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Safety of Live Oral *Salmonella typhi* Vaccine Strains with Deletions in *htrA* and *aroC aroD* and Immune Response in Humans

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A single-dose, oral *Salmonella typhi* vaccine strain has been sought as a carrier or vector of cloned genes encoding protective antigens of other pathogens. Such a hybrid vaccine, administered orally, would stimulate immune responses both at the mucosal surface and in the systemic compartment and would potentially provide protection against multiple pathogens. *S. typhi* CVD 908 and CVD 906, which harbor deletions in *aroC* and *aroD*, were further engineered by deletion in *htrA* to produce strains CVD 908-*htrA* and CVD 906-*htrA*, which are unable to sustain growth and are severely impaired in their ability to survive in host tissues. These strains were fed to humans at doses of 5×10^7 to 5×10^9 CFU with buffer, and safety and immune responses were assessed. CVD 908-*htrA* and CVD 906-*htrA* were well tolerated in volunteers; mild diarrhea in 3 of 36 volunteers and mild fever in 1 volunteer were the only notable adverse responses. The vaccine strains were not detected in blood cultures and only transiently detected in stool. Serum immune responses to *S. typhi* lipopolysaccharide and H antigens were observed in 75 to 100% of volunteers who received 5×10^8 to 5×10^9 CFU, and cells secreting *S. typhi*-specific antibodies were found in all volunteers after ingestion of either strain. Sixty-three percent to 83% of volunteers developed lymphoproliferative responses to *S. typhi* flagellar and particulate antigens after the higher doses. These studies demonstrate the potential of CVD 908-*htrA* as a live vector for the delivery of heterologous genes, and a clinical trial of such a construct is planned.

Salmonellae may be attenuated in a number of ways for use as oral vaccine strains; for example, the licensed oral *Salmonella typhi* vaccine strain Ty21a was prepared by chemical mutagenesis (5). More recently, *S. typhi* has been attenuated for human use by deleting genes encoding adenylate cyclase and the cyclic AMP receptor protein (Δ*cya* Δ*crp*) (18) or by deleting genes of the *phoP-phoQ* virulence regulon (8). Another strategy for attenuating salmonellae has been by introducing defined deletions into the genes encoding enzymes of the aromatic amino acid biosynthesis pathway, thereby rendering the bacteria auxotrophic for *para*-aminobenzoic acid (PABA) and dihydroxybenzoate; these are substrates that the organism cannot scavenge in sufficient quantity in mammalian tissues to sustain growth. Such *aro*-deleted mutants of *Salmonella typhimurium* are safe and immunogenic as live oral vaccines in mice and cattle (1, 9, 13, 15). Analogous auxotrophic mutants of *S. typhi* have been prepared as typhoid vaccines for humans. These strains will ultimately be used as live vectors for cloned genes encoding protective antigens of other pathogens. Several attenuated *S. typhi* oral vaccines have been constructed to serve as vectors for heterologous antigens, including antigens of *Shigella sonnei* (4), *Escherichia coli* (3, 6), *Vibrio cholerae* (17), *Clostridium tetani* (2), *Bordetella pertussis* (20), and *Plasmodium falciparum* (7).

In recent studies at the Center for Vaccine Development, two vaccine strains harboring deletion mutations in *aroC* and *aroD* (10) have been evaluated as candidate live oral vaccines in adult volunteers (11, 18, 19). One of these strains, CVD 908, a derivative of the wild-type strain *S. typhi* Ty2, was well tolerated and highly immunogenic when given to volunteers in phase 1 studies after having been freshly harvested from solid agar plates and washed (18, 19). These *aroC aroD* deletion mutants were significantly more immunogenic after a single oral dose than is the currently licensed live oral vaccine strain Ty21a after multiple doses. However, at a dose of 5×10^7 CFU, CVD 908 caused clinically silent, self-limiting vaccine bacteremias between days 4 and 8 after vaccination in about half of the volunteers. Another Δ*aroC* Δ*aroD* strain, CVD 906, a derivative of wild-type strain ISP1820, was highly immunogenic but caused fever in a significant proportion of vaccinees (11). Additional attenuating mutations in CVD 908 and CVD 906 were sought.

