INFECTION AND IMMUNITY, June 1990, p. 1620–1627
0019-9567/90/061620-08$02.00/0

Vol. 58, No. 6

Safety, Immunogenicity, and Efficacy against Cholera Challenge in Humans of a Typhoid-Cholera Hybrid Vaccine Derived from *Salmonella typhi* Ty21a

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Received 3 November 1989/Accepted 5 March 1990

A live oral vaccine consisting of attenuated *Salmonella typhi* Ty21a expressing *Vibrio cholerae* O1 Inaba lipopolysaccharide (LPS) O antigen was constructed and tested in volunteers for safety, immunogenicity, and efficacy. Fourteen adults ingested three doses of 10^{10} viable organisms with buffer. One month later, 8 vaccinees and 13 unimmunized controls were challenged with 10^6 pathogenic *V. cholerae* O1 E1 Tor Inaba organisms. No significant adverse reactions to vaccination were observed. All volunteers had significant rises in serum immunoglobulin G (IgG) antibody to *S. typhi* LPS. Only 2 (14%) of 14 had significant rises in serum IgA or IgG antibody to Inaba LPS, and 5 (36%) of 14 had fourfold rises in vibriocidal antibody. In the challenge study, diarrhea occurred in 13 of 13 controls and 6 of 8 vaccinees (vaccine efficacy, 25%; $P = 0.13$). The vaccine significantly reduced the severity of the clinical illness ($P < 0.05$) and caused decreased excretion of challenge vibrios ($P < 0.05$). Although the typhoid-cholera hybrid vaccine did not provide significant protection overall against experimental cholera, this study demonstrates the importance of antibody to *V. cholerae* O antigen in ameliorating clinical illness and illustrates the use of an *S. typhi* carrier vaccine strain expressing a foreign antigen.

Endemic cholera remains an important threat to millions of people in Asia, Africa, and Oceania who have inadequate sanitation. Natural infection clearly provides protective immunity which could be reproduced by vaccination (20, 35, 47). Since the likelihood of widespread correction of inadequate sanitation in the near future is remote, prevention of this disease by vaccination is a more practical approach.

A variety of vaccine development strategies, many involving biotechnology, have been pursued. These include bacterial subunit vaccines (27, 48, 52), inactivated whole vibrio vaccines (8), and attenuated *Vibrio cholerae* O1 strains as live vaccines (32, 39, 40, 45). Yet another approach has been to use an attenuated *Salmonella* strain as a carrier for the expression of *V. cholerae* antigens. Salmonellae have been used as carriers to express other heterologous antigens, such as colonization factor fimbriae of enterotoxigenic *Escherichia coli* (75), subunits of *E. coli* enterotoxins (9), *Shigella sonnei* O antigen (2), streptococcal virulence antigens (11), and *Plasmodium* circumsporozoite protein (62).

A prototype *Salmonella* carrier vaccine against cholera was developed by using live oral *S. typhi* vaccine strain Ty21a harboring a plasmid carrying the genes responsible for biosynthesis of *V. cholerae* serotype Inaba lipopolysaccharide (LPS) O antigen. The rationale for this construct is that (i) Ty21a is a safe and effective live oral typhoid vaccine (15, 18, 37, 41, 72), (ii) anti-LPS antibody contributes significantly to the vibriocidal antibody response (57) which correlates with protection against cholera (51), and (iii) locally produced mucosal antibody plays a role in protection against enteric infections (61, 71, 74). This hybrid vaccine was safe and immunogenic in early studies in volunteers in Australia (17, 46). We describe here the hybrid Ty21a-Inaba bivalent vaccine, designated EX645, and the results of volunteer studies to assess its safety, immunogenicity, and efficacy against *V. cholerae* O1 challenge.

