected systemically by increases in anti-LPS IgA but not in titers of vibriocidal antibodies.

Salivary IgA antibodies against the Owaga LPS were evaluated at days 0 and 9 (Tabla 4). Postchallenge responses of IgA salivary antibodies against the Ogawa LPS were seen in 8 of 9 (89%) challengees of the placebo group but not in any of 12 volunteers of the vaccine group.

DISCUSSION

This study demonstrates that a single oral dose containing 10^9 CFU of freshly cultured *V. cholerae* 638 is well tolerated, immunogenic and able to afford short-term protection (at 1 month) against cholera in a volunteer model that uses strain *V. cholerae* 3008 as the challenge agent. This model has been previously used at the Center for Vaccine Development (Maryland) to assess protection conferred by El Tor Ogawa strain CVD111 (25). Clinical and bacteriological responses to *V. cholerae* 3008 in challengees of the placebo group in the present study were similar to those seen by Tacket et al. (25), in unimmunized North American volunteers. The attack rate of diarrhea in North Americans was 7 of 8, while in Cuba it was 7 of 9; the mean diarrheal stool volume in North Americans was 2,534 ml (984 to 7,703), and in Cuba it was 2,231 ml (330 to 5,731). The mean number of diarrheal stools was 14 in both studies, ranging from 6 to 46 in the United States and from 3 to 34 in Cuba. The mean peak stool excretion levels were 9.3×10^6 CFU/g in Maryland and 2.3×10^6 CFU/g in Cuba. In both studies, all nonvaccinated volunteers exposed to 3008 seroconverted with vibriocidal antibodies with mean peak reciprocal titers of 2,177 (United States) and 2,240 (Cuba). We interpret from this analysis that Cuban volunteers are similarly susceptible to infection and cholera produced by wild-type strain 3008.

In this model of challenge with strain 3008, *V. cholerae* 638 provided significant protection against fecal shedding of the challenge agent and complete protection against cholera (any diarrhea) (Table 2). Protection against severe and moderate cholera could not be rated since only two subjects in the pla-

TABLE 4. LPS-specific salivary IgA and IgA-ASC in volunteers challenged with *Vibrio cholerae* 3008

Group	Mean no. of ASC/10^6 PBMC (range) and response rate			Mean optical density in salivary IgA ELISA (range) and response rate		
	Day 0[a]	Day 7	No. responding/no. tested	Day 0[a]	Day 9	No. responding/no. tested
Vaccine	0 (0–0)	15 (4–35)	12/12	0.21 (0.11–0.39)	0.27 (0.15–0.48)	7/12
Placebo	0 (0–0)	1,063 (35–2,600)	9/9	0.23 (0.107–0.754)	0.49 (0.24–0.689)	7/9

[a] Challenge day, 1 month after vaccine or placebo intake.

cebo group met the definition of severe cases. As an additional criterion for protection, challenge did not boost the titers of vibriocidal antibodies in the vaccinees but induced 100% seroconversion in challengees of the placebo group (Tables 3 and 4). These results agree with previous data in which the titers of vibriocidal antibodies of volunteers orally inoculated with the live *V. cholerae* O1 vaccine strain CVD 103-HgR were not significantly increased after second administration of the same strain one year later (27) or after challenge with fully virulent strains of *V. cholerae* within 4 months (17).

In contrast, the counts of IgA-ASC in all volunteers from the vaccine group had slight but detectable postchallenge increases (Table 4). Since the challenge agent was not fecally shed by most of the vaccinees (75%), this likely represents a limited process of antigen sampling at mucosal priming sites during gut passage of the challenge agent that lead to low responses. These results contrast with previous findings (27) in which volunteers primed with CVD103-HgR and boosted 418 days later with the same strain did not respond with IgA-ASC following reexposure. One important difference here is that reexposure was done with the wild-type challenge agent *V. cholerae* 3008 and not with an attenuated strain. Because of the lack of extensive work addressing this topic, it merits further analysis in future studies. In correspondence with the increase in IgA-ASC in blood, the level of circulating anti-LPS IgA also increased by 2-fold or greater in 7 of 12 challengees of the vaccine group (Table 4).

