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Protective Efficacy in Humans of Killed Whole-Vibrio Oral Cholera Vaccine with and without the B Subunit of Cholera Toxin

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Natural protection from cholera is associated with local intestinal antibacterial and antitoxic antibodies, which appear to act synergistically. Although current parenteral cholera vaccines offer insufficient protection, new vaccines administered orally have more promise. Killed *Vibrio cholerae*, alone or given with the B subunit of cholera toxin, was evaluated in adult volunteers. Vaccinees, who received three doses of either vaccine, and unvaccinated controls ingested 10^6 *V. cholerae* organisms to determine the protective efficacy of the vaccines. The combination vaccine provided 64% protection, and the whole vibrio vaccine given alone provided 56% protection. In addition, illnesses in vaccines were milder than those in controls, and both vaccines gave complete protection against more severe disease. This substantial level of protection against a dose of *V. cholerae* that caused cholera in nearly 90% of controls suggests that these vaccines might provide at least as high a level of protection if given to the population of an endemic area. Indeed, a field efficacy trial is underway in Bangladesh, and preliminary data indicate a protective efficacy of 85% for a killed whole vibrio plus B subunit vaccine similar to that tested in volunteers and an efficacy of 58% for the killed whole vibrio vaccine alone. Thus, the studies in human volunteers were successful in predicting the substantial protection afforded by the vaccines in a cholera endemic area.

Cholera confers lasting immunity against subsequent disease due to either the homologous or heterologous serotype of *Vibrio cholerae* (12, 14). In contrast, currently available killed whole vibrio or purified toxoid cholera vaccines given parenterally have not provided a similar level of protection when evaluated in field trials (5, 14, 22). Recent evidence indicates that the parenteral route is not optimal for immunizing against enteric infections (14). Furthermore, cholera stimulates both antibacterial and antitoxic antibodies in the intestine, and data from animal models suggest that these two responses act synergistically to result in enhanced protection (25).

Killed whole vibrios, given orally in multiple doses, were previously evaluated in adult volunteers and found to provide 61% protection against a challenge dose of *V. cholerae* that resulted in diarrhea in 80% of unvaccinated controls (1). Lipopolysaccharide (LPS) present in the killed vaccine probably served as a major protective antigen. In addition, other antigens of *V. cholerae*, such as outer membrane proteins (21) and hemagglutinins (HA) (9), may stimulate an immune response which contributes to protection. Since these antigens are heat labile, vibrios must be inactivated with Formalin or some other procedure, rather than heat, for better preservation of the antigens in vaccine preparations.

Cholera toxin is comprised of one enzymatically active A subunit and five B subunits, which are responsible for binding of the toxin to mucosal receptors (6; J. Holmgren, I. Lonnroth, O. Ouchterlony, and A.-M. Svennerholm, J. Gen. Microbiol. 73:29a, 1972). Purified B subunit, which eliminates the risk of reversion to toxicity previously seen with Formalin-treated toxoids but retains immunogenicity, is a logical toxoid immunogen for an oral cholera vaccine (7, 10). Antibodies to the B subunit are more active in neutralizing cholera toxin activity than antibodies to the A subunit (23). The B subunit may be particularly good as an oral immunogen because it is able to bind to the intestinal epithelium, which is important for stimulating mucosal immunity in animals (18). The B subunit has been shown to be more immunogenic than chemically inactivated toxoids, but still less immunogenic than cholera toxin (19).

A combined killed whole vibrio and B subunit oral cholera vaccine has been evaluated in adults in an endemic area (Bangladesh), and the mucosal antibacterial and antitoxic immune response has been determined (26). A single dose containing 2.5 mg of the B subunit and 5×10^{10} killed vibrios stimulated a local immunoglobulin A (IgA) antitoxin response and also in most of the volunteers a local IgA antibody response to the LPS of *V. cholerae*. Immunization induced responses, including memory responses, that were comparable to those induced by cholera. These promising findings, along with those from animal models, prompted us to evaluate in volunteers the protective efficacy of an oral killed whole vibrio vaccine, alone and in combination with the B subunit.

