INFECTION AND IMMUNITY, Feb. 1984, p. 515-522
0019-9567/84/020515-08$02.00/0

Vol. 43, No. 2

Evaluation in Humans of Attenuated *Vibrio cholerae* El Tor Ogawa Strain Texas Star-SR as a Live Oral Vaccine

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Received 16 August 1983/Accepted 8 November 1983

Texas Star-SR, an A^-B^+ mutant derived by nitrosoguanidine treatment from *Vibrio cholerae* El Tor Ogawa strain 3083, was fed to 68 volunteers as an oral vaccine in doses of 10^5 to 5×10^{10} organisms with $NaHCO_3$. Sixteen (24%) vaccinees experienced some loose stools (unrelated to vaccine dose), but in only one did the total stool volume exceed 1.0 liter. The vaccine strain was cultured from duodenal fluid of 35 of 46 (76%) persons who ingested doses of 10^8 organisms or greater. No A^+B^+ toxinogenic revertants were found among 456 clinical isolates tested. Sixty-three vaccinees (93%) manifested seroconversions of vibriocidal antibody, whereas only 20 (29%) had significant rises in serum antitoxin titers. Paired intestinal fluids from 41 volunteers showed significant rises of secretory immunoglobulin A against lipopolysaccharide (29%), Ogawa outer membrane preparation (29%), and toxin (12%) antigens. In challenge studies with pathogenic *V. cholerae* El Tor Ogawa and El Tor Inaba, the attack rate in vaccinees (7 of 25) was significantly lower than in controls (18 of 25) (vaccine efficacy, 61%); furthermore, the diarrheal stool volume in vaccinees was significantly less than that in controls ($P < 0.01$). Texas Star-SR served as a prototype to investigate the concept of immunoprophylaxis by means of attenuated strains as oral vaccines. These observations provide an invaluable background for planning future studies with newly developed attenuated strains prepared by recombinant DNA techniques.

Cholera vaccines which have been tested in the past two decades, including parenterally administered killed whole cell preparations and parenteral and enteral toxoids (7–10, 17, 28, 38), have generally demonstrated incomplete protection of relatively short duration. In contrast, studies in North American volunteers have shown that a single clinical infection with *Vibrio cholerae* provides 90 to 100% protection against rechallenge with cholera vibrios of either the homologous or heterologous serotype (4, 21, 23, 25, 26, 28). This infection-induced immunity persists for at least 3 years, the longest duration tested (23). Epidemiological data from Bangladesh, an endemic area, confirm the potency and persistence of acquired immunity (13, 33).

The greatly diminished isolation of *V. cholerae* from stool or jejunal fluid cultures and the low rate of seroconversions of serum immunoglobulin G (IgG) or intestinal secretory IgA (SIgA) antitoxin in rechallenged volunteers imply that antibacterial immune mechanisms impeded proliferation of *V. cholerae* before they could elaborate and release significant quantities of cholera enterotoxin (4, 21, 23, 25, 26, 28). These observations give promise that one successful approach toward immunoprophylaxis of cholera may be by means of attenuated, non-enterotoxigenic *V. cholerae* strains utilized as oral vaccines.

In 1979, Honda and Finkelstein (16) described Texas Star-SR, an attenuated *V. cholerae* strain that was derived by chemical mutagenesis from El Tor Ogawa 3083, an enterotoxinogenic strain highly adherent for rabbit intestinal mucosa (35). In contrast with the parent 3083 strain, Texas Star-SR elaborates only the nontoxic immunogenic B (binding) subunit oligomer (choleragenoid) of cholera enterotoxin but not holotoxin. In animal studies, Texas Star-SR was nonpathogenic, genetically stable, and conferred protection against experimental challenge (3, 16). Accordingly, clinical studies were undertaken in humans to explore the feasibility of immunoprophylaxis of cholera by use of this attenuated strain as a live oral vaccine.

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MATERIALS AND METHODS

Volunteers. Participants in vaccination and efficacy challenge studies consisted of college students and other healthy adults from the Baltimore community. The methods of medical screening, care of the volunteers, and informed consent have been previously described (21, 23–25, 26, 28). To ensure the informed nature of consent, volunteers had to pass a written examination containing approximately 30 multiple choice and true-false questions on all aspects of the study including risks, benefits, procedures, bacteriology, and immunology. Both vaccination and challenge studies were carried out under legal quarantine in the 22-bed isolation ward maintained by the Center in the University of Maryland Hospital.