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MATERIALS AND METHODS

Construction of EX645 typhoid-cholera vaccine. Hybrid typhoid-cholera vaccine strain EX645, developed jointly by investigators at Enterovax, Ltd., and at the University of Adelaide, is a rifampin-resistant, thymine-dependent derivative of *S. typhi* vaccine strain Ty21a into which a 30.4-kilobase plasmid containing the genes for the LPS O antigen of *V. cholerae* O1 serotype Inaba was inserted. In addition, the *rfa* chromosomal region of Ty21a was replaced with the homologous region from *E. coli* K-12 to allow expression of *V. cholerae* O antigen on the cell surface. The strain has a mutation in *thyA* to select for organisms possessing the plasmid (with the complementing proficient *thyA* gene) and rifampin resistance encoded on the chromosome to facilitate selection of the vaccine strain from stools.

The steps in developing the strain were as follows. Ty21a is an attenuated *S. typhi* strain extensively treated with nitrosoguanidine and contains multiple defined and undefined mutations which collectively account for its attenuation (18, 29). One of the mutations is *galE*; this mutation results in a block of the enzyme UDP-galactose-4-epimerase such that the organism does not express LPS O antigen when grown in the absence of galactose. When grown in vitro in the presence of galactose, smooth O is produced, but the accumulation of intermediate products leads to bacterial death. A spontaneous rifampin-resistant derivative of Ty21a was selected by plating on rifampin-containing medium and was designated *S. typhi* E759. A plasmid, pPM1004, encoding tetracycline resistance and carrying *V. cholerae* Inaba O-antigen biosynthesis genes (44) was introduced by conju-

gation into strain E759, yielding a new strain designated *S. typhi* V487. However, V487 did not produce detectable *V. cholerae* O antigen.

The lack of expression of *V. cholerae* O antigen in Ty21a was due to the inability of the *S. typhi* LPS core to serve as a substrate for *V. cholerae* O antigen polymerization. Therefore, *S. typhi* strain EX210 was constructed, in which the chromosomal *rfa* region of V487 was replaced with the homologous region of *E. coli* K-12 strain EX170 by conjugation. EX170 was the result of transduction of *E. coli* K-12 PK3 (Hfr PO131) with bacteriophage P1 *vir* grown on *E. coli* K-12 NK6701 (*mtl*::Tn*9*). One such transconjugate, *S. typhi* EX210, produced *V. cholerae* O antigen in the absence of galactose and both *V. cholerae* and *S. typhi* O antigens in the presence of galactose.

EX210 was unsuitable as a vaccine because of its tetracycline and chloramphenicol resistance. To remove the tetracycline resistance, EX210 was cured of plasmid pPM1004, resulting in strain *S. typhi* EX233, with replacement by another plasmid, pEVX22, described below. To remove the chloramphenicol resistance property, *mtl*$^+$ revertants of EX233 were obtained. One such strain was designated EX256. A nonreverting *thyA* mutation was introduced into *S. typhi* EX256 for selection purposes and designated EX259.

Plasmid pEVX22 contained a 20-kilobase *SacI* fragment from the chromosome of *V. cholerae* 569B (classical biotype, Inaba serotype) which encoded O-antigen biosynthesis genes (73) and a *Hin*dIII fragment encoding *thyA*$^+$ of *E. coli* K-12 (3, 4). The plasmid with the *thyA*$^+$ gene repaired the thymine deficiency of the host strain and therefore applied selective pressure against loss of the plasmid. pEVX22 was introduced into EX259 by mating with an *E. coli* K-12 Hfr KL96 strain (EX484) carrying pEVX22, and the final strain was designated *S. typhi* EX645. The vaccine was lyophilized, shipped on dry ice, and stored frozen at −80°C until used.

When grown in the absence of galactose, EX645, like Ty21a, produced no *S. typhi* LPS. In the presence of limiting galactose, EX645 had 6.25 to 12.5% of the activity of *S. typhi* Ty2 (the parent of Ty21a) and Ty21a had 25 to 50% of the activity of Ty2 in a hemagglutination inhibition assay using sheep erythrocytes sensitized with *S. typhi* LPS. Regardless of galactose concentration, EX645 had 25 to 50% of the activity of *V. cholerae* Inaba strain 569B in a hemagglutination inhibition assay using sheep erythrocytes sensitized with *V. cholerae* Inaba LPS. EX645 exhibited a retarded rate of growth in the presence of galactose, but unlike Ty21a, did not lyse.

