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Comparison of the Safety and Immunogenicity of Δ*aroC* Δ*aroD* and Δ*cya* Δ*crp Salmonella typhi* Strains in Adult Volunteers

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Three attenuated *Salmonella typhi* strains have been constructed by introducing deletions in *aroC* and *aroD* or deletions in *cya* and *crp* into one of two wild-type parent strains, Ty2 or ISP1820. These mutant strains were designated CVD 906 (ISP1820 Δ*aroC* Δ*aroD*), CVD 908 (Ty2 Δ*aroC* Δ*aroD*), and χ3927 (Ty2 Δ*cya* Δ*crp*). Two studies were conducted with 36 healthy adult inpatient volunteers to determine in a double-blind fashion the safety and immunogenicity of approximately 5×10^4 and 5×10^5 CFU of each of these three vaccine candidates given as a single dose. No statistically significant difference in the incidence of reactions among vaccinees was observed. Fever (oral temperature ≥ 38.2°C) occurred in 2 of 12 volunteers who received CVD 906, in 0 of 12 who received CVD 908, and in 1 of 12 who received χ3927. Vaccine bacteremia without symptoms occurred in 1 of 12 vaccinees who received CVD 906, in 0 of 12 who received CVD 908, and in 2 of 12 who received χ3927. Overall, 19 (53%) of 36 vaccinees developed immunoglobulin G antibody to *S. typhi* lipopolysaccharide after vaccination, with no statistically significant differences in the rate of seroconversion among volunteers in the three groups. We conclude that defined mutations in the aromatic biosynthetic pathway and in the cyclic AMP global regulatory system attenuate *S. typhi*. Mutant strains CVD 906, CVD 908, and χ3927 are highly (and approximately equally) immunogenic but possibly differ in their propensity to induce fever. Further studies are needed to document the apparent relative safety of CVD 908 as a typhoid vaccine and as a vaccine carrier of foreign antigens.

Live oral attenuated *Salmonella typhi* vaccines are likely to replace the parenteral killed whole-cell vaccine as safer and possibly more effective vaccines against typhoid fever for use in healthy adults and children (19). Ingestion of attenuated bacteria mimics natural enteric infection and potentially improves the intensity and duration of the immune response compared with parenterally administered vaccines. These strains may also serve as vaccine carriers of cloned protective antigens of other pathogens. A prototype attenuated *S. typhi* vaccine strain, Ty21a, developed in the early 1970s by chemical mutagenesis before the advent of recombinant DNA technology (7), has been licensed in the United States and used to prevent typhoid fever. Ty21a has also been used as a carrier of foreign antigens, with variable success (1, 26). However, Ty21a requires multiple doses to achieve acceptable immunogenicity and is a difficult background in which to perform genetic manipulations. Therefore, new oral typhoid vaccines have been sought to serve as immunoprophylaxis against typhoid and as a recombinant vaccine carrier.

One approach to attenuating *S. typhi* has been to develop nutritional auxotrophs by interrupting the pathway for biosynthesis of aromatic metabolites (8) which renders salmonella auxotrophic (i.e., nutritionally dependent) for *p*-aminobenzoic acid (PABA) and 2,3-dihydroxybenzoate, substrates not available to bacteria in mammalian tissues. Such mutants are unable to synthesize chorismic acid (precursor of aromatic compounds PABA and 2,3-dihydroxybenzoate), and no other pathways that can overcome this deficiency in salmonella exist. As a consequence of this auxotrophy, the *aro*-deleted bacteria should not sustain proliferation within mammalian cells (8). Yet they reside and grow intracellularly long enough to stimulate protective immune responses. Pathogenic *Salmonella typhimurium* and *Salmonella dublin* have been successfully attenuated by inactivation of *aro* genes (4, 14, 17, 24, 25). Such *aro*-deleted strains of *S. typhimurium* used as live oral vaccines are safe in mice and calves and protect these animals against otherwise lethal challenges with virulent *S. typhimurium* (14, 24, 25). In calves and mice, both antibody and cell-mediated immune responses are stimulated by the vaccines.