Cohorts of up to 14 volunteers were given a single dose of vaccine and observed for 5 days, after which they received 5 days of tetracycline therapy before being discharged. Two other cohorts received a second dose of vaccine 7 days after the first dose; after 5 additional days of observation they received a course of tetracycline and were discharged.

Vaccine and challenge strain. *V. cholerae* strains utilized in the volunteer studies, including Texas Star-SR, El Tor Ogawa 3083 (the parent from which Texas Star was derived), and two pathogenic strains, El Tor Ogawa E7946 and El Tor Inaba N16961, were stored in vials of skim milk at −70°C. Inocula were prepared as previously described and were given to volunteers with 2.0 g of $NaHCO_3$ to neutralize gastric acid (21, 23–25, 26, 28); vaccination or challenge was

TABLE 1. Clinical response of volunteers after ingestion of attenuated *V. cholerae* El Tor Ogawa strain Texas Star-SR

Vaccine dose (with $NaHCO_3$)	No. of volunteers	No. with diarrhea (%)	Mean no. of diarrheal stools per ill volunteer[a]	Mean diarrheal stool volume (ml)[b]
10^5	14	3 (21)	4.7 (2–6)	871 (340–1,464)
10^6	8	0	0	0
10^8	5	2 (40)	4.0 (3–5)	349 (339–358)
10^{10}	6	1 (17)	2.0	310
5×10^{10}	9	2 (22)	3.0 (2–4)	536 (413–659)
10^9 (two doses 1 week apart)	8	2 (25)[c]	2.0	268 (261–275)
2×10^{10} (two doses 1 week apart)	18	6 (33)[c]	3.8 (2–6)	439 (233–651)

[a] Ranges are shown in parentheses. Mean for all volunteers, 3.6 (2–6).
[b] Ranges are shown in parentheses. Mean for all volunteers, 491 ml (233–1,464).
[c] Diarrhea followed the first dose only.

always carried out at 3 p.m., with volunteers fasting for 90 min before and after.

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

Volunteers were examined and interviewed at least once daily. During challenge studies, volunteers with diarrhea were given oral glucose-electrolytes solution to maintain hydration (34). For each volume of diarrheal stool passed, volunteers were given 1 1/2 volumes of glucose-electrolytes solution to drink. During challenge studies, ill volunteers received tetracycline 24 h after onset of diarrhea; in a few controls with cholera, intravenous fluids were required to maintain hydration, whereupon tetracycline was also initiated.

Bacteriology. Specimens of all stools were cultured. If no stool specimen was available in a 24-h period, a rectal swab was obtained. Stool and swabs were inoculated directly onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (BBL Microbiology Systems, Cockeysville, Md.) and inoculated into alkaline peptone water and sodium-gelatin-phosphate enrichment broths (42). After overnight incubation, the enrichment broths were subcultured onto TCBS agar. Quantitative cultures were obtained by diluting 1.0 g (or 1.0 ml) of stool 10-fold serially in phosphate-buffered saline (pH 7.2), inoculating 0.1 ml onto plates of TCBS agar, and counting colonies. Suspicious colonies for TCBS 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.).

Gelatin string capsule devices (Enterotest; HEDECO, Mountain View, Calif.) were employed to obtain duodenal fluid for culture (12). Fasting volunteers ingested string capsule devices at 7:00 a.m. on 1 or 2 consecutive days immediately after vaccination. Strings were removed 4 h later, and the distal portions were cultured as above.

Serology. Sera were collected from all volunteers before and 10, 21, and 28 days after ingestion of the *V. cholerae* strains. Vibriocidal antibody was measured by a microtiter technique (6), IgG cholera antitoxin was measured by enzyme-linked immunosorbent assay (ELISA) (43, 48), and neutralizing antitoxin was measured by Y-1 adrenal cell methodology (27) as previously described.