The *htrA* gene locus was chosen as the site of genetic deletion to further attenuate CVD 908 and CVD 906. The gene *htrA* encodes a heat shock protein in *S. typhimurium*. When this gene is deleted, the resulting mutant is less virulent because of impaired ability to survive and/or replicate in host tissues (12). In vitro, *htrA* mutants of *S. typhimurium* are more susceptible to oxidative stress than the wild type, suggesting that the mutant may be less able to withstand oxidative killing within macrophages. The purpose of this study was to assess the safety in humans of two new strains derived from CVD 908 and CVD 906, CVD 908-*htrA* and CVD 906-*htrA*, which were expected to be further attenuated by deletions in the *htrA* gene. In addition, studies of the humoral, cellular, and mucosal immune responses to these vaccines in humans were conducted.

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MATERIALS AND METHODS

Study 1. A group of 13 adult volunteers, age 18 to 40 years, was admitted to the Center for Vaccine Development Research Isolation Ward, located in the University of Maryland Hospital, to participate in a randomized, double-blind study. Following appropriate screening, a detailed explanation of the protocol, and obtaining of informed consent, baseline collections of blood and jejunal fluid were obtained for measurement of immune responses. Two days after admission, the volunteers were randomized to receive a single oral dose of (i) 5×10^7 CFU of CVD 908-*htrA* with 2 g of $NaHCO_3$ ($n = 7$) or (ii) 5×10^7 CFU of CVD 906-*htrA* with 2 g of $NaHCO_3$ ($n = 6$).

Study 2. A second study was conducted with another cohort of 26 volunteers who were admitted to the Isolation Ward and randomized to receive in a double-blind manner either (i) 5×10^8 CFU of CVD 908-*htrA* with 2 g of $NaHCO_3$ ($n = 8$), (ii) 5×10^9 CFU of CVD 908-*htrA* with 2 g of $NaHCO_3$ ($n = 7$), (iii) 5×10^8 CFU of CVD 906-*htrA* with 2 g of $NaHCO_3$ ($n = 8$), or (iv) a placebo consisting of 2 g of $NaHCO_3$ ($n = 3$). This study began after the results of the first study had been reviewed by an unblinded clinical monitor. The placebo group was included in the second study to provide controls for immunologic assays and to begin to assess the potential for person-to-person spread of vaccine.

Preparation of vaccine inocula. Stock cultures of *S. typhi* CVD 908-*htrA* and CVD 906-*htrA* vaccines were thawed and plated onto Aro agar supplemented with 0.1% (wt/vol) PABA and 0.1% (wt/vol) *p*-hydroxybenzoic acid 2 days before challenge as previously described (18). The growth on these plates was harvested and washed twice with sterile phosphate-buffered saline. Dilutions were made in phosphate-buffered saline to approximate the concentration of salmonellae required. Microscopic examination and slide agglutination with *S. typhi* O, H, and Vi antisera were performed before use. Replica spread plate quantitative cultures were made of the inocula before and after vaccination to confirm viability and inoculum size.

Vaccination. The vaccines were administered by the oral route with $NaHCO_3$. Volunteers had nothing to eat or drink for 90 min before and after vaccination. Two grams of $NaHCO_3$ was dissolved in 5 oz of distilled water. The volunteers drank 4 oz of the bicarbonate solution; 1 min later, the volunteers ingested the vaccine suspended in the remaining 1 oz of bicarbonate solution.

Clinical and bacteriologic surveillance. The volunteers in both studies were closely observed for 14 days after vaccination. During the period of clinical observation, all stools were graded on a scale of 1 to 5 according to whether they were formed (grades 1 or 2) or liquid (grade 3, stool that takes the shape of its container; grade 4, opaque watery stool; grade 5, rice water stool) and cultured to detect excretion of the vaccine strain. Stools were also cultured on days 21, 28, and 60. On days 7, 10, and 13, each volunteer swallowed a gelatin string device (Enterotest) to collect duodenal fluid and bile for culture. Multiple blood cultures to detect vaccine organisms were obtained on days 4, 5, 7, 8, 10, 12, 21, 28, and 60 after vaccination to detect vaccine bacteremia. Stools, rectal swabs, and the distal 15 cm of bile-stained duodenal string were inoculated directly onto supplemented *Salmonella-Shigella* agar and into gram-negative enrichment broth supplemented with PABA and *p*-hydroxybenzoic acid. After overnight incubation at 37°C, subcultures were made onto *Salmonella-Shigella* agar. Suspicious colonies were transferred to triple sugar iron slants, and confirmation was made by agglutination with *S. typhi* O, H, and Vi antisera. Quantitative cultures were done on stool samples collected on days 0 to 4. Blood cultures were performed by inoculating 7 ml of blood into 50 ml of Septachek broth.