Another approach to attenuating *S. typhi* is by creating deletions (Δ) in *cya* (adenylate cyclase) and *crp* (cyclic 3′,5′-AMP [cAMP] receptor protein) genes. cAMP and cAMP receptor protein, the products of these genes, are transcription regulators of many genes and operons concerned with the transport and breakdown of catabolites (3). Systems used for transporting carbon sources and several amino acid permeases are all under positive control by cAMP. The concentration of cAMP in cells also influences lysogenization by temperate phages and the expression of fimbriae, flagella, and at least one outer membrane protein (3). Although cAMP is present in mammalian cells, the concentrations present in gastrointestinal tissues and fluids and other cells which *S. typhi* invades are below the concentration necessary to allow *cya* mutants to exhibit a wild-type phenotype (3).

Mutants of *S. typhi* which are attenuated as a result of deletions in both *aroC* and *aroD* (10, 21) or deletions in both *cya* and *crp* (3, 16) have been constructed. Inactivation of either *aroC* or *aroD*, which are widely separated on the

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salmonella chromosomal map, independently results in attenuation, but deletions in both provide a high level of safety against restoration of pathogenicity by recombination. Similarly, inclusion of a *crp* deletion mutation in a *cya*-deleted strain prevents the organism from utilizing any available tissue cAMP, since the cAMP-binding protein is not synthesized. The *cya* and *crp* mutations are 11 min apart on the 100-U linkage map of salmonella. The *aroC aroD* deletions were made in two pathogenic *S. typhi* parent strains, Ty2 (the parent of Ty21a) and ISP1820 (a recent isolate from Santiago, Chile); the *cya crp* deletions were produced in strain Ty2. These strains provide an opportunity to study the same attenuating mutations (*aroC aroD*) in two different background *S. typhi* strains (Ty2 and ISP1820) and attenuation of the same background strain (Ty2) by two different types of deletion mutations (*aroC aroD* and *cya crp*).

MATERIALS AND METHODS

Volunteers and study design. The clinical protocols were approved by the Human Volunteers Research Committee of the University of Maryland at Baltimore. Healthy adults, 18 to 35 years of age, from the Baltimore community were admitted to the Research Isolation Ward of the Center for Vaccine Development, located within the University of Maryland Hospital. The study was explained in detail, and written, informed consent was obtained. Volunteers were screened to confirm their good health by medical histories and physical examinations, a battery of clinical hematology and chemistry tests, electrocardiograms, and serologic tests for syphilis, hepatitis B surface antigen, and human immunodeficiency virus type 1. Before vaccination, stools were examined for bacterial pathogens, ova, and parasites.

Two cohorts of volunteers were recruited for two studies in which different doses of vaccine were given. In the first study, 17 volunteers were randomized in a double-blind fashion to receive approximately 5×10^5 CFU of CVD 906 ($n = 6$), CVD 908 ($n = 5$), or χ3927 ($n = 6$). In the second study, 19 volunteers were randomized in a double-blind fashion to receive approximately 5×10^4 CFU of CVD 906 ($n = 6$), CVD 908 ($n = 7$), or χ3927 ($n = 6$). These doses were chosen because in a previous study, a dose of 5×10^7 CVD 906 organisms had caused fever (11). Volunteers were closely monitored on the isolation ward for 15 days (first study) or 24 days (second study). Vital signs were measured every 6 h during the period of observation. All stools from each volunteer were collected in plastic containers, examined, and graded on a five-point scale (18), and the volume was measured when the stool was loose. Volunteers were interviewed daily by a physician and asked about symptoms. Fever was defined as oral temperature ≥38.2°C; diarrhea was defined as two or more loose stools within 48 h totalling at least 200 ml in volume or a single loose stool ≥300 ml in volume. Antibiotic therapy (amoxicillin, 1 g given orally every 6 h for 10 days, and ciprofloxacin, 750 mg every 12 h for 14 days) was given to volunteers who developed fever or positive blood cultures.