Before and 8 to 14 days after ingestion of vaccine or pathogenic vibrios, volunteers ingested polyvinylchloride intestinal tubes to collect jejunal fluid for measurement of specific SIgA to vibrio antigens (21, 23, 28). 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 (100 ml) from each volunteer was centrifuged (8,000 × *g*) to remove particulate material. SIgA was measured by radial immunodiffusion (29) as previously described (21, 23). Portions of fluid were dispensed into polystyrene tubes and lyophilized. Fluids were reconstituted to 20 mg of SIgA per 100 ml before testing for specific antibody.

Cholera toxin (Schwarz/Mann, Orangeburg, N.Y.), Ogawa lipopolysaccharide (LPS) (kindly provided by Ann-Mari Svennerholm, Goteborg, Sweden), or an outer membrane preparation (OMP) (19) from El Tor Ogawa strain E7946 (kindly provided by Kathleen Richardson and Charlotte Parker, Columbia, Mo.) was utilized as antigen in microtiter ELISA (48) to measure specific SIgA antibody. One hundred microliters of toxin (10 μg/ml), LPS (50 μg/ml), or OMP (10 μg/ml) were applied to microtiter wells. After washing the antigen-coated plates, twofold serial dilutions of jejunal fluids were applied. After incubation and washing, goat anti-human IgA conjugated to alkaline phosphatase (Kirkegaard and Perry, Co., Gaithersburg, Md.) was added. Further washing, addition of substrate, and plate readings were performed by standard ELISA procedure as previously described (23, 48).

The endpoint titer was considered the last dilution that gave an optical density (OD) ≥0.20 and was at least 0.05 OD units below the preceeding dilution. Fourfold rises were considered significant.

RESULTS

Reactogenicity. Single doses of 10^5, 10^6, 10^8, 10^{10}, or 5×10^{10} Texas Star-SR organisms were ingested by a total of 42 volunteers (Table 1); eight additional volunteers received two doses of 10^9 organisms 1 week apart, and 18 others were fed 2×10^{10} organisms 1 week apart (Table 1). Passage of a few loose stools meeting our definition of diarrhea occurred in 16 of the 68 vaccinees (24%). Neither the frequency nor volume of diarrhea correlated with the dose of vaccine organisms. The diarrhea was not accompanied by malaise, nausea, fever, or anorexia; two vaccinees (who received 2×10^{10} organisms) had notable abdominal cramps. Among the

TABLE 2. Clinical pattern in 16 vaccinees who developed diarrhea after ingestion of attenuated *V. cholerae* Texas Star-SR

Vaccine dose	Incubation period (h)	Diarrheal stool volume (ml)	No. of loose stools	No. of loose stools on observation day:					Significant rise in serum antitoxin (ELISA)
				0	1	2	3	4	
10^5	46	1,464	6	0	2	4	0	0	+
10^5	24	808	6	1	1	0	2	2	−
5×10^{10}	42.5	659	4	0	2	1	0	1	−
2×10^{10}[a]	16.5	651	4	4	0	0	0	0	−
2×10^{10}	18	603	6	5	1	0	0	0	−
2×10^{10}[a]	45.5	498	5	3	2	0	0	0	−
5×10^{10}	17	453	2	2	0	0	0	0	+
10^8	21	358	5	2	2	1	0	0	+
2×10^{10}[a]	19	348	3	2	1	0	0	0	−
10^5	67	340	2	0	0	1	1	0	−
10^8	48	339	4	0	0	3	0	1	−
10^{10}	44	310	2	0	1	0	1	0	−
2×10^{10}[a]	16	303	3	3	0	0	0	0	+
10^9[a]	92	275	2	0	0	0	1	1	+
10^9[a]	99	261	2	0	0	0	0	2	−
2×10^{10}[a]	19	233	2	2	0	0	0	0	+

[a] Diarrhea followed the first of two doses.

26 recipients of two doses of vaccine, 8 developed loose stools but only after the first dose.

In only one instance did the diarrheal stool volume exceed 1.0 titer; this person (who received 10^5 organisms, the lowest dose of vaccine) passed six loose stools over a 2-day period totalling 1.46 liters (Table 2). In the remaining 15 vaccinees diarrhea was mild, totalling less than 0.50 liters in 11 vaccinees and typically consisting of one or two loose stools for 1 or 2 days (Table 2).

Significant rises in serum antitoxin measured by IgG-ELISA were recorded with comparable frequency in vaccinees with diarrheal reactions (6 of 16) and in those who did not have diarrhea (14 of 52).