MATERIALS AND METHODS

Volunteers. Participants in these studies were healthy adults 18 to 35 years old from the Baltimore area who passed a medical and psychological screening and provided informed consent, as previously described (11–13).

Vaccines. The whole vibrio vaccine was composed of 5×10^{10} each of heat-killed classical Inaba (strain Cairo 48) and Ogawa (strain Cairo 50) and 1×10^{11} Formalin-treated El Tor Inaba (strain Phil 6973) *V. cholerae* organisms in 8 ml of phosphate-buffered saline (PBS). The Formalin-treated El

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Tor vibrios were included to provide heat-labile El Tor-associated antigens (e.g., the mannose-sensitive, cell-bound HA [9]) in the vaccine. The classical components of the vaccine were prepared as for the parenteral whole vibrio vaccine produced by the National Bacteriological Laboratory, Stockholm, Sweden. The two classical strains were grown to a high density in a fermentor, harvested by centrifugation, and inactivated by heating at 56°C for 30 min. Merthiolate was added to a concentration of 0.01%. The El Tor organisms were grown in a fermentor, centrifuged, and inactivated by 0.025 M formaldehyde at room temperature for 24 h and at 4°C for 48 h. The vaccine strains were diluted to proper concentrations and stored at 4°C until use.

Purified B subunit was prepared at the Institut Merieux, Lyon, France, by affinity chromatography purification with a lyso-GM_1 ganglioside column followed by gel filtration in acid buffer on a Sephadex column and dialysis against PBS buffer as previously described (10, 27). The B subunit preparation (M_r, ~58,500) was diluted to 1 mg/ml in PBS buffer, and one 5-ml vial was utilized for each dose.

Before use the vaccine preparations were tested for sterility, LPS antigen composition (8), cell-bound HA (9), and B subunit pentamers (10), lack of cholera toxin activity, and safety and protective immunogenicity in animals (24).

Immunization schedule. Volunteers received three oral doses at 2-week intervals. Three hours before ingesting the vaccine, they took 300 mg of cimetidine, and 1 min before vaccination they ingested 2 g of sodium bicarbonate in 150 ml of distilled water; both were taken to reduce gastric acid that might have otherwise damaged the vaccine. Volunteers fasted for 90 min before and after ingesting the vaccine dose. In one study the whole vibrio vaccine alone was ingested, and in the other it was taken in combination with 5 mg of B subunit.

Immune response. Sera were collected from all volunteers before and 13, 21, 28, 42, and 56 days after the first dose of vaccine. Jejunal fluid was collected by aspiration from polyvinylchloride intestinal tubes before and 12 and 28 days after the first dose of vaccine (before and 2 weeks after the second dose, respectively). Localization of the tubes in the jejunum (130 cm) was documented by the appearance of bile-stained fluid of pH 6.0 or above. Jejunal fluid (70 ml) from each volunteer was centrifuged (8,000 × *g*) to remove particulate material. Secretory (sIgA) was measured by radial immunodifusion (11, 16). Measured volumes of fluid were lyophilized in polystyrene tubes and later reconstituted to 20 mg of sIgA per 100 ml before testing for specific antibody.

For sera, vibriocidal antibody was measured by a microtiter technique in which a fourfold or greater increase in titer is significant (4). IgG cholera antitoxin was measured by an enzyme-linked immunosorbent assay (ELISA) as previously described (15). Antibodies to *V. cholerae* LPS were measured by an ELISA which used a pool of anti-IgG, anti-IgA, and anti-IgM conjugates (26). IgG antibodies to an outer membrane preparation (OMP) were measured by an ELISA (21).