Vaccines. *S. typhi* strains attenuated in one of two ways were constructed in one of two parent strains to produce three vaccine candidates, designated CVD 906, CVD 908, and χ3927 (Table 1). Their construction is described elsewhere (10, 15). CVD 906 is derived from *S. typhi* ISP1820, a strain isolated in the early 1980s from a Chilean child with uncomplicated acute typhoid fever, by introducing deletions in *aroC* and *aroD*. CVD 908 is a Δ*aroC* Δ*aroD* derivative of *S. typhi* wild-type strain Ty2, isolated in 1916. Strain χ3927 is a Δ*cya* Δ*crp* mutant of *S. typhi* Ty2.

TABLE 1. Parent and attenuated *S. typhi* strains

Vaccine strain	Wild-type parent strain	Genes with deletions	LD_{50} in mice (CFU)[a]
CVD 906	ISP1820	*aroC aroD*	2.9×10^7
CVD 908	Ty2	*aroC aroD*	2.7×10^6
χ3927	Ty2	*cya crp*	1.8×10^4
	ISP1820	None	1,050
	Ty2	None	<10

[a] Intraperitoneal injection with hog gastric mucin (10). LD_{50}, 50% lethal dose.

Stock cultures of CVD 906 and CVD 908, maintained at −70°C in aro broth with 50% glycerol, were thawed and plated onto aro agar consisting of L broth supplemented with 0.2% (wt/vol) Casamino Acids (Difco), 0.25% (wt/vol) glucose (Sigma, St. Louis, Mo.), 0.01% (wt/vol) PABA, 0.01% (wt/vol) *p*-hydroxybenzoate (Sigma), 0.01% (wt/vol) 2,3-dihydroxybenzoate (Sigma), 100 μg of ferric ammonium citrate (Sigma) per ml, and 1.5% (wt/vol) Bacto-agar (Difco) and onto salmonella-shigella (S-S) agar supplemented with 0.1% (wt/vol) PABA and 0.1% (wt/vol) *p*-hydroxybenzoate. After incubation at 37°C overnight, 20 to 30 typical colonies of the vaccine strains were picked from aro agar, suspended in saline, and inoculated again onto aro agar. After overnight incubation at 37°C, the bacteria were harvested with 3 ml of sterile phosphate-buffered saline (PBS), and the concentration of bacteria was standardized turbidometrically. Stock cultures of χ3927 were maintained on Trypticase soy broth with 15% glycerol at −70°C. To make the inoculum, the organism was grown on supplemented aro agar and prepared in the same way as described above for CVD 906 and CVD 908. Dilutions of the suspensions were made in PBS to achieve the desired concentration of viable organisms per milliliter. The identity of the inoculum was confirmed by microscopic examination and by slide agglutination with *S. typhi* O, H, and Vi antisera. Replica spread plate quantitative cultures were made of the inocula before and after vaccination to confirm viability and the inoculum size.

Vaccination. The vaccine strains were administered by the oral route with sodium bicarbonate. Sodium bicarbonate (2 g) was dissolved in 150 ml of distilled water, and volunteers drank 120 ml of the solution to neutralize gastric acid. One minute later, volunteers drank the vaccine suspended in the remaining 30 ml of bicarbonate solution. Volunteers had nothing to eat or drink for 90 min before and after vaccination.

Bacteriology. Every stool passed by volunteers (and rectal swabs if no stool was passed) was cultured daily for the vaccine strain. Stool was inoculated into Gram Negative Broth (BBL, Cockeysville, Md.) supplemented with 0.1% PABA and 0.1% *p*-hydroxybenzoate and directly onto S-S agar with supplements. After incubation overnight at 37°C, subcultures were made onto supplemented S-S agar. To quantitate the shedding of the vaccine strains, 1 g of stool was serially diluted 10-fold in saline and each dilution was plated onto S-S agar supplemented as described above. Suspicious colonies were transferred to triple-sugar–iron agar slants, and the identities of the itolates were confirmed by agglutination with *S. typhi* O, H, and Vi antisera.