Bacteriological isolation of vaccine strain. Texas Star-SR was recovered from stool cultures of only 4 of 22 individuals (22%) who ingested 10^5 or 10^6 vaccine organisms but from at least 60% of those who ingested 10^8 or more Texas-Star SR (Table 3); quantitative cultures revealed 10^2 to 10^3 vibrios per g of stool. The vaccine strain grew very slowly from clinical specimens; colonies were pinpoint at 24 h and small (2 to 4 mm) at 48 h.

Duodenal fluid was obtained for culture 20 and 44 h after ingestion of the vaccine from volunteers who received higher doses (10^8 or more organisms). Texas Star-SR was recovered from 35 of these 46 vaccinees (76%) (Table 3). Among the 26 recipients of two doses of vaccine spaced 1 week apart, Texas Star-SR was isolated in duodenal fluid cultures from 21 of 26 (81%) after the initial dose but from 0 of 26 after the second dose ($P < 0.0001$), suggesting that the first dose had already stimulated antibacterial immune mechanisms. Quantitative cultures of duodenal fluid revealed 10^2 to 10^6 vibrios per ml.

A total of 456 isolates of Texas Star-SR from stool and duodenal fluid specimens of vaccinees were examined for cholera enterotoxin by Y-1 adrenal cell assay; all isolates were negative.

Immune response to Texas Star-SR (i) serum responses. At all dosage levels Texas Star-SR induced prominent serum vibriocidal antibody responses, with 93% of all vaccinees manifesting significant seroconversions (Tables 4 and 5). The distribution of vibriocidal titers and the geometric mean titer were compared with those seen after experimental infection with pathogenic *V. cholerae* El Tor Ogawa (Table 5). Although Texas Star stimulated prominent vibriocidal antibody responses, they were not quite equal to infection with pathogenic El Tor Ogawa.

In contrast, although almost all volunteers manifested significant rises in circulating antitoxin after infection with pathogenic *V. cholerae* (Table 5), such rises were seen in only 11 of 42 recipients of one dose of Texas Star (26%) and

TABLE 3. Bacteriological findings in recipients of various doses of attenuated *V. cholerae* strain El Tor Ogawa Texas Star-SR

Vaccine dose (with 2 g of $NaHCO_3$)	No. immunized	Stool cultures		Duodenal fluid cultures		
		No. positive (%)	Vibrios per g	No. positive (%)		Vibrios per g
				Day 1	Day 2	
10^5	14	2 (4)				
10^6	8	2 (25)		0		
10^8	5	5 (100)		3 (60)		
10^{10}	6	1 (17)		3 (50)		
5×10^{10}	9	6 (67)	10^2–10^3	7 (78)	8 (89)	10^2–10^5
10^9 (two doses 1 week apart)	8	Dose 1, 5 (63); dose 2, 1 (13)	10^2–10^6	Dose 1, 6 (75)[a]	Dose 2, 0[a]	10^2–10^5
2×10^{10} (two doses 1 week apart)	18	Dose 1, 12 (67); dose 2 0	10^2–10^4	Dose 1, 15 (83)[b]	Dose 2, 0[b]	10^2–10^5

[a] Significantly different at $P = 0.007$.
[b] Significantly different at $P < 0.0001$.

TABLE 4. Immunologic response of volunteers following ingestion of varying doses of attenuated *Vibrio cholerae* strain Texas Star-SR

Vaccine dose (with $NaHCO_3$)	No. inoculated	No. with significant rises in (%):					
		Serum vibriocidal antibody	Serum antitoxin		Intestinal SIgA antibody to:		
			Y-1	ELISA	Toxin	LPS	OMP
10^5	14	12 (86)	4 (33)	4 (33)	NA[a]	NA	NA
10^6	8	7 (88)	2 (29)	1 (14)	NA	NA	NA
10^8	5	5 (100)	2 (40)	2 (40)	1/4[b]	0/4	2/4
10^{10}	6	4 (67)	1 (17)	2 (33)	0/6	0/6	1/6
5×10^{10}	9	9 (100)	1 (11)	2 (22)	0/8	4/8	4/8
10^9 (two doses 1 week apart)	8	8 (100)	3 (38)	4 (50)	0/8	3/8	3/8
2×10^{10} (two doses 1 week apart)	18	18 (100)	4 (22)	5 (28)	4/15	5/15	2/15

[a] NA, Intestinal fluids not available for testing.
[b] Number positive/number of volunteers tested. Intestinal fluids were not available from some individuals.

in only 9 of 26 (35%) who received two doses. Furthermore, the mean peak level of antitoxic antibody (0.53 OD units) in the 20 vaccinees who manifested significant rises was significantly lower ($P < 0.02$) than the mean peak level (1.07) encountered after challenge with pathogenic *V. cholerae* El Tor.