Antibodies to the mannose-sensitive cell-bound HA, which was present on the Formalin-treated El Tor vibrios in the vaccine, were measured as follows. A crude preparation of the cell-bound HA was prepared from the *V. cholerae* El Tor strain, Phil 6973. Bacteria were cultured in Trypticase soy broth (BBL Microbiology Systems) for 7 h at 37°C with shaking; after centrifugation at 8000 × *g* for 30 min, the sediment was mixed for 1 min at a high speed in a Sorvall omnimixer. The supernatant obtained after repeated centrifugation of the mixed bacteria at 10,000 × *g* for 10 min was used as crude cell-bound HA. The preparation had a HA titer of 1/64 to 1/256 when titered against chicken erythrocytes (9). Chicken erythrocytes were coated with the HA by incubating the crude HA preparation diluted in Krebs-Ringer solution (pH 7.4) with washed chicken erythrocytes at 37°C for 1 h; 1 ml of crude HA was mixed with 50 μl of concentrated erythrocyte suspension. After incubation, the coated cells were washed twice in PBS, centrifuged at 600 × *g* for 5 min, and kept diluted in a small volume of PBS. The HA-coated erythrocytes, 50 μl of a 0.2% HA-coated cell suspension, were coupled to the inner surface of ELISA plates. Before use, the HA-erythrocyte-coated plates were washed twice in PBS and then incubated with a 1% bovine serum albumin–PBS solution at 37°C for 30 min, 120 μl per well, and then washed twice with PBS containing 0.05% Tween 20 (PBS-Tween). For titration of antibodies, sera were absorbed with chicken erythrocytes by incubating equal volumes of washed, packed erythrocytes and serum at 37°C for 60 min with intermittent shaking and then testing the absorbed sera serially diluted fivefold in PBS-Tween in the coated plates (0.1 ml per well). After incubation with serum at room temperature for 4 h, the plates were washed three times in PBS-Tween and then incubated with anti-human IgG alkaline phosphatase conjugate diluted in PBS-Tween (0.1 ml per well) at room temperature for 18 h. After the plates were washed three times in PBS-Tween, 100 μl of nitrophenylphosphate, diluted in 1 M ethanolamine buffer (pH 9.8) to a final concentration of 1 mg of nitrophenylphosphate per ml, was reacted in each well for 100 min. The enzyme substrate reactions were read spectrophotometrically with a Titertek Multiscan ELISA reader at 405 nm; titers were determined as the reciprocal of the interpolated dilution resulting in an optical density of 0.3 above background. Rabbit high titer immune sera against *V. cholerae* O1 LPS did not bind significantly (i.e., they did not when diluted 1/5 result in an optical density of >0.3 in the test) to the HA-coated erythrocytes, whereas rabbit sera against whole live El Tor vibrios or against crude mannose-sensitive, cell-bound HA gave high titers in the HA ELISA.

Antibodies against the fucose-sensitive cell-bound HA associated with classical vibrios (9) or antibodies against *V. cholerae* soluble HA were not assayed since these antigens were not present in the whole cell vaccine preparation given to volunteers.

For jejunal fluids, specific sIgA antibodies to cholera toxin (Schwarz/Mann, Orangeburg, N.Y.) and to OMP from El Tor Ogawa strain E7946 were measured in a microtiter ELISA (18). The endpoint titer was considered the last dilution that gave an optical density of >0.20 and was at least 0.05 optical density units below the preceding dilutions. Fourfold rises were considered significant. Also for jejunal fluids, specific sIgA antibodies to *V. cholerae* LPS (Ogawa and Inaba) were determined by ELISA as previously described (26).

Challenge studies. Vaccinated and unvaccinated volunteers were admitted to the isolation ward and were challenged with 2 × 10^6 El Tor Inaba *V. cholerae* (N16961) organisms. The challenge study to determine vaccine efficacy was done 4 weeks after completion of vaccination with the killed whole vibrio vaccine alone and 5 weeks after completion of vaccination with the combination whole vibrio and B subunit. Sera were collected from all volunteers before and 10, 21, and 28 days after ingestion of *V. cholerae*. Jejunal fluids were collected before and 8 days after challenge.