Study design. The clinical protocol was approved by the University of Maryland Human Volunteers Research Committee. A cohort of volunteers was admitted to the isolation ward of the Center for Vaccine Development, University of Maryland Hospital, and the volunteers were vaccinated with vaccine strain EX645. Duodenal fluids and stools were collected to detect excretion of the vaccine, and sera, peripheral blood lymphocytes, and jejunal fluids were collected to measure the immune response. One month later, vaccinees and unimmunized control volunteers were readmitted and challenged with *V. cholerae*.

Vaccination. Fourteen healthy adult volunteers were admitted to the isolation ward. The lyophilized vaccine packaged in single-dose vials was reconstituted in 30 ml of 0.8% NaCl. Volunteers ingested three doses of 10^{10} viable organisms with the plasmid (about 10^{11} total organisms) on days 0, 2, and 4 with sodium bicarbonate as previously described (34, 36). They were kept under clinical surveillance for 9 days thereafter.

Challenge. Eight of the 14 vaccinees agreed to return for challenge. Four weeks after vaccination, these 8 vaccinees and 13 unvaccinated control volunteers were admitted to the isolation ward and challenged with 1.1×10^6 *V. cholerae* O1 strain N16961 (biotype El Tor, serotype Inaba) organisms. The inoculum was prepared as previously described (36) and was given to volunteers with sodium bicarbonate to neutralize gastric acidity. Volunteers fasted for 90 min before and after challenge.

Clinical surveillance. Volunteers were monitored in the isolation ward for 8 days after challenge to record symptoms, to treat illness with appropriate hydration and support, and to collect specimens for bacteriologic and immunologic studies. Volunteers with diarrhea were given an oral glucose-electrolyte solution to maintain hydration (55). All volunteers received tetracycline (500 mg four times a day) for 5 days to eradicate challenge organisms from their stools before discharge.

All stools after vaccination and challenge were collected and graded. The consistency of stools was graded on a five-point scale: grade 1, formed stool; grade 2, soft but formed stool; grade 3, thick liquid; grade 4, opaque watery liquid; grade 5, rice water. Diarrhea was defined as a single liquid stool (grade 3, 4, or 5) of a weight ≥300 g or at least two liquid stools with a total weight of 200 g passed within 48 h.

Clinical specimens. All stools obtained after vaccination and challenge were cultured (59). Gelatin string capsule devices (Enterotest; HEDECO, Mountain View, Calif.) were swallowed about 20 and 44 h after each dose of vaccine and after challenge to obtain duodenal fluid for culture for *S. typhi* vaccine strain EX645 and *V. cholerae* O1 as previously described (36).

Serum specimens were collected before vaccination, on days 7, 14, and 28 after vaccination, before challenge, and on days 10, 21, and 28 after challenge. Volunteers swallowed polyvinyl chloride intestinal tubes to collect jejunal fluid for local antibody production on days 7 and 14 after vaccination and after challenge (36).

Bacteriology. All stools and duodenal string fluids obtained after vaccination with the Ty21a-Inaba hybrid vaccine were inoculated onto nutrient agar plates (Oxoid, Ltd., Basingstoke, Hants, England) containing 100 μg of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml and 50 μg of thymine (Sigma) per ml and incubated for 40 h at 37°C to recover the vaccine strain. Samples were also enriched by using nutrient broth no. 3 (Oxoid) containing 100 μg of rifampin per ml and 50 μg of thymine per ml and incubated for at least 16 h at 37°C. Each broth was sampled and plated. Colonies were identified as EX645 by slide agglutination with antisera against *Salmonella* group D (Difco Laboratories) and *V. cholerae* O1 serotype Inaba (Vibrio Reference Laboratory).

To recover the *V. cholerae* O1 challenge strain, all stools and fluids from duodenal strings after challenge were plated directly onto TCBS agar (BBL Microbiology Systems) and inoculated into alkaline peptone water enrichment broth for overnight incubation before plating onto TCBS as described previously (59).