Humoral and local immunologic responses. The humoral and local intestinal immune responses to the vaccine strains were assessed. Blood was collected before vaccination and on days 7, 21, 28, and 60 after vaccination to measure serum antibodies to *S. typhi* O and H antigens by enzyme-linked immunosorbent assay (ELISA) (14). For lipopolysaccharide (LPS) immunoglobulin G (IgG) antibody, a change in optical density at 405 nm of ≥0.2 from before to after sample collection was considered positive at a 1:100 dilution of serum, as previously described (14). For H and Vi antibodies, a fourfold rise in titer was considered positive. Before and on day 13 after vaccination, approximately 70 ml of jejunal fluid were collected by intubation to measure secretory IgA (sIgA) responses to typhoid antigens O, H, and Vi (14). Total IgA in the jejunal fluids was measured by enzyme-linked immunosorbent assay, and each specimen was standardized to contain 20 mg of IgA per 100 ml of jejunal fluid by lyophilizing the fluid and resuspending in the appropriate volume (14). A fourfold rise in sIgA titer was reported as positive.

Before and on days 7 and 10 after vaccination, peripheral blood lymphocytes were collected and separated on Lymphocyte Separation Medium (Organon Teknika, Durham, N.C.) to detect antibody-secreting cells by ELISPOT assay (19); cells secreting antibody to *S. typhi* LPS O antigen were sought. The presence of four or more antibody-secreting cells (ASC) per 10^6 peripheral blood mononuclear cells (PBMC) was considered positive.

Cellular immune responses. PBMC from volunteers before vaccination and on day 28 (for study 1) and on days 12 and 28 (for study 2) after vaccination were isolated by density gradient centrifugation on Lymphocyte Separation Medium and immediately used for studies of proliferative responses to *S. typhi* antigens as previously described (16). *S. typhi* flagellar antigen was purified from the *aroA S. typhi* 541Ty strain by standard procedures and used at 4 μg/ml (16). The purity of this preparation was confirmed by the presence of a single band of approximately 50 kDa by silver staining of sodium dodecyl sulfate-polyacrylamide gels. This flagellar preparation was found to contain less than 24 pg of LPS per ml, as determined by the E-Toxage *Limulus* amebocyte lysate assay (Sigma, St. Louis, Mo.). Particulate *S. typhi* consisted of Ty2 heat-phenol-killed whole-cell bacteria (Wyeth typhoid vaccine; Wyeth Laboratories, Marietta, Pa.) prepared as described previously and used at an *S. typhi* particle/PBMC ratio of 3:1 (16). Bovine serum albumin (BSA) (Fraction V; Sigma) was used as a control antigen in these studies at a concentration of 4 μg/ml. Phytohemagglutinin (PHA) (HA-17; Wellcome Diagnostics, Beckenham, United Kingdom) was used at a concentration of 2 μg/ml to confirm the ability of PBMC obtained from immunized volunteers to proliferate in response to mitogenic stimulation. Cultures were pulsed with [^{3}H]thymidine 6 days after stimulation with specific *S. typhi* antigens or 2 days after stimulation with PHA and harvested 18 h later as previously described (16).

Statistical analysis. Mouse antibody titers were compared by the Wilcoxon rank-sum test. For PBMC proliferative responses, the mean counts per minute of triplicate wells from cells obtained 12 or 28 days after immunization in response to antigen were compared with the mean counts per minute on day 0 for each volunteer by one-tailed *t* tests with Bonferroni corrections for multiple comparisons. Mean changes in proliferation for each group before and after stimulation were analyzed by one-tailed paired *t* tests with Bonferroni corrections. Correlations of the magnitudes of immunologic responses to vaccination were evaluated by Spearman's method. Magnitudes of responses were assessed for serological responses as the fold rise of pre- to peak postimmunization responses and for lymphocyte replication as mean counts per minute. Comparison of the proliferative responses to *S. typhi*-specific antigens among the different groups (*S. typhi* vaccine strains CVD 908-*htrA* and CVD 906-*htrA* at the various doses) were evaluated by repeated-measures analysis of variance (the dependent variable was counts per minute; the independent variables were vaccines, day, and their interaction; repeated across subjects). Statistical tests for which the resulting probability was <5% were considered significant. *P* values reported here are Bonferroni corrected where appropriate and should be evaluated at the 5% level.