(ii) Intestinal antibody. Paired specimens of jejunal fluid were collected from 41 of the 46 volunteers who ingested Texas Star in doses of 10^8 or higher. One specimen was collected before vaccination and a second at either 7 or 8 days (in those who received one dose) or 11 to 14 days (in those who received two doses) after ingestion of the first dose of vaccine.

Significant rises in SIgA antibody were recorded (Table 4) against cholera toxin in 5 vaccinees (12%), against Ogawa LPS in 12 (29%), and against Ogawa OMP in 12 (29%). Intestinal fluids of eight persons manifested significant rises in antibody titer against both Ogawa LPS and OMP.

Efficacy challenge with El Tor Ogawa 3083. Four to 6 weeks after ingestion of a single dose of 10^8 or 10^{10} Texas Star-SR vibrios, eight vaccinees and four control volunteers were given 10^6 El Tor Ogawa 3083 organisms, the enterotoxigenic, presumably pathogenic, strain from which Texas Star was derived by chemical mutagenesis. Surprisingly, although the four control volunteers excreted strain 3083 and had significant rises in serum vibriocidal and antitoxic antibody, none developed diarrhea. In comparison, among the eight Texas Star vaccinees, only two excreted strain 3083 and only one manifested a rise in serum vibriocidal and antitoxic antibody ($P < 0.05$). Of other presumably pathogenic (i.e., isolated from coprocultures of patients with cholera) El Tor *V. cholerae* strains utilized in volunteer studies, all but one have caused diarrhea in 70 to 100% of individuals when an inoculum of 10^6 is administered with $NaHCO_3$. Thus, we must tentatively conclude that the parent strain 3083 is diminished in pathogenicity, despite the fact that it elaborates cholera holotoxin.

Challenge with pathogenic El Tor Ogawa. To assess the efficacy of Texas Star-SR against challenge with an El Tor strain of the homologous serotype (Ogawa) and of known pathogenicity, seven vaccinees who ingested a single dose of 5×10^{10} Texas Star-SR 2 months earlier and 10 control volunteers were given 10^6 El Tor Ogawa E7946. Diarrhea occurred in 7 of 10 controls and in 2 of 7 vaccinees (Table 6). The two ill vaccinees had minimal diarrhea; one vaccinee had one loose stool of 0.4 liter, whereas the other passed three small loose stools of 0.2 liter. The diarrheal stool volume in the ill vaccinees was significantly less than that in the ill controls ($P < 0.01$) (Table 6).

Challenge with pathogenic El Tor Inaba. Two studies were carried out to assess the efficacy of Texas Star-SR against challenge with El Tor vibrios of the heterologous (Inaba) serotype, and the results were pooled. Eighteen volunteers who received two doses of Texas Star-SR (10^9 or 2×10^{10}) 5 to 7 weeks earlier and 15 unimmunized controls were challenged with 10^6 El Tor Inaba N16961 (Table 6). Diarrhea occurred in 11 of 15 controls (73%) but in only 5 of 18 vaccinees (28%) ($P < 0.02$); the five vaccine failures included recipients of both 10^9 and 2×10^{10} Texas Star-SR. The severity of diarrhea in the five ill vaccinees, as measured by

TABLE 5. Comparison of serum vibriocidal and antitoxin responses of volunteers after ingestion of Texas Star-SR or pathogenic *V. cholerae* El Tor Ogawa E7946

Group	*n*	Vibriocidal antibody				Antitoxin[a]		
		% With significant rises	Peak geometric mean titer	% With titers:		% with significant rises	Mean net OD	
				≥2,560[b]	≥10,240[b]		Before	Peak
Controls who ingested pathogenic El Tor Ogawa E7946	21[c]	95	3,764[b]	81	43	90	0.15	1.07[d]
All Texas Star vaccinees	68	93	1,635	51	28	29		
Vaccinees who had significant rises in serum antitoxin	22					—	0.12	0.53[d]

[a] Measured by IgG-ELISA
[b] Reciprocal titers against Ogawa antigen.
[c] Control volunteers from two challenge studies who ingested 10^6 E7946; all had positive coprocultures and 20 developed diarrhea.
[d] Significantly different at $P < 0.02$.