Serology. Vibriocidal responses were measured by two separate laboratories, one at the Center for Vaccine Development, University of Maryland, and the other at Enterovax, Ltd. At the Center for Vaccine Development, sera were assayed for vibriocidal antibodies against serotype Inaba strain 89 and Ogawa strain 79 by a microtechnique

after vaccination and challenge, as previously described (10). At Enterovax Ltd., sera were assayed for vibriocidal antibodies against classical Inaba *V. cholerae* 569B. The sera were heat inactivated (56°C for 30 min) and diluted in 0.1% (wt/vol) peptone in saline. Serum dilutions were mixed with equal volumes (0.4 ml) of a suspension of the indicator bacteria. Ty2Vi (ca. 4×10^3/ml) in the same diluent containing 20% (vol/vol) guinea pig serum served as a complement source. After 60 min at 37°C, the tubes were transferred to an ice water bath and 0.1-ml aliquots were spread on nutrient agar plates to determine residual viability. By plotting the viability as a function of reciprocal serum dilutions, vibriocidal titers were obtained by interpolation and expressed as the reciprocal serum dilution killing 50% of the added bacteria.

V. cholerae LPS O antibodies (25) and *S. typhi* LPS O antibodies (68) were measured by enzyme-linked immunosorbent assay. Sera of volunteers and appropriate control sera were diluted in phosphate-buffered saline-Tween containing 1% heat-inactivated fetal bovine serum. Immunoglobulin G (IgG) and IgA seroconversion to *V. cholerae* LPS and IgA seroconversion to *S. typhi* LPS were defined as fourfold rises in titers. IgG seroconversion to *S. typhi* LPS was defined as an increase in net optical density (OD) of $\geq$0.15. The relationship between titer and OD was established with a specific titration curve (data not shown). The change of OD of $\geq$0.15 was statistically derived by testing paired sera from 30 Maryland residents who received oral cholera or *E. coli* vaccines and represents a value equal to the mean rise in net OD of these paired sera plus 3 standard deviations (SD).

Sera were diluted 1:50 and assayed for IgA and IgG anti-cholera toxin as previously described (42). IgG seroconversion to cholera toxin was defined as an increase in net OD of $\geq$0.20 from prechallenge to postchallenge sera (>3 SD from the mean net OD for 30 individuals not challenged or vaccinated with *V. cholerae* strains). IgA seroconversion was defined as a fourfold rise in titer from prechallenge to postchallenge sera.

Jejunal fluids were processed as previously described (36). Intestinal secretory IgA antibodies to *V. cholerae* LPS and *S. typhi* LPS were measured by enzyme-linked immunosorbent assay. Fourfold rises in titer were considered significant.

Detection of antibody-secreting cells. Before and 7 days after vaccination, peripheral blood lymphocytes were collected to measure antibody-secreting cells by a modified Elispot assay as previously described (13, 16, 31). This assay detects IgA-producing lymphocytes stimulated by oral immunization as they traffic from the gut-associated lymphoid tissue to mucosal lymphoid tissue elsewhere. A positive IgA, IgG, or IgM response to *V. cholerae* LPS or *S. typhi* LPS was defined as a postvaccination OD which was greater than 3 SD of the mean above the prevaccination OD.

RESULTS

Characterization of strain EX645. Strains Ty21a and EX645 were identical in colony morphology, inability to ferment galactose, inability to produce H_2S, auxotrophies, antimicrobial sensitivity pattern, outer membrane protein profiles on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and identity on the API 20E Enterobacteriaceae Typing System (Analytab Products). In the presence of galactose, EX645 expressed both *Salmonella* O9 and O12 and *V. cholerae* Inaba O antigens as determined by slide agglutination; in the absence of galactose, expression of *Salmonella* O9 and O12 LPSs was undetected (as it is for Ty21a) but *V. cholerae* Inaba LPS was expressed.

Clinical acceptability. In general, the large doses (10^{10} organisms) of vaccine were well tolerated. One of the 14 vaccinees had anorexia, malaise, nausea, and mild cramps on day 2 about 2 h after the second dose, which lasted for 3 h. Two volunteers had mild nausea on day 2, and one volunteer had cramps beginning minutes after the first dose, lasting about 1.5 h.