RESULTS

Clinical and bacteriologic results. The dose response to CVD 908-*htrA* is shown in Table 1. For comparison, data from previous studies of CVD 908 (18, 19) are also displayed. The major difference between CVD 908-*htrA* and CVD 908 is the lack of vaccine bacteremia observed after vaccination with CVD 908-*htrA*. In addition, two volunteers who received either 10^8 or 10^9 CFU of CVD 908-*htrA*, but not CVD 908, had mild diarrhea after vaccination. One volunteer who received 5×10^9 CFU had low-grade fever (maximum temperature, 38.3°C) with mild headache for one day only on day 1. All duodenal fluid cultures were negative. None of the three placebo recipients shed vaccine organisms in their stools or developed *S. typhi*-specific immune responses during the study.

The dose response to CVD 906-*htrA* is shown in Table 2 along with previously published data for vaccination with CVD 906 for comparison (11). Fever was not observed in any volunteer who received CVD 906-*htrA* at a dose of 5×10^7 or 5×10^8 CFU, while two of nine volunteers who had received CVD 906 at a dose of 5×10^7 CFU in a previous study (11) had fever. Blood cultures, which were positive in five of nine volunteers who had received CVD 906 in the previous study (11), were negative in the volunteers who received CVD 906-*htrA*, even in those volunteers who received 10-fold more organisms of CVD 906-*htrA*. All adequate duodenal fluid specimens were culture negative.

Humoral and local immunologic responses. The immune responses measured by seroconversion of IgG anti-LPS after vaccination with CVD 908-*htrA* were similar to those seen after vaccination with CVD 908 (Table 1); the range of peak optical densities among seroconverters after approximately 5×10^8 CFU of CVD 908-*htrA* was 0.39 to 2.87, and that after a similar dose of CVD 908 was 1.46 to 2.87. Serum IgG responses to H antigen were also observed after vaccination with CVD 908-*htrA* in five of seven volunteers who received 5×10^7 CFU (reciprocal geometric mean titer [GMT] of 400 for all volunteers), in six of eight volunteers who received 5×10^8 CFU (reciprocal GMT, 1,234), and in five of seven volunteers who received 5×10^9 CFU (reciprocal GMT, 1,189). The serum

TABLE 1. Clinical, bacteriologic, and immunologic responses to CVD 908-*htrA* and CVD 908

Response	No. positive/total no. after vaccination with strain:				
	CVD 908-*htrA*			CVD 908[a]	
	5×10^7 CFU	5×10^8 CFU	5×10^9 CFU	5×10^7 CFU	5×10^8 CFU
Fever (maximum)	0/7	0/8	1/7 (38.3°C, day 1)	0/12	1/6 (38.7°C, day 5)
Diarrhea (total vol)	0/7	1/8 (403 ml, days 9–11)	1/7 (1,055 ml, day 0)	0/12	0/6
Positive blood cultures	0/7	0/8	0/7	6/12 (days 4, 5, 7, 8)	6/6 (days 4, 5, 6, 7)
Positive stool cultures	4/7 (days 0–1)	6/8 (days 0–2)	7/7 (days 0–2)	7/12 (days 0–2)	6/6 (days 0–1)
Serum IgG anti-LPS	5/7	8/8	7/7	11/12	6/6
Serum IgG anti-H (GMT[b])	5/7 (400)	6/8 (1,234)	5/7 (1,189)	4/6 (504)	6/6 (5,701)
ASC IgA anti-LPS (mean no. of spots)[c]	7/7 (99)	8/8 (122)	7/7 (169)	11/12 (357)	6/6 (1,062)
Lymphocyte proliferation to *S. typhi* flagella	5/7	4/6	5/7	5/6	ND[d]
Lymphocyte proliferation to *S. typhi* particulate antigen	ND	5/6	5/7	5/6	ND

[a] Data are from references 18 and 19 and unpublished data.
[b] Peak reciprocal GMT.
[c] Number of ASC per 10^6 PBMC measured by ELISPOT assay.
[d] ND, not determined.