TABLE 1. Immunologic response of adult volunteers after ingestion of three doses of killed whole vibrio vaccine with or without B subunit

Vaccine	No. of vaccinees	No. (%) with significant rise in serum antibodies to:					No. (%) with significant rise in intestinal sIgA to:		
		Vibrios	LPS	OMP	HA	Toxin	LPS	OMP	Toxin
Whole vibrio-B subunit	19	17 (89)	13 (68)	11 (58)	6 (32)	19 (100)	10 (53)	0 (0)	14 (74)
Whole vibrio	14	10 (71)	8 (57)	8 (57)	6 (43)	0 (0)	0 (7)	0 (0)	0 (0)

The challenge inocula were prepared as previously described (2) and were given with 2.0 g of sodium bicarbonate to neutralize gastric acid; volunteers fasted for 90 min before and after ingesting the vibrios. Volunteers were questioned daily about relevant symptoms for 4 days after challenge before receiving the 5-day course of oral tetracycline (500 mg every 6 h). All stools were collected in plastic pans that fit on the commode, graded for consistency, and weighed. Diarrhea was defined as passage of two or more loose stools within 48 h with a least 200 ml of total volume or passage of a single loose stool of 300 ml or greater.

Volunteers with diarrhea took oral glucose-electrolyte solution to maintain hydration. Ill volunteers also received tetracycline 24 h after the onset of diarrhea. For a small proportion of illnesses, intravenous fluids were required to maintain hydration; in such cases, volunteers were immediately treated with tetracycline.

Bacteriology. All stools (if no stool was passed in a 24-h period, a rectal swab was obtained) were plated directly onto thiosulfate-citrate-bile salts-sucrose agar and inoculated into alkaline peptone water and sodium-gelatin phosphate enrichment broths. After 18 to 24 h the enrichment broths were subcultured onto thiosulfate-citrate-bile salts-sucrose agar (20). Quantitative cultures were obtained by diluting 1.0 g (or 1.0 ml) of stool serially 10-fold in PBS (pH 7.2), inoculating 0.1 ml onto plates of thiosulfate-citrate-bile salts-sucrose agar, and counting colonies. Suspicious colonies were confirmed as *V. cholerae* and serotyped by agglutination with Ogawa or Inaba typing sera (kindly provided by Harry Smith, Jr., Vibrio Reference Laboratory, Jefferson Medical College, Philadelphia, Pa.).

Statistical analyses. Antibody titers and quantitative counts of *V. cholerae* in stool were compared by Student's *t* test. Protection in challenge studies was determined by Fisher's Exact Test and Student's *t* test.

RESULTS

Killed whole vibrio-B subunit vaccine. Nineteen volunteers received three oral doses of the combination vaccine without adverse reactions; 17 (89%) had significant rises in serum vibriocidal antibody levels, and 13 (68%) had rises in antibodies to *V. cholerae* LPS (Table 1). Eleven (58%) of the vaccinees had a significant increase in antibodies to the outer membrane preparation, whereas six (32%) had a >2-fold rise in antibodies against the mannose-sensitive, cell-bound HA of *V. cholerae*. All had rises in serum IgG ELISA antitoxin, and substantial antitoxin titers persisted until day 56, the time of challenge.

Ten (53%) volunteers manifested significant rises in sIgA anti-LPS antibody in intestinal fluids; eight of these responses were to both Ogawa and Inaba LPS, and two were only due to Ogawa LPS. Five of the rises occurred by day 13 after the first dose, and five occurred by day 28 (14 days after the second dose). By day 56 (time of challenge), 6 of the 10 still had elevated titers. Fourteen volunteers had rises in sIgA antitoxin; nine rises occurred by day 13, and the remainder occurred by day 28. By day 56, all sIgA antitoxin titers had returned to baseline levels. None of the volunteers developed a rise in sIgA to *V. cholerae* OMP.