On days 7, 10, and 13 after vaccination, fasting volunteers swallowed gelatin capsules containing string devices (Entero-Test; HDC, Mountain View, Calif.) to collect samples

of bile-stained duodenal fluid. After 4 h, the strings were removed and the color and pH of the distal 15 cm were recorded. Duodenal fluid was squeezed from the end of the string and cultured as described above.

Blood for culture of the vaccine organisms was systematically collected on days 4, 5, 7, 8, 10, 12, and 15 after vaccination and again when fever occurred. A sample of 5 ml of blood was inoculated into 50 ml of supplemented aro broth.

In addition, tonsillar cultures were obtained on days 1, 2, 4, 5, 7, 8, 10, 12, and 15 in an attempt to detect the vaccine strain (6, 14). Swabs applied to the tonsils were inoculated into Gram Negative Broth with supplements for 24 h and then onto supplemented S-S agar.

Immunology. (i) Serology. Serum samples were obtained before vaccination and on days 7, 21, 28, and 60 after vaccination. Jejunal fluids were collected before vaccination and on day 14 after vaccination as previously described (20). The total immunoglobulin A (IgA) contents of the fluids were measured by enzyme-linked immunosorbent assay (ELISA), and each specimen was standardized to contain 20 mg of IgA per 100 ml. Antibodies to *S. typhi* lipopolysaccharide (LPS), H, and Vi antigens were measured in serum and jejunal fluids.

(ii) LPS O antibody. IgG antibody to LPS O antigen (Difco) was detected by ELISA as previously described (20). The range of net optical density (OD) measured in prevaccination serum was 0.21 to 0.59. An increase in net OD of $\geq$0.20 between pre- and postvaccination sera tested at a 1:100 dilution was considered significant (20). In previous studies, this change in net OD has correlated with a fourfold or greater change in titer. The positive-control serum used with each microtiter plate contained a high level of LPS O antibody and represented a pool of serum specimens from 12 healthy Chileans who had strong IgG anti-LPS O antibody responses after immunization with Ty21a vaccine (19). IgA antibody to LPS O antigen was measured as previously described (20). Twofold dilutions of serum were made, starting with a 1:25 dilution. An IgA titer was considered significant when a fourfold increase between pre- and postvaccination specimens occurred.

Intestinal secretory IgA antibody to *S. typhi* LPS O antigen was also measured by ELISA as previously described (20). Fourfold increases were considered significant.

(iii) H antibody. H-d flagellar antigen was prepared from *S. typhi* 541Ty. Serum and jejunal fluid were tested for H-d antibody by ELISA (11). A fourfold increase in titer was considered significant.

(iv) Widal test. The Widal tube agglutination test for H antibody was also performed as previously described (20) by using *Salmonella virginia*, which shares the flagellar antigen d with *S. typhi* but no other antigen.

(v) Vi antibody. Vi antibody in serum and jejunal fluid was measured by ELISA as described (22, 27); a fourfold increase was considered significant.

ASC assays. Gut-derived, trafficking antibody-secreting cells (ASC) that secrete IgG, IgA, or IgM antibody against *S. typhi* O, H, or Vi antigen were measured by a modification of the method of Forrest (5) by using both ELISA and ELIS POT as previously described (11, 28). Heparinized blood was drawn before vaccination and on days 7 and 10 after vaccination. Briefly, peripheral blood lymphocytes separated by a Ficoll gradient (Organon Teknika, Durham, N.C.) were added to antigen-coated plates. In the ELISA, binding of antibody secreted by lymphocytes was measured by the change in OD produced by reaction of the substrate with bound anti-IgA conjugate. Significant responses to LPS, H, and Vi antigens were determined by using the differences in OD plus 3 standard deviations generated from preimmunization and day 4 cells taken from volunteers participating in these studies (11). In the ELISPOT, specific IgA secreted by individual lymphocytes was detected by adding an agarose overlay to each well and counting colored spots produced by reaction of the substrate with bound anti-human IgA conjugate (28). Detection of four or more spots per four wells (i.e., four spots per 4 million cells) after vaccination was defined as a positive response; this number is based on the mean number of spots counted before vaccination plus 2 standard deviations.