IgG anti-H antigen GMT before vaccination was 144. No volunteer developed a rise in serum IgG, IgA, or IgM against Vi antigen. The rate of ASC responses after vaccination with CVD 908-*htrA* was also similar to that seen after CVD 908, although vaccination with CVD 908 resulted in higher numbers of ASC per 10^6 PBMC (Table 1). Since the number of ASC which reflects protective immunity is not known, the significance of these lower numbers of ASC is not certain. One of seven volunteers who received 5×10^7 CFU of CVD 908-*htrA* developed a rise in jejunal fluid sIgA anti-LPS and sIgA anti-H; another volunteer of eight volunteers who received 5×10^8 CFU developed sIgA anti-LPS (data not shown).

The seroconversion rates and ASC response rates were similar after vaccination with CVD 906-*htrA* and CVD 906, although the total number of ASC per 10^6 PBMC was less in the CVD 906-*htrA* group than in the CVD 906 group (Table 2). No secretory anti-*S. typhi* antibodies were detected in jejunal fluids from these vaccinees (data not shown).

Cellular immune responses. Since *S. typhi* is an intracellular pathogen, cellular immune responses, including cytokine production, proliferation, and cytotoxic T-cell activity, are important effector mechanisms in the response to *S. typhi*. The relative importance of each is as yet unknown. PBMC obtained before and 28 days after immunization with CVD 908-*htrA* and CVD 906-*htrA* (in study 1) and before and at 12 and 28 days after immunization (in study 2) were studied in lymphoproliferative assays. Cells isolated from the first vaccine trial were evaluated against purified *S. typhi* flagella, while PBMC obtained from the second study were evaluated against purified *S. typhi* flagella and particulate heat-phenol-killed whole-cell *S. typhi*. In study 1, significant increases in proliferative responses to *S. typhi* flagella were observed after immunization with either CVD 908-*htrA* or CVD 906-*htrA* at 5×10^7 CFU (data not shown). In study 2 (Fig. 1), increases in proliferative responses to *S. typhi* flagella (Fig. 1A) and particulate whole-cell *S. typhi* (Fig. 1B) were observed as early as 12 days after immunization with CVD 908-*htrA* (administered at a dose of 5×10^8 or 5×10^9 CFU) and CVD 906-*htrA* (administered at a dose of 5×10^8 CFU). Comparable proliferative responses were observed 28 days after immunization (Fig. 1A and B). No significant differences were observed in the proliferative responses to *S. typhi* antigens among placebo recipients at either time point (Fig. 1A and B) or to BSA antigen by any of the groups (Fig. 1C). PBMC from all volunteers and at all time points exhibited vigorous proliferative responses to PHA stimulation (range, 78,659 to 250,100 cpm; data not shown). The rates of significant responses to *S. typhi* antigens in both studies are shown in

TABLE 2. Clinical, bacteriologic, and immunologic responses to CVD 906-*htrA* and CVD 906

Response	No. positive/total no. after vaccination with strain:		
	CVD 906-*htrA*		CVD 906 (5×10^7 CFU)[a]
	5×10^7 CFU	5×10^8 CFU	
Fever	0/6	0/8	2/9
Diarrhea (total vol)	0/6	1/8 (396 ml, day 6)	0/9
Positive blood cultures	0/6	0/8	5/9
Positive stool cultures	4/6 (days 0–2)	6/8 (days 0–2)	9/9 (days 0–3, 10, 11)
Serum IgG anti-LPS	4/6	6/8	8/9
Serum IgG anti-H (GMT[b])	3/6 (635)	6/8 (436)	7/9 (2,352)
ASC IgA anti-LPS (mean no. of spots)[c]	6/6 (121)	8/8 (81)	9/9 (1,751)
Lymphocyte proliferation to *S. typhi* flagella	3/6	5/8	7/9
Lymphocyte proliferation to *S. typhi* particulate antigen	ND[d]	6/8	5/9

[a] Data are from reference 11.
[b] Peak reciprocal GMT.
[c] Number of ASC per 10^6 PBMC measured by ELISPOT assay.
[d] ND, not determined.