TABLE 6. Studies in volunteers to assess the efficacy of Texas Star-SR to protect against challenge with pathogenic *V. cholerae* El Tor Ogawa or Inaba serotype

Challenge strain	Group	Clinical attack rate	Mean diarrheal stool volume (liters) per ill volunteer (range)	Positive coprocultures	Geometric mean excretion of *V. cholerae* (per g of stool)
El Tor Ogawa	Control	7/10[a]	2.1 (0.7–3.4)	9/10	5.8×10^6
	Vaccinees	2/7[a]	0.3 (0.2–0.4)[b]	6/7	4.2×10^4
El Tor Inaba	Control	11/15[c]	4.4 (0.4–10.3)	14/15	2.3×10^7
	Vaccinees	5/18[c]	0.8 (0.3–1.2)[b]	14/18	2.5×10^5

[a] $P = 0.15$.
[b] Significantly different from controls ($P < 0.01$).
[c] Significantly different at $P < 0.02$.

diarrheal stool volume, was significantly less than that in the ill controls ($P < 0.01$) (Table 6).

Excretion of pathogenic *V. cholerae*. Quantitative cultures performed during the challenge studies with pathogenic *V. cholerae* demonstrated a partial interference with proliferation of *V. cholerae* in the vaccinees versus the control volunteers. In both the homologous and heterologous challenge studies, the geometric mean excretion of pathogenic *V. cholerae* (organisms per gram of stool) was 100-fold lower among the vaccinees in comparison with the controls (Table 6).

DISCUSSION

Four notable observations have come from these clinical studies. (i) Mild diarrhea occurred in one-fourth of the recipients of Texas Star-SR, although it does not elaborate holotoxin. (ii) Evidence showed that Texas Star-SR colonized the duodenum and stimulated local and circulating antibody responses. (iii) Within 7 days after just one dose of vaccine, antibacterial mechanisms were evident. (iv) One or two doses of vaccine provided a moderate degree of protection (61% vaccine efficacy) against experimental challenge with high inocula of *V. cholerae* El Tor of either serotype which caused cholera in 70 to 80% of control volunteers.

The observation that the A^-B^+ Texas Star-SR mutant induced mild diarrhea in approximately 25% of the recipients was somewhat surprising, although it had been pointed out earlier (16) that Texas Star-SR caused some intestinal secretion in the infant rabbit model. It is conceivable that the mere act of colonization of the proximal small intestine may elicit some diarrheal response. In fact, Smith and Linggood (45) found that an *Escherichia coli* strain that produced K-88 fimbriae but not heat-labile or heat-stable enterotoxin nevertheless induced mild diarrhea when fed to neonatal piglets. Similarly, Levine et al. (22; *in* E. C. Boedeker, ed., *Attachment of Microorganisms to the Gastrointestinal Mucosal Surface*, in press) noted that 2 of 19 volunteers developed mild diarrhea when orally immunized with an attenuated *E. coli* strain that produces colonization factor antigen/II fimbriae but not heat-labile or heat-stable enterotoxin. One common denominator among these strains is the ability to readily adhere to and colonize the mucosa of the proximal small intestine. It has also been claimed that *E. coli* and *V. cholerae* can elaborate cytotoxins and other "toxic materials" distinct from cholera toxin and heat-labile and heat-stable enterotoxin that have the ability to induce net water secretion when tested in closed or perfused animal intestinal loop or whole animal models (20, 30, 36, 37, 39, 44). It is thus possible that the presence of such factors in highly adherent, small bowel colonizing vaccine strains of *V. cholerae* and *E. coli* could induce intestinal secretion and mild diarrhea in some human recipients of the vaccine strains. If evidence accumulates, then such cytotoxins/enterotoxins may also have to be deleted from attenuated *V. cholerae* and *E. coli* vaccine candidates to render them completely non-reactogenic. The fact that the mild diarrheal response was clearly not dose related suggests that factors in the host, possibly genetic, could be more responsible than the vibrio.