RESULTS

Clinical responses. The clinical signs of volunteers after vaccination were evaluated in a double-blind fashion. The results for each volunteer are summarized in Table 2.

(i) CVD 906. Of 12 volunteers who received CVD 906 (*S. typhi* ISP1820 Δ*aroC* Δ*aroD*), 2 developed fever, 1 at the higher dose and 1 at the lower dose. Volunteer 13004-4 had a temperature of 38.6°C on day 1 after vaccination; on day 2, his temperature rose to 39.7°C, associated with anorexia and cramps requiring bedrest. On day 3, he remained febrile with continued mild cramps and headache but generally felt better. Blood cultures were negative. Volunteer 13003-5, who received CVD 906, also had a febrile illness. Beginning on day 6, this volunteer had headache, malaise, cramps, chills, body aches, cough, and sore throat, with fever beginning on day 7. His maximum temperature was 39.1°C on day 9. Blood cultures were negative.

No other vaccinees had adverse reactions defined by objective criteria, but three additional volunteers (13004-2, 13003-3, and 13003-6) reported subjective symptoms such as headache, anorexia, malaise, and abdominal cramps during the period of surveillance. None of these volunteers had an illness resembling typhoid fever.

(ii) CVD 908. In contrast, none of 12 volunteers who received 6×10^4 or 6×10^5 CFU of CVD 908 (*S. typhi* Ty2 Δ*aroC* Δ*aroD*) had fever during the observation period. Two volunteers (14002-6 and 14002-12) had subjective symptoms such as anorexia, malaise, cramps, headache, and sore throat, without fever, beginning on days 1 and 3, respectively.

(iii) χ3927. Of 12 volunteers who received strain χ3927 (*S. typhi* Ty2 Δ*cya* Δ*crp*), 1 had fever. Volunteer 14001-10 developed fever with a maximum temperature of 40.1°C on day 22 after vaccination. This volunteer had severe abdominal cramps, malaise, anorexia, headache, and vomiting on days 4 to 13, but his fever did not begin until day 22 and required readmission to the isolation ward for treatment. His symptoms then included dizziness, muscle and body aches, constipation, insomnia, and cough productive of brown sputum. His chest radiograph and sputum Gram stain were unremarkable; paired serum samples examined by the Maryland Department of Health and Mental Hygiene showed no changes in the titers of antibodies to influenza virus, parainfluenza virus, and enteroviruses.

Another volunteer (14002-14) in this group had malaise, cramps, headache, and nausea during the inpatient surveillance period.

Bacteriology. (i) CVD 906. One of six volunteers (13004-6) who received 6×10^4 CFU and none of six who received 6×10^5 CFU of CVD 906 had positive blood cultures (Table 2). The positive cultures occurred on days 4 and 5 after vaccination and were not associated with symptoms. None of the 12 recipients of CVD 906 at either dose had positive stool, tonsillar, or duodenal string cultures.

TABLE 2. Clinical, bacteriologic, and immune responses to vaccination with a single oral dose of attenuated *S. typhi*

Vaccine strain (CFU) and patient no.	Presence or absence of fever (≥38.2°C)	Presence or absence of *S. typhi* in:		Seroconversion with IgG anti-LPS
		Blood culture	Stool culture	
CVD 906 (6×10^5)				
13003-1	−	−	−	+
13003-2	−	−	−	+
13003-3[a]	−	−	−	−
13003-4	−	−	−	+
13003-5	+ (days 7–9)	−	−	−
13003-6[a]	−	−	−	−
CVD 906 (6×10^4)				
13004-1	−	−	−	+
13004-2[a]	−	−	−	−
13004-3	−	−	−	−
13004-4	+ (days 1–3)	−	−	+
13004-5	−	−	−	+
13004-6	−	+ (days 4 and 5)	−	+
CVD 908 (6×10^5)				
14001-2	−	−	−	−
14001-4	−	−	−	+
14001-7	−	−	+	+
14001-8	−	−	−	−
14001-9	−	−	+	−
CVD 908 (6×10^4)				
14002-1	−	−	−	−
14002-3	−	−	−	+
14002-4	−	−	−	+
14002-5	−	−	−	−
14002-6[a]	−	−	−	+
14002-10	−	−	−	−
14002-12[a]	−	−	−	+
χ3927 (6×10^5)				
14001-1	−	−	+	−
14001-3	−	−	−	−
14001-5	−	+ (days 8 and 12)	−	+
14001-6	−	−	−	−
14001-10	+ (days 22–25)	−	−	+
14001-11	−	−	−	−
χ3927 (5×10^4)				
14002-2	−	+ (day 15)	−	+
14002-7	−	−	−	+
14002-9	−	−	−	−
14002-11	−	−	−	−
14002-13	−	−	−	+
14002-14[a]	−	−	−	+