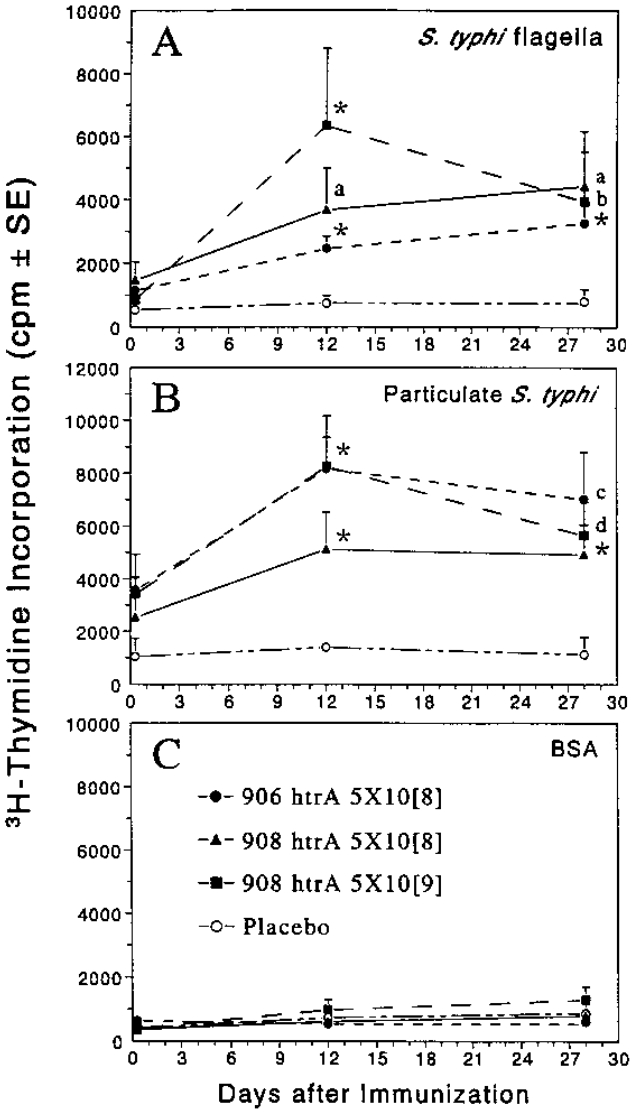

FIG. 1. Proliferative responses by volunteers immunized orally with the CVD 908-*htrA* and CVD 906-*htrA* attenuated strains of *S. typhi*. PBMC from volunteers immunized orally with CVD 908-*htrA* (administered at a dose of 5×10^8 or 5×10^9 CFU), CVD 906-*htrA* (administered at a dose of 5×10^8 CFU), or a placebo were obtained before and 12 and 28 days after immunization. Cells were evaluated for proliferative responses to *S. typhi* specific antigens or BSA as described in Materials and Methods. Results are expressed as mean counts per minute ± standard error (SE) for all volunteers in each group. Comparison of values before and after immunization in each group: *, $P < 0.05$; a, $P = 0.16$; b, $P = 0.07$; c, $P = 0.06$; d, $P = 0.11$ (Bonferroni corrected).

Tables 1 and 2, along with previously published data describing responses to CVD 908 and CVD 906 (16) for comparison.

Significant positive correlations (by Spearman's analysis) were observed between the magnitudes of proliferative responses to *S. typhi* flagella and particulate whole-cell *S. typhi* in volunteers immunized with CVD 908-*htrA* administered at a dose of 5×10^8 or 5×10^9 CFU. This observation is similar to our previous findings for volunteers immunized with CVD 908 (16). Proliferative responses to *S. typhi* flagella and particulate whole-cell *S. typhi* were significantly higher (by repeated-measures analysis of variance) in volunteers immunized with either CVD 908-*htrA* or CVD 906 *htrA* than in placebo recipients. Furthermore, proliferative responses to *S. typhi* flagella were significantly higher ($P < 0.05$) in volunteers immunized with CVD 908-*htrA* administered at the 5×10^9 CFU dose than in those immunized with 5×10^8 CFU of either CVD 908-*htrA* or CVD 906-*htrA*.

DISCUSSION

The use of live attenuated bacterial vectors for delivery of vaccine antigens continues to be a promising method to stimulate systemic and mucosal immune responses. The first step in this strategy is to develop a well-tolerated and highly immunogenic live vector strain. *S. typhi* CVD 908-*htrA*, at doses of 5×10^7 to 5×10^9 CFU, meets these requirements in these phase 1 studies with volunteers in the United States. CVD 908-*htrA* offers an advantage compared to CVD 908 in that detectable vaccine bacteremia does not occur after vaccination with CVD 908-*htrA*. However, vaccine bacteremia with CVD 908-*htrA* may occur in low numbers not detected by routine culture methods and may lead to seeding of the deep organs of the reticuloendothelial system (such as the spleen, liver, and bone marrow). In fact, this may be a desirable feature, especially in stimulating cell-mediated immune responses against cloned heterologous antigens of pathogens such as *P. falciparum* (7) or pathogens that inhabit intracellular niches. In addition, mutation in the *htrA* gene may be desirable in that such mutants have reduced proteolytic degradation of aberrant periplasmic proteins (12), such as certain cloned foreign antigens. CVD 906-*htrA* was also well tolerated compared to its parent strain CVD 906; however, this strain is less desirable as a vaccine vector since only a single mutation in *htrA* distinguishes CVD 906-*htrA* from the reactogenic CVD 906. The immunogenicities of the *htrA* mutants compared to those of their parent strains may have been somewhat less, but whether this difference would affect the level of protective immunity is unknown.