Isolation of vaccine strain from clinical specimens. All 14 vaccinees shed small numbers (about 10^1 organisms per g) of the vaccine strain in their stools, and the mean number of days on which the vaccine strain could be recovered during the 9-day observation period was 3.2 days. For only 1 of 14 volunteers could the vaccine strain be recovered by culturing duodenal strings. The recovered vaccine organisms agglutinated with *V. cholerae* O1, *S. typhi* group D, and Inaba LPS antisera.

Immunogenicity of vaccine. The immune responses of volunteers after vaccination are shown in Tables 1 and 2.

The vibriocidal antibody responses measured by two assays at two different institutions were consistent. By the Center for Vaccine Development technique, 5 (36%) of 14 recipients of EX645 had at least fourfold rises in vibriocidal antibody to *V. cholerae* O1 Inaba at 14 days (Table 1). Among volunteers who participated in the challenge, prevaccination reciprocal titers ranged from <20 to 80; the reciprocal titers at day 14 postvaccination ranged from <20 to 320 in the Center for Vaccine Development assay. An additional seroconverter was identified in the vibriocidal assay performed at Enterovax Ltd., making the seroconversion rate 43%. One of 14 volunteers had a significant rise in vibriocidal antibodies against serotype Ogawa (data not shown).

After vaccination, only 1 (7%) of 14 vaccinees had a fourfold rise in serum IgG against Inaba LPS and 1 (7%) of 14 had a fourfold rise in IgA against Inaba LPS (Table 1). In contrast, 14 (100%) of 14 vaccinees had significant rises in IgG anti-*S. typhi* LPS ($P < 0.0001$, Fisher's exact test, two tailed versus Inaba LPS seroconversion rate) and 10 (71%) of 14 had fourfold rises in IgA anti-*S. typhi* LPS ($P < 0.001$, Fisher's exact test, two tailed versus Inaba LPS seroconversion rate) (Table 1).

Immunologic studies of jejunal fluid aspirates of vaccinees revealed that only 1 (7%) of 14 vaccinees had a rise in jejunal fluid IgA anti-Inaba LPS while 12 (86%) of 14 had rises in jejunal fluid IgA anti-*S. typhi* LPS ($P < 0.0001$, Fisher's exact test, two tailed) (Table 1).

Detection of antibody-secreting cells. Cells secreting IgA, IgM, and IgG in response to *S. typhi* LPS and *V. cholerae* LPS were sought 7 days after vaccination (Table 2). Cells secreting IgA against *S. typhi* LPS were detected in 13 (93%) of 14 volunteers, cells secreting IgG against *S. typhi* LPS were detected in 10 (71%) of 14 volunteers, and cells secreting IgM against *S. typhi* LPS were detected in 13 (93%) of 14 volunteers (Table 2). The rate of response to *V. cholerae* LPS measured as antibody-secreting cells on day 7 was lower. One (7%) of 14 volunteers developed IgA-secreting cells, 2 (14%) of 14 had IgG-secreting cells, and 2 (14%) of 14 had IgM-secreting cells against *V. cholerae* LPS (Table 2).

Challenge study. Eight vaccinees and 13 nonvaccinated controls were challenged with 1.1×10^6 organisms of *V. cholerae* O1 N16961 (biotype E1 Tor, serotype Inaba). The geometric mean titer (GMT) of vibriocidal antibody of vol-

TABLE 1. Immune responses after vaccination with Ty21a-Inaba hybrid typhoid-cholera vaccine