[a] Volunteers with subjective symptoms. See text for details.

(ii) CVD 908. None of 12 volunteers who received CVD 908 at either dose had positive blood cultures detected by the systematic sampling (Table 2). Two of five recipients of 5×10^5 CFU of CVD 908 had positive stool cultures. Vaccine was detected in the stool of one volunteer on day 0 ($<10^2$ CFU/g) and on day 1 (2×10^4 CFU/g); for the other volunteer, vaccine was detected in the stool on day 0 only (1.4×10^3 CFU/g). None of the CVD 908 vaccinees had positive tonsillar or duodenal string cultures.

TABLE 3. Immunologic responses to vaccination with oral attenuated *S. typhi* vaccine strains

Antibody test	No. positive/no. tested with indicated CFU of vaccine strain:					
	CVD 906		CVD 908		χ3927	
	6×10^4	6×10^5	6×10^4	6×10^5	5×10^4	6×10^5
S. typhi LPS						
IgG	4/6[a]	3/6[b]	4/7[c]	2/5[d]	4/6[c]	2/6[c]
IgA	3/6	0/6	2/7	0/5	1/6	2/6
H						
IgG	3/6	0/6	1/7	2/5	0/6	0/6
IgA	0/6	0/6	0/7	1/5	0/6	0/6
Vi						
IgG	0/6	0/6	1/7	0/5	0/6	0/6
IgA	0/6	0/6	0/7	1/5	0/6	0/6
Widal H	1/6	1/6	0/7	3/5	0/6	0/6
Jejunal fluid sIgA[e]						
LPS	0/6	1/6	0/7	2/5	0/6	1/6
H	0/6	0/6	0/7	0/5	0/6	1/6
Vi	0/6	0/6	0/7	0/5	0/6	0/6
ASC-LPS[f]						
IgG	3/6	3/6	5/6	2/5	1/6	2/6
IgA	4/6	3/6	5/6	4/5	2/6	3/6
IgM	4/6	2/6	3/6	4/5	0/6	4/6
ASC-H						
IgG	3/5	2/6	0/6	0/5	0/5	1/6
IgA	2/5	5/6	3/6	3/5	3/5	3/6
IgM	3/5	2/6	3/6	2/5	0/5	0/6
ASC-Vi						
IgG	0/6	1/6	0/6	2/5	0/6	1/6
IgA	1/6	1/6	0/6	0/5	0/6	0/6
IgM	0/6	0/6	0/6	0/5	0/6	0/6

[a] Ratio of mean post- to mean prevaccination OD = 2.1.
[b] Ratio of mean post- to mean prevaccination OD = 2.2.
[c] Ratio of mean post- to mean prevaccination OD = 1.6.
[d] Ratio of mean post- to mean prevaccination OD = 1.8.
[e] sIgA, secretory IgA.
[f] ASC detected by either ELISA or ELISPOT.

The blood and stool isolates of Δ*aroC* Δ*aroD* strains recovered from volunteers retained their auxotrophic phenotype when growth on minimal media was compared with growth on supplemented media.

(iii) χ3927. One (14002-2) of six volunteers who received 5×10^4 CFU and one (14001-5) of six volunteers who received 5×10^5 CFU of χ3927 had positive blood cultures. These occurred on day 15 and days 8 and 12, respectively. Neither of these volunteers had any symptoms. Of the 12 volunteers who received χ3927, 1 had one colony of vaccine organisms detected in the stool on day 1. None of these volunteers had positive tonsillar or duodenal string cultures. The χ3927 isolates recovered from the blood and stool specimens of volunteers retained all expected phenotypes associated with the presence of the Δ*cya* Δ*crp* mutations.