Eleven vaccines and seven unimmunized controls were challenged with 2×10^6 *V. cholerae* organisms. Whereas all seven controls developed cholera, only four vaccinees became ill, indicating a vaccine efficacy of 64% (Table 2). The severity of diarrhea in the ill vaccines was significantly less than that in controls, based on a comparison of stool volumes and numbers of diarrheal stools (Table 2). Furthermore, vaccinees were completely protected from more serious disease; no vaccinee had diarrhea exceeding 2.0 liters, whereas four of seven controls had diarrheal stool volumes of 2.0 liters or more ($P = 0.01$). Ten of the 11 vaccinees had positive coprocultures, as did all of the controls. The mean peak level (highest number of organisms) of excretion for each infected volunteer was 3.2×10^6 *V. cholerae* organisms per g of stool for vaccinees and 1.0×10^8 organisms per g for controls ($P < 0.05$).

There was no clear-cut relationship between any of the serum or local antibody responses after immunization or antibody levels before challenge and protection from illness. After challenge with *V. cholerae*, the 11 vaccinees had serum antibody rises by vibriocidal (82%), anti-OMP (82%), anti-LPS (55%), anti-HA (29%), and antitoxin (36%) assays. The seven controls likewise had antibody rises by vibriocidal (86%), anti-OMP (100%), anti-LPS (86%), anti-HA (57%),

TABLE 2. Clinical response of vaccinees and controls to challenge with 2×10^6 El Tor Inaba *V. cholerae* organisms

Immunization status	No. of volunteers	No. with diarrhea	Mean incubation period (h)	Diarrheal stool vol (liters)		Mean no. of diarrheal stools
				Mean	Range	
Controls	7	7[a]	28.8	3.5[b]	0.3–7.7	13.8[c]
Whole cells-B subunit	11	4[a]	48.6	0.7[b]	0.4–1.0	6.8[c]
Controls	8	6[d]	27.0	2.1[e]	0.9–3.4	9.8[f]
Whole cells	9	3[d]	23.0	0.9[e]	0.5–1.4	6.0[f]

[a] $P = 0.01$ (Fisher's exact test).
[b,c] $P < 0.05$ (*t* test).
[d] $P = 0.11$ (Fisher's exact test).
[e,f] $P < 0.05$ (*t* test).

and antitoxin (86%) assays. None of the vaccinees and only one of five controls tested had a rise in sIgA in jejunal fluid to cholera toxin after the challenge.

Killed whole vibrio vaccine. Fourteen volunteers ingested three oral doses of killed whole vibrio vaccine without adverse reactions. Ten (71%) of them developed fourfold or greater rises in serum vibriocidal antibodies, and eight (57%) had a rise with each of the anti-LPS and anti-OMP assays; six (43%) had a rise against the HA (Table 1). Only one of 13 had a significant increase in local sIgA anti-LPS antibody (to both Ogawa and Inaba) by day 34 after the first vaccine dose.

Nine vaccinees and eight controls participated in the challenge study. Three vaccinees became ill, as did six controls, suggesting a vaccine efficacy of 56%. Although this vaccine efficacy was not appreciably different from the 64% observed with the combination vaccine, the lower attack rate in controls precluded the possibility of statistical significance. Since the two challenge studies utilized the same *V. cholerae* strains, dose, and method of administration, the efficacy of the combination and killed whole vibrio vaccines was also calculated by using the pooled controls from both studies. By this analysis, both vaccines had a protective efficacy of approximately 60% (both $P < 0.025$).

In recipients of the killed whole vibrio vaccine, the severity of diarrhea was significantly less than in the controls, based on stool volume and frequency. As with the combined vaccinees, whole vibrio vaccinees were completely protected from illnesses having 2.0 liters or more of diarrheal fluid loss compared with controls, of whom three had diarrhea of this severity ($P = 0.08$). Seven of the vacinees had positive coprocultures, as did all eight controls. The mean peak number of *V. cholerae* organisms per g of stool was 9.9×10^5 in vaccinees and 5.1×10^7 in controls ($P < 0.05$).