Antibody	Vaccinees who later participated in a challenge study (n = 8)			All vaccinees (n = 14)		
	Titers[a]		% Seroconversion[b]	Titer[a]		% Seroconversion[b]
	Pre	Peak		Pre	Peak	
Vibriocidal (Inaba)						
CVD laboratory[c]	21.8	40.0	25	24.4	51.2	36
Enterovax laboratory	23.8	79.4	38	29.7	133.5	43
V. cholerae Inaba LPS						
IgG	80.0	87.2	0	65.6	88.3	7
IgA	28.3	40.0	13	26.9	34.5	7
S. typhi LPS						
IgG	0.43[d]	1.48[d]	100	0.35[c]	1.26[c]	100
IgA	43.6	246.8	75	38.1	215.3	71
Jejunal fluid *V. cholerae* Inaba LPS IgA	2.4	2.4	0	2.6	2.4	7
Jejunal fluid *S. typhi* LPS IgA	4.8	83.0	100	5.4	58.0	86

[a] GMT, except where indicated otherwise. Pre, Prevaccination.
[b] Seroconversion defined as a fourfold rise in titer for all assays except *S. typhi* LPS IgG, for which seroconversion is defined as a net change in OD ≥0.15.
[c] CVD, Center for Vaccine Development.
[d] Arithmetic mean net OD.

unteers who returned for challenge was less than that of the group as a whole (Table 1). Overall, the vaccine did not significantly protect against challenge. Thirteen (100%) of 13 controls and 6 (75%) of 8 vaccinees developed diarrhea (vaccine efficacy, 25%; P = 0.13, Fisher's exact test, two tailed) (Table 3). The two vaccinees who did not become ill were the same two who seroconverted as determined by the vibriocidal assay performed at the Center for Vaccine Development (Table 1). The average incubation period was 20.5 h after challenge for controls and 25.6 h for vaccinees.

The mean stool volume was significantly less for vaccinees than for controls. For controls, the mean stool volume was 2,603 ± 1,752 ml, and for vaccinees, it was 867 ± 622 ml (P = 0.05, Student's t test; P = 0.01, Wilcoxon rank sum test, two tailed). Moreover, although protection against all diarrhea was not significant, protection against moderate (total stool volume, ≥2.0 liters) to moderately severe (total stool volume, ≥3.0 liters) diarrhea approached statistical significance (Table 4).

TABLE 2. Antibody-secreting cells after vaccination with a Ty21a-Inaba hybrid typhoid-cholera vaccine

Antibody	Vaccinees who later participated in a challenge study (n = 8)			All vaccinees (n = 14)		
	Titer (OD)[a]		% Responders[b]	Titer (OD)[a]		% Responders[b]
	Day 0	Day 7		Day 0	Day 7	
V. cholerae Inaba LPS						
IgA	0.02	0.06	38	0.03	0.05	7
IgG	0.00	0.02	25	0.00	0.01	14
IgM	0.05	0.10	25	0.06	0.09	14
S. typhi LPS						
IgA	0.02	1.2	100	0.02	0.94	93
IgG	0.02	0.38	100	0.02	0.26	71
IgM	0.06	0.46	100	0.06	0.42	93

[a] Mean net optical density.
[b] Responder defined as having change in net OD greater than 3 SD from the mean.

Immunological studies in relation to challenge. The GMT of vibriocidal (Inaba) antibodies before challenge was 40.0 for the vaccinees and 30.0 for the controls. After challenge, 13 of 13 controls and 8 of 8 vaccinees had fourfold or greater rises in serum vibriocidal antibodies to *V. cholerae* Inaba. The peak GMT after challenge was 3,044 for vaccinees and 3,255 for controls. Eleven of 12 controls and 5 of 8 vaccinees had significant rises in Ogawa vibriocidal antibodies.

Antitoxin responses were typical of those seen after experimental cholera infection. Ten (83%) of 12 controls and 7 (88%) of 8 vaccinees had significant rises in serum IgA anti-cholera toxin antibody, and 11 (92%) of 12 controls and 7 (88%) of 8 vaccinees had significant rises in serum IgG anti-cholera toxin antibody. Most volunteers also had significant rises in anti-LPS titers after challenge. Seven (58%) of 12 controls and 6 (75%) of 8 vaccinees had serum IgA anti-Inaba LPS antibody, and 2 (25%) of 8 vaccinees and 1 (8%) of 12 controls had significant rises in serum IgG anti-Inaba LPS antibody after challenge.