Immunology. The immunologic responses to vaccination are shown in Table 3. Overall, 33 (92%) of 36 volunteers showed an immune response by one or more serologic tests. The rate of seroconversion of serum IgG anti-LPS antibodies has been shown to correlate with protective efficacy, as

measured in field trials with individuals who received typhoid vaccine strain Ty21a (19). Although very low numbers of vaccine organisms were given in this study and in a single dose, 19 (53%) of 36 volunteers who received any vaccine at either dose seroconverted with IgG anti-LPS antibody. The rates of seroconversion did not vary significantly among recipients of the three vaccines.

(i) CVD 906. Of 12 recipients of CVD 906, 7 (58%) developed IgG antibody responses to *S. typhi* LPS. Overall, 3 of 12 vaccinees developed IgG anti-H antigen, and none had antibodies to Vi antigen after vaccination.

Only one of the volunteers developed secretory IgA antibody to LPS detectable in jejunal fluid. Secretory antibodies to H and Vi antigen in jejunal fluid were not detected after vaccination.

Development of circulating *S. typhi*-specific ASC after vaccination represents a priming of the mucosal secretory immune system. Of 12 volunteers, 7 had detectable circulating B cells secreting IgA antibody to LPS. The range of prevaccination spots was 0 to 2, and the range of spots counted after vaccination was 6 to 175 among all vaccine recipients. Table 3 shows the rates of responses detected by either ELISA or ELISPOT.

(ii) CVD 908. Of 12 recipients of CVD 908, 6 (50%) developed IgG antibodies to LPS. Of the 12 volunteers, 3 had IgG antibody to H antigen; 2 volunteers in this group developed Vi antibodies.

Of 12 recipients of CVD 908, 2 developed secretory IgA anti-LPS. Secretory antibodies to H and Vi antigen were not detectable after vaccination.

Of 11 vaccinees, 9 had detectable cells secreting IgA antibody to LPS.

(iii) χ3927. Of 12 vaccinees who received χ3927, 6 (50%) developed IgG anti-*S. typhi* LPS responses. No antibody to H or Vi antigen was detected in any of the 12 volunteers.

Only 1 of the 12 volunteers developed secretory IgA against LPS in the jejunal fluid. Secretory IgA antibody responses to H antigen occurred in only 1 volunteer, and no volunteer had secretory anti-Vi antibody after vaccination.

Of the 12 volunteers, 5 developed circulating cells secreting IgA against LPS detected by ELISA or ELISPOT.

Correlation of symptoms with immune response. The occurrence of fever or symptoms in vaccinees did not correlate with subsequent seroconversion with IgG anti-LPS antibodies (Table 2). Two (67%) of three volunteers with fever and 17 (52%) of 33 volunteers without fever seroconverted (*P*, not significant); 5 (56%) of 9 volunteers with any symptoms and 14 (52%) of 27 volunteers without symptoms seroconverted. Even recovery of the vaccine strains in blood cultures did not statistically correlate with seroconversion, although the sample size is small. Three of three vaccinees from whom vaccine was isolated from the blood and 16 (48%) of 33 with negative blood cultures seroconverted with IgG antibodies against LPS ($P = 0.14$, Fisher's exact test, one-tailed).

DISCUSSION

In this comparative study of three live oral *S. typhi* vaccine candidates, one strain emerges as a desirable candidate for further studies. CVD 908 (Ty2 Δ*aroC* Δ*aroD*) did not produce fever or vaccine bacteremia in the small number of volunteers studied, yet it was as immunogenic as CVD 906 and χ3927, which did cause fevers or bacteremia in some volunteers. Further studies with more volunteers are needed to confirm the apparent safety of CVD 908, since these differences among strains are not statistically significant. Each vaccine produced approximately 50% IgG seroconversion to *S. typhi* LPS, a parameter which may correlate with protective efficacy (19). This rate of seroconversion compares favorably with that seen in volunteers after three doses of much higher numbers of oral typhoid vaccine strain Ty21a (2, 19).