Infection with Texas Star-SR elicited significant vibriocidal antibody responses in nearly all the volunteers, although the mean titers observed were not as high as those in people convalescing from the disease (Table 5). It is clear from studies of Mosley et al. (32, 33) that serum vibriocidal antibody levels correlate closely with resistance to infection, whether the antibody is acquired by natural infection or by vaccination with whole cells or isolated LPS. It is also evident that infection with Texas Star generated an operative antibacterial immune response in that vibrios were not recoverable from duodenal fluid cultures of any of the 26 individuals receiving a second dose of vaccine.

Significantly fewer vaccinees developed rises in serum antitoxin, and the titers, likewise, were lower than those in convalescent patients (Table 5). It is not clear whether the relatively poor serum antitoxin response stems from reduced elaboration of B subunit oligomers in vivo (although Texas Star is, compared with most *V. cholerae* El Tor strains, a hyper-producer of B-subunit antigen in vitro) or from a lesser antigenicity of choleragenoid in comparison with the holotoxin (40).

Because of logistical considerations, jejunal fluids from most volunteers were collected only 8 days after vaccination (shortly before discharge), which is short of the period (15 to 21 days) when peak local antibody responses are expected (M. M. Levine, unpublished data). Nevertheless, 12 to 29% of vaccinees from whom paired specimens were available manifested significant rises in SIgA antibody to cholera toxin, LPS, or OMP antigen. This is less than that observed in convalescents from experimental cholera in whom 50 to 60% have SIgA rises to these antigens in testing of paired specimens collected before and 8 days after challenge (M. M. Levine, unpublished data). Although the Texas Star vaccine strain was not recovered in high numbers from stool cultures of vaccinees, evidence that it colonized effectively in the small bowel is derived indirectly by the fact that serum vibriocidal antibody levels were similar in the group which received only 10^5 organisms to those receiving higher levels of inocula (Table 4) and directly by recovery of organisms from the small bowel (Table 3).

Immunization with Texas Star provided significant protec-

TABLE 7. Protective efficacy conferred by prior infection with pathogenic *V. cholerae* of classical or El Tor biotype or attenuated *V. cholerae* El Tor strain Texas Star-SR

V. cholerae strain used for immunization	Attack rate for diarrhea[a]		Vaccine efficacy (%)	Isolation of *V. cholerae* from vaccinees	
	Controls (%)	Vaccinees (%)[b]		Direct coprocultures	Enrichment coprocultures
Pathogenic classical vibrios	24/27 (89)	0/16	100	0/16	1/16
Pathogenic El Tor vibrios	32/37 (86)	2/22 (9)	90	8/22	11/22
Texas Star attenuated El Tor	18/25 (72)	7/25 (28)	61	17/25	20/25

[a] Challenge with 10^6 pathogenic *V. cholerae*; number positive/number of volunteers challenged.
[b] Includes both serotype-homologous and -heterologous challenges.

tion against cholera due to the Ogawa or Inaba serotype of El Tor biotype. The overall vaccine efficacy was 61% (Table 7) against challenge doses which induced clinical disease in 70 to 80% of control recipients. However, Texas Star-SR was less effective than pathogenic vibrios in protecting against cholera. The relative efficacy of Texas Star-SR is put into perspective in Table 7 by comparison with earlier results obtained with volunteers rechallenged after an initial clinical infection due to pathogenic El Tor or classical *V. cholerae*. It was possible to isolate *V. cholerae* from 80% of Texas Star-SR vaccinees who were challenged. However, *V. cholerae* were also recovered from 50% of rechallenged El Tor veterans. In contrast, classical vibrios not only provided 100% clinical protection, but upon rechallenge *V. cholerae* were virtually never recovered from stool cultures (only one coproculture was positive by enrichment on only 1 day). It should be recognized that these studies were performed, for practical reasons, with challenge doses which were known to be capable of causing disease in more than 70% of the subjects; protection against lower, more natural, challenge doses may be even higher.