DISCUSSION

One strategy in the search for a live oral cholera vaccine has involved the use of an attenuated *Salmonella* strain as a carrier bearing vibrio genes encoding protective antigens. The advantage of using an attenuated *Salmonella* strain as a vaccine carrier is that these organisms are selectively taken up by the gut-associated lymphoid tissue of the intestinal mucosa. *S. typhi* organisms traverse the intestinal epithelium without disrupting it and reach the immune cells of the lamina propria where they deliver the foreign antigen for immune recognition and response (12). As a result, secretory and serum immunoglobulins are stimulated, as are potent cellular responses. Oral Ty21a stimulates cell-mediated immunity as measured by several assays, including leukocyte migration inhibition (63), natural antibacterial activity mediated by CD4 cells armed with IgA (a form of antibody-dependent cellular cytotoxicity) (69, 70), and lymphocyte

TABLE 3. Clinical and bacteriologic responses of recipients of Ty21a-Inaba hybrid typhoid-cholera vaccine and of controls after challenge with *V. cholerae* El Tor Inaba N16961

Group	Attack rate for diarrhea	Total diarrheal stool vol (ml)	No. of diarrheal stools	Incubation period (h)	Peak *V. cholerae* excretion
Vaccinees (n = 8)	6/8 (75%)	867[a]	6	25.6	3.5×10^6 CFU/g[b]
Controls (n = 13)	13/13 (100%)	2,603	11.6	20.5	6.2×10^7 CFU/g

[a] $P < 0.05$, comparing total stool volumes of vaccinees and controls.
[b] $P < 0.05$, comparing peak *V. cholerae* excretion by vaccinees and controls.

proliferation in response to LPS and particulate *S. typhi* antigen (53).

Vaccine strain EX645 is only the second carrier vaccine tested in humans that uses an attenuated *Salmonella* strain (*S. typhi* vaccine strain Ty21a) as the host for a heterologous antigen. Ty21a was previously used to carry *S. sonnei* form I O-polysaccharide antigen encoded on a plasmid. In studies of this typhoid-shigellosis hybrid vaccine (designated *S. typhi* 5076-1C), the vaccine was well tolerated and provided significant protection against *Shigella* challenge (6). EX645 is another example of the feasibility of this approach.

In EX645, however, the foreign polysaccharide is present in a form different from that of the foreign shigella polysaccharide in 5076-1C. Like the LPSs of other gram-negative organisms, the LPSs of *V. cholerae* O1 and *S. sonnei* have a lipid A moiety, which is responsible for endotoxin activity, as well as the O polysaccharide linked to a core oligosaccharide (23, 30, 33). The typhoid-shigella hybrid vaccine did not express a complete *Shigella* LPS, since it was not covalently bound to core lipid A but rather surrounded the organism like a capsule (65). In the typhoid-cholera vaccine we describe, the organisms express a complete Inaba-like LPS; the O polysaccharide is covalently anchored to a core which is the product of the inserted *E. coli rfa* genes. The construct also produces complete *S. typhi* LPS.

The ability to detect stool excretion of EX645 and the typhoid-shigella hybrid vaccine 5076-1C appears different from that of the parent Ty21a because of the ability to select the hybrid vaccine strains on antimicrobial agent-containing media. EX645 may be selected by rifampin resistance, and 5076-1C may be selected by streptomycin resistance. Ty21a, which has no antimicrobial resistance for selection, is not found in the stools of vaccinees who receive $\leq 10^9$ CFU per dose; at doses $\geq 10^{10}$CFU, Ty21a is recovered in the stools of some vaccinees on the first day after vaccination (19). The typhoid-shigella vaccine given at a dose of 10^9 CFU was recovered from the stools of some vaccinees in the first 2 days after vaccination (6), while the typhoid-cholera vaccine at 10^{10} CFU was detected in the stools of all vaccinees for about 3 days after the first dose.