These vaccine candidates were designed on the basis of clinical behavior in animal models of analogous mutants constructed in *S. typhimurium* (3, 4, 14, 17, 24, 25). *aro*-deleted *S. typhimurium* is widely considered a safe and effective veterinary vaccine (4, 14, 24, 25). It should be noted that in calves, fever and vaccine bacteremia were occasionally detected in some studies in which appropriate monitoring was done (14, 23, 24). Although animal models of typhoid using attenuated *S. typhimurium* do not always predict responses in humans given attenuated *S. typhi* (9), our experience with volunteers in whom occasional febrile responses and bacteremia occurred is actually very similar to the reported experience with calves vaccinated with Δ*aro S. typhimurium* (14, 24).

The degree of attenuation conferred by deletions in the aromatic amino acid biosynthetic pathway and in the cAMP regulatory pathway cannot be strictly measured without simultaneous challenge of volunteers with mutant and parent strains. However, on the basis of historical experience with volunteers given similar doses of wild-type strains (12), it is likely that both types of deletions confer attenuation to *S. typhi*, just as these deletions attenuate *S. typhimurium* in animal models. When wild-type *S. typhi* Ty2 was fed to six volunteers at a dose of 10^7 CFU without bicarbonate, 83% developed typhoid fever (defined as temperature ≥103°F [ca. 39.4°C] for >36 h) or infection (defined as low-grade fever, significant serologic response, positive blood culture, or excretion of *S. typhi* in stools for >5 days) (12). Of 116 volunteers given 10^5 CFU of the Quailes *S. typhi* strain without bicarbonate, 28% developed disease (12). In contrast, among the 24 volunteers of the present study who received vaccines derived from Ty2 at a dose of 10^4 or 10^5 CFU with bicarbonate (equivalent to a much higher dose without bicarbonate), fever occurred in only 1 volunteer and blood cultures were positive for only 2 volunteers.

The availability of these vaccines provided a unique opportunity to compare strategies for attenuating *S. typhi*. By comparing the same mutations in two different background *S. typhi* strains and by comparing different mutations in the same strain, some preliminary impressions about the relative virulence of the parent strains and the relative degree of attenuation conferred by the mutations can be formed. By comparing CVD 906 (ISP1820 Δ*aroC* Δ*aroD*) and CVD 908 (Ty2 Δ*aroC* Δ*aroD*) it appears that the modern isolate ISP1820 may be a more virulent strain than venerable Ty2. Fevers and vaccine bacteremia occurred in a few volunteers who received CVD 906 but not in those who received CVD 908. These differences may disappear when larger numbers of volunteers are vaccinated. Ty2 may have somewhat diminished virulence consequent to in vitro passage and storage over the decades since its original isolation. Nevertheless, the wild-type Ty2 parent is known from volunteer studies to be pathogenic (12), and other attempts at attenuation of Ty2 in which a Δ*galE* Δ*viaA* mutant was given at a dose of 7×10^8 CFU completely failed (9). Conversely, the *cya crp* deletions appear to be less attenuating than *aroC aroD* deletions when the responses to CVD 908 vaccination are compared with responses to χ3927 vaccination. Of the small number of volunteers studied, fever and vaccine bacteremia occurred in a few volunteers who received χ3927 but not in those who received CVD 908.

The clinical consequences of vaccine bacteremia, if any, will be determined in future studies. Ty21a vaccine strain has set a high standard of typhoid vaccine safety which future vaccine strains must match. Further studies with more volunteers are needed to document and quantify this difference in clinical response to these strains.

This study is the first to describe the direct comparison of carefully constructed vaccine strains with well-defined mutations introduced into an identical *Salmonella* background. We are confident that the application of biotechnology that allows defined mutations to be introduced into *S. typhi* will lead to improved vaccines against typhoid fever and, more importantly, to a carrier strain to express protective antigens of other organisms.

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