The protection afforded by vaccination with 638 was accompanied by a 96% seroconversion rate for vibriocidal antibodies, the best indirect marker of protection. Equally, high percentages of specific IgA-ASC (100%) and salivary IgA (75%) producing individuals were detected among the vaccinees. However, the numbers of specific IgA-ASC were higher after feeding the virulent strain than after immunization with attenuated strain 638 (mean numbers of ASC per 10^6 PBMC, 1,063 and 248, respectively). This may result from the positive effect exerted by cholera toxin on the immune response to *V. cholerae* 3008 or from the level of attenuation attained during construction of 638.

The immune response to vaccination with 638 resulted from colonizing activity of the attenuated agent as revealed by detection of fecal shedding of vibrios. Shedding did not begin the day after vaccination in all volunteers, which led to a median incubation period to excretion of 3 days. This result contrasted with the one obtained for the progenitor strain *V. cholerae* 81 and the toxigenic challenge strain 3008 for which the incubation period to shedding was 1 day in all volunteers, and most subjects kept sustained excretion for the entire 5-days follow-up period. It is not completely clear whether the longer incubation period to excretion seen with 638 in humans is due to the presence of an inactivated *hapA* gene and the consequently lower detachase activity (3, 10) or due to an occult spontaneous mutation that may have occurred during construction of strain 638. The wild-type *hapA* gene has been restored to strain 638 in order to test the effect of Hap on safety and vibrio excretion in volunteers (unpublished data).

Strain 638 induced systemic responses of vibriocidal antibodies in 96% of the volunteers without inducing relevant adverse events. These results are in agreement with previous volunteer studies that addressed the safety and immunogenicity of the attenuated strain (3). Here, the most important symptom elicited was grade 3 diarrhea in 4 (16%) of 24 vaccinees in comparison with 2 (10%) of 21 placebo recipients, which resulted in nonsignificant differences in the numbers of episodes or their intensity rates. In contrast with strain 638, its progenitor strain, *V. cholerae* 81 induced diarrhea in 46% of the subjects. Thus, the safety of strain 638 is not a common feature in all C7258 derivatives, but an inherent attribute of strain 638, presumably due to inactivation of *hapA* with the marker gene *celA*.

In this work, we exploited the high rates of fecal shedding and reactions induced by strain 81 to make an exploratory challenge with this attenuated agent to subjects vaccinated with strain 638. Our findings indicated that vaccinees with strain 638 were protected from infection and symptoms elicited by 81 and allowed us going into the wild-type challenge studies. This small study is of interest, since in the future one could consider using an attenuated but still colonizing and reactogenic mutant as a more convenient challenge strain to measure protection to infection without the need to induce cholera in subjects of the placebo group. This would be possible because it is widely accepted, although not conclusively established, that protective immunity to cholera is mainly mediated by secretory IgA antibodies directed against bacterial antigens more than an antitoxic response (14, 15).

The results of the present study indicate that strain 638 is safe, immunogenic, and protective in healthy volunteers. Future studies with lots prepared under good manufacturing practices for clinical trials should address the protective capacity conferred by 638 3 months and later after vaccination of volunteers. Subsequent field studies should evaluate whether the final vaccine preparation is protective in areas of endemicity.

ACKNOWLEDGMENTS

We are indebted to Richard A. Finkelstein for presenting these results at the 38th USA-Japan Joint Panel Meeting on Cholera and Other Related Enteric Infections at the National Institutes of Health, Bethesda, MD, 8 to 10 October 2003, and for suggesting revisions of the manuscript. We thank James B. Kaper and Carol O. Tacket for providing *Vibrio cholerae* 3008, the challenge strain used in this work. We also appreciate the technical assistance of Arlenis Moreno and Daily Serrano. We are indebted to Luis Izquierdo and Marlene Armesto for randomization of the subjects and mathematical processing of the data as well as to Laura Bravo for screening of *V. cholerae* excretion. We also thank the infirmary staff of the isolation ward at the Institute for Tropical Medicine Pedro Kouri for their professional work. We especially acknowledge the voluntary participation of all of the subjects enrolled in the trial.

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Editor: V. J. DiRita