This study of a typhoid-cholera hybrid vaccine demonstrates that anti-Inaba LPS contributes to the protective immune response against cholera. Since Inaba O antigen is indisputably the only cholera antigen in this vaccine, the 25% protective efficacy afforded by EX645 and the reduction in excretion of challenge *V. cholerae* organisms by vaccinees can be attributed to anti-Inaba LPS alone. This finding supports previous observations about the importance of anti-LPS immunity against cholera. Purified LPS-protein extract, administered parenterally, has previously been evaluated in vaccine trials in Bangladesh, where it produced significant but short-lived protection in some age groups (5, 51, 52). The protection conferred by this Inaba LPS-protein parenteral vaccine was as high as that stimulated by a killed Inaba whole cell parenteral vaccine (48, 52). In epidemiologic studies of breast-fed children in Bangladesh, an increased titer of secretory IgA against LPS in breast milk was associated with a decreased risk of cholera (21). Whether anti-LPS alone can provide the high-level and long-lasting protection stimulated by natural infection is unknown. It is possible that more solid immunity is stimulated by combinations of antigens; for example, LPS and toxoid elicit synergistic protective responses in experimental models (58, 60, 67).

Anti-LPS is a significant part of the vibriocidal immune response (26, 38, 56, 57), although other responses such as anti-outer membrane protein antibody (1, 56, 57, 64) may also contribute. In seroepidemiologic studies in an endemic area, elevated vibriocidal titers correlated with protection against infection and disease caused by *V. cholerae* (38, 47, 49–51). In another study of North American volunteers, however, no association of vibriocidal antibody with immunity could be made (7). Since cholera is caused by organisms acting at the mucosal surface without invasion, it is likely that local immune responses at the mucosa are protective and the serum vibriocidal response is simply a marker of that local response.

The modest protective efficacy afforded by EX645 may be related to the relative expression of *S. typhi* LPS and Inaba LPS. Clearly *S. typhi* LPS expression occurred in vivo, since all vaccines had rises in IgG anti-*S. typhi* LPS. It is not known whether the poor response to Inaba LPS (7% of volunteers responded) was because it was expressed in a small amount or was poorly immunogenic when presented on the surface of *S. typhi*. In a previous study of EX645 given to Australian volunteers at three doses of 6×10^{10} CFU per dose, 30 to 40% of the volunteers developed IgA or IgG against Inaba LPS (17). The differences in immune responses between our study and this Australian study may have been due to the different doses of vaccine or to differences between laboratories performing the immunoassays or may have been related to the small number of volunteers in each study.

EX645 is the prototype of a *Salmonella*-based cholera vaccine. In nature, *S. typhi* organisms do not commonly carry plasmids (22, 43, 54); the foreign gene in the hybrid vaccine would be more stably maintained if integrated into

TABLE 4. Total diarrheal stool volumes among Ty21a-Inaba hybrid typhoid-cholera vaccine recipients and controls challenged with 10^6 pathogenic *V. cholerae* O1 El Tor Inaba N16961 organisms

Total diarrheal stool vol (liters)	Vaccinees (n = 8)	Controls (n = 13)	P[a]
≥5.0 (severe)	0	1	1.0
≥3.0 (moderately severe)	0	5	0.11
≥2.0 (moderate)	1	7	0.08
Any diarrhea	6	13	0.13

[a] Fisher's exact test, two tailed.

the *Salmonella* chromosome. A system has been developed in which heterologous DNA encoding genes for an antigen from another pathogen may be recombined into an attenuated *Salmonella* carrier (28). Application of this system to a typhoid-cholera vaccine would be a further refinement. Further vaccines based on this approach might also use a *Salmonella* carrier with precisely defined attenuating mutations such as.nutritional auxotrophies (14, 24, 66). A future vaccine should also have better expression of *V. cholerae* O1 LPS in vivo and include other vibrio antigens. Ideally, the hybrid vaccine should be immunogenic after a single dose of a smaller number of organisms. These refinements may lead to a more effective and practical typhoid-cholera hybrid vaccine.

ACKNOWLEDGMENTS

This study was supported by Enterovax, Ltd., Salisbury, South Australia, and National Institute of Allergy and Infectious Diseases contract NO1 AI62528.

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