There was again no apparent relationship between serum or local antibody responses or titers before challenge and protection from illness. After challenge, the nine vaccinees had serum titer rises by vibriocidal (56%), anti-OMP (56%), anti-LPS (33%), anti-HA (33%), and antitoxin (67%) assays. The eight controls had antibody rises by vibriocidal (75%), anti-OMP (50%), anti-HA (50%), and antitoxin (75%) assays.

DISCUSSION

Both the oral killed whole vibrio vaccine alone and the combination whole vibrio and B subunit vaccine were shown to be completely safe in U.S. adult volunteers. The combination vaccine provided substantial (64%) protection against a challenge dose of *V. cholerae* that caused illness in all of the unvaccinated controls. The whole vibrio vaccine given alone provided 56% protection, not significantly lower in these studies than the combination vaccine. Futhermore, with both vaccines, the illnesses that did occur in vaccinees were significantly milder than those in controls.

The killed vibrios given orally in three doses, alone or in combination, stimulated fourfold or greater serum vibriocidal antibody responses in 71 and 89% of volunteers, respectively. The geometric mean titers after oral vaccination were, however, notably lower than those seen after cholera (2) or immunization with parenteral vaccines (4). Serum vibriocidal antibody levels have been correlated with protection from illness in Bangladesh, an endemic area for cholera (17). Additionally, the whole cell vaccine stimulated a serum immune response to *V. cholerae* LPS in more than half of each group of vaccinees. Half of the vaccinees with the combination vaccine also had a rise in sIgA antibodies to LPS, but inexplicably only one of 14 recipients of the whole vibrio vaccine alone had a local immune response. This vaccine also appears to have stimulated serum antibodies to OMP and HA, but local antibodies to OMP could not be demonstrated. It appears that the vaccine had an antibacterial effect since the peak excretion of *V. cholerae* in infected vaccinees was 30- to 50-fold lower than in infected controls.

The B subunit component of the oral combination vaccine stimulated a serum antitoxin response in all of the vaccinees and an intestinal response in 74%. These levels of serum and local antibody response to the vaccine approach those stimulated by cholera itself in previous volunteer studies (14). With the vaccine, as with cholera, local antitoxin responses were short lived, returning to baseline levels after 2 months.

The mechanisms of protective immunity are unknown, but it is likely that local intestinal IgA is a critical component. There is evidence that antibacterial and antitoxic antibodies work synergistically in animal models. Due to the limitations of the volunteer model (small number of individuals and variability in response to challenge dose) it was not possible to demonstrate synergy in these studies, although the combination vaccine appeared to result in somewhat enhanced protection in comparison to the whole vibrio vaccine alone. Cimetidine was used to reduce gastric acid and avoid possible damage to the ingested vaccines. Cimetidine also has immunomodulating effects, including demonstration in in vitro and animal models of increased cell-mediated cytotoxicity, proliferative responses to mitogens and antigens, and enhanced antibody production and some evidence in humans of enhanced delayed hypersensitivity. It is unknown whether cimetidine affected the immune response or protection from these oral cholera vaccines.

The ultimate test of the level of protection afforded by the whole vibrio vaccine, alone or with B subunit, is a field trial for efficacy in a cholera endemic area. Such a trial, now underway in Bangladesh, is evaluating vaccine preparations similar to those studied in volunteers; the B subunit dose used was 1 mg rather than 5 mg, and the whole vibrio vaccine contained Formalin-killed *V. cholerae* cells of the Ogawa classical biotype in addition to the components in the vaccine evaluated in volunteers. Preliminary results from this trial indicate a 6-month protective efficacy against cholera of 85% for the combined vaccine and 58% for the killed-whole vibrio vaccine alone (3). Thus, the volunteer challenge studies were successful in predicting vaccine efficacy in an endemic area and appear to be a useful model for the evaluation of candidate cholera vaccines.

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