Curiously, CVD 908-*htrA* appeared to cause mild diarrhea in a few volunteers. Loose stools occurred on days 9 to 11 in one volunteer and on day 0 (first 24 h after vaccination) in another; this difference in timing of the onset of diarrhea further obscures the pathogenic mechanism. One hypothesis to explain these data is that genetic mutation of *S. typhi* changes the interaction of the organism with the mucosa, resulting in release of cytokines that might contribute to the development of diarrhea. This response is similar to the mild diarrhea observed after vaccination with strain χ4073, a Δ*cya* Δ*crp* Δ*cdt* derivative of wild-type *S. typhi* Ty2 (16a), and after administration of strain Ty800, a Δ*phoP*/*phoQ* derivative of Ty2 (8). Whether loose stools after vaccination will be a significant event will be determined in future studies of CVD 908-*htrA* conducted with outpatient adults and, ultimately, with children in developing countries. It is encouraging that loose stools were not observed after the lowest dose (5×10^7 CFU) and yet the immune response was maintained at this dose.

Without a randomized trial and the use of standardized immunologic assays to measure immune responses, it is difficult to draw conclusions about the relative immunogenicities of CVD 908-*htrA*, Ty800, and χ4073. Nevertheless, it appears that all three of these strains when given to small numbers of subjects at similar dosage levels are able to elicit intestinal immune responses (detected by IgA ASC) and systemic antibody responses. A more detailed comparison must await further clinical trials.

The ability of PBMC isolated from volunteers immunized with the newly developed CVD 908-*htrA* and CVD 906-*htrA* vaccine strains to proliferate in response to specific *S. typhi* flagella and whole-cell *S. typhi* particles confirms and extends

similar observations we have previously made with volunteers immunized orally with CVD 908 and CVD 906 (16). Despite the conservative statistical analytic approach and modest sample size, the majority of volunteers exhibited significantly increased proliferative responses to *S. typhi*-specific antigens compared to preimmunization levels. In the present studies we found that proliferative responses to these specific *S. typhi* antigens persisted at similar levels up to 28 days after oral immunization with CVD 908-*htrA* and CVD 906-*htrA* at doses of 5×10^8 to 5×10^9 CFU. These results differ somewhat from our previous observations showing moderately decreased proliferative responses to flagella and particulate *S. typhi* antigens 28 days after vaccination with CVD 908 expressing the circumsporozoite protein of *P. falciparum* as compared to the responses observed 14 days after immunization (16). This decline in proliferative responses to *S. typhi* antigens 28 days after immunization could have been a consequence of the presence of a foreign gene in the CVD 908-*P. falciparum* CSP constructs or of the fact that two vaccine doses (administered at days 0 and 8) were used in the CVD 908-CSP trial (16) while only one dose was given in the present study. Alternatively, since both studies involved a relatively small number of volunteers and only two time points were evaluated, the difference in kinetics between them might be the result of individual variations among different donors. In any event, the fact that PBMC obtained from CVD 908-*htrA*-immunized volunteers still exhibited maximal proliferative responses to *S. typhi* antigens 28 days after immunization supports the potential of these attenuated vaccine constructs as single-dose typhoid vaccine candidates and as carriers of foreign antigens.

CVD 908-*htrA* will soon be used as an orally administered carrier of the heterologous antigen tetanus toxin fragment C in upcoming phase 1 clinical studies. These studies, to be conducted with volunteers with negative or low titers of antitetanus toxin, will test the principle that a mucosally delivered vaccine vector can stimulate circulating serum immunoglobulin in humans. If successful, this strategy will advance the development of other orally administered *S. typhi*-based vaccines against enteric, respiratory, and systemic infections.

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