The clear-cut demonstration in volunteer studies in recent years that an initial clinical infection due to pathogenic *V. cholerae* (of either classical or El Tor biotypes) stimulates potent, long-lived immunity against subsequent rechallenge with *V. cholerae* of either Ogawa or Inaba serotype (4, 21, 23, 25, 26, 28) has accelerated research on development of new cholera vaccines and has provided a standard against which to measure success. Cholera infection in humans stimulates both antibacterial and antitoxic immune responses. Some investigators are attempting to mimic infection-derived immune responses and to induce protection by oral vaccination of humans with combinations of toxoid (such as B subunit oligomer or procholeragenoid) and killed whole vibrios (14, 40, 41, 46, 47). This approach has the advantage of virtual complete safety but the disadvantage of requiring multiple oral doses of a relatively expensive vaccine to prime and boost immunity in the intestine.

The alternative approach to immunoprophylaxis is by means of attenuated, non-enterotoxinogenic strains employed as live oral vaccines. Earlier work with naturally occurring non-enterotoxinogenic *V. cholerae* O1 strains from environmental sources showed that these strains were useless as oral vaccines; although they caused no adverse reactions, they colonized minimally, if at all, stimulated meager antibody responses, and did not confer protection against experimental challenge (5, 24). These observations led us to conclude that a successful oral vaccine would have to be derived by modification of a pathogenic *V. cholerae* strain that readily colonizes the human intestine.

There are three inherent drawbacks of Texas Star-SR. First, the precise genetic lesion responsible for the inability to elaborate holotoxin is not presently known; thus, reversion is theoretically possible. Since reversion has not been detected during multiple direct and indirect serial passages of large and moderate inocula in experimental animals (16), if it can occur, it must be at a relatively low frequency. The fact that the parent strain did not cause cholera in volunteers perhaps provides an additional margin of safety. Second, nitrosoguanidine, the agent responsible for deriving this A^-B^+ mutant, induces other changes in the genome as well, not all of which may be recognized. Texas Star-SR, for example, although it has no additional growth requirements (16), grows notably more slowly than the parent 3083 strain. Finally, it is not clear why immunity induced by Texas Star is less strong than that induced by virulent vibrios; it should be emphasized that the immunizing capacity of the parent strain, 3083, has not been tested in humans.

It is important to determine how persons living in cholera endemic areas will respond clinically after immunization with colonizing, attenuated vaccine strains such as Texas Star-SR. It is conceivable that in such populations, in which serological evidence of antitoxic immunity is present early in childhood (1), subclinical *V. cholerae* infections are common (32, 33), and children in the first 2 years of life experience on the average six or seven separate episodes of diarrheal disease per year anyway (2), the mild diarrhea that can occur in some recipients of Texas Star-SR may not be readily apparent. In contrast, it must also be considered that because of malnutrition accompanied by altered small intestinal physiology and morphology, children in endemic areas may manifest even more diarrhea than North American adults after ingestion of Texas Star-SR or comparable vaccine strains. Only careful clinical safety tests in populations in endemic areas will provide an answer to this critical question. Nevertheless, it can be argued that, as observed in studies with Texas Star-SR, the occurrence of mild, non-inconveniencing diarrhea in one-fourth of recipients may be an acceptable price to be paid for stimulation of substantial immunity against cholera by means of an inexpensive, one-dose, live oral vaccine.

Recombinant DNA techniques have recently been applied to develop attenuated *V. cholerae* oral vaccine strains with none of these drawbacks (18, 31; J. B. Kaper, M. M. Baldini, H. A. Lockman, and M. M. Levine, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B55, p. 32). Precise deletions of the genes encoding the cholera toxin (or the A subunit) have been achieved in strains whose pathogenicity and ability to confer protection were documented in volunteer studies. These recombinant DNA techniques can predictably and

repeatedly yield non-enterotoxigenic variants for use as potential live oral vaccines. Nevertheless, Texas Star-SR, which appeared in the period just before the advent of the genetically engineered strains, has played a pathfinding role as a prototype, allowing us to explore the feasibility of mimicking infection-derived immunity by means of an oral attenuated vaccine.

ACKNOWLEDGMENTS

These studies were supported in part by Public Health Service research contract NO1AI12666 (M.M.L.) and grant AI17312 (R.A.F.) from the National Institute of Allergy and Infectious Diseases.

We thank Kathleen Richardson and Charlotte Parker, University of Missouri, for generously providing the outer membrane protein preparation used.

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