

# Evaluation in Volunteers of a Candidate Live Oral Attenuated *Salmonella typhi* Vector Vaccine

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## Abstract

Candidate vector vaccine strain CVD 906 (*aroC*<sup>-</sup> and *aroD*<sup>-</sup> derivative of virulent *Salmonella typhi* strain ISP1820) was evaluated in phase 1 clinical trials. The first nine volunteers ingested a single dose of  $5 \times 10^7$  CVD 906 bacilli. At this dose CVD 906 stimulates remarkable systemic and mucosal immune responses, inasmuch as 89% of volunteers developed marked serum antibody levels to *S. typhi* antigens and high numbers of antigen-specific gut-derived antibody-secreting cells. Four (44%) volunteers developed asymptomatic vaccinemia 4–10 d after immunization and all volunteers excreted CVD 906 on at least one occasion. However, two volunteers developed febrile adverse reactions, one on the day of vaccination and the other on day 4. Of 11 volunteers who ingested a single dose of  $5 \times 10^3$  CVD 906 bacilli, none displayed side effects but 27% developed significant serum responses to *S. typhi* LPS. In vitro, CVD 906 replicates for only nine generations in pooled human serum, indicating that CVD 906 growth is limited in this physiologically relevant medium. In phorbol myristate acetate-induced U937 human macrophage-like cells, CVD 906 replicates intracellularly to a lesser extent than parent strain ISP1820. Although, strain CVD 906 is attenuated and highly immunogenic, the occasional febrile reactions at high doses indicate that further attenuation of this strain is necessary. (*J. Clin. Invest.* 1992. 90:412–420.) Key words: auxotroph • macrophage • *Salmonella* • typhoid • U937 monocytes

## Introduction

To apply greater control over typhoid fever, which currently results in about 30 million cases and 500,000 deaths each year (1), the World Health Organization has given a high priority to the improvement of vaccines against *Salmonella typhi* (2). The development of a safe but highly immunogenic live oral typhoid vaccine for use in such programs would have advantages over the currently available reactogenic whole-cell parenteral typhoid vaccines and the well-tolerated but modestly immunogenic live oral typhoid vaccine strain Ty21a (3–5). In addition, a highly immunogenic live oral typhoid vaccine would ideally be suited as a carrier of genes that express protec-

tive antigens, cloned from other pathogens (6–9). Such hybrid recombinant *Salmonella* vaccines are expected to invoke protective immunity in humans against both the carrier strain as well as the foreign antigen.

In the 1950s, Bacon and co-workers (10) showed that a *p*-aminobenzoic acid (PABA)<sup>1</sup>-dependent strain of *S. typhi* was attenuated in mice (10). This work did not receive much attention until recently when Hoiseth and Stocker (11) showed that an *aroA* mutant of *Salmonella typhimurium*, dependent on aromatic amino acids, PABA, and 2,3-dihydroxybenzoate (DHB; restores ability to synthesize the iron chelator enterochelin) for growth, was attenuated but immunogenic in mice.

Building on this earlier observation, Edwards and Stocker (12) carefully constructed a nonreverting aromatic-dependent mutant (*aroA*) of *S. typhi* strain CDC 10-80. Because there exists a very remote chance that a single mutation can be lost and the wild-type allele restored as a result of in vivo DNA exchange with other organisms, a nonreverting *purA* deletion mutation (causing adenine or adenosine dependence) was introduced into their aromatic-dependent derivative for added safety (12). In clinical trials two such *S. typhi aroA purA* strains were remarkably well tolerated by volunteers and elicited cell-mediated immunity in nearly all volunteers (4). Unfortunately, *S. typhi aroA purA* strains induced only meager humoral responses in a small percentage of volunteers even when two  $10^9$ -organism doses were given (4). The noneffectiveness of the *S. typhi aroA, purA* mutants suggests that *S. typhi* mutants with only a single auxotrophic requirement should be evaluated as live oral typhoid vaccines.

There are data showing that *aro* mutants of *Salmonella* are attenuated in animals but retain high levels of immunogenicity (11, 13–18). This finding, taken together with a study of *S. typhimurium* auxotrophic mutants in mice showing that the *purA* mutation either alone or in combination with *aroA* results in poorly immunogenic vaccine strains (15, 18), suggests that evaluation of *S. typhi aro* mutants is a direction of choice. The *aro* genes are well separated on the *Salmonella* chromosome (19) and several individual *aro* genes have been shown to be equally attenuating (12, 20–22).

## Methods

### Bacterial strain and media

Strain CVD 906, a derivative of wild-type *S. typhi* ISP1820, carries two well-separated, genetically well-defined *aro* mutations,  $\Delta$ *aroC*1019 and  $\Delta$ *aroD*1013 (22). Strains ISP1820 (O9, 12; Vi<sup>+</sup>, phage type 46; H-d) and CVD 906 (O9, 12; Vi<sup>+</sup>; H-d) were grown at 37°C aerobically on Aro-agar, which includes 0.5% (wt/vol) yeast extract (Difco Labora-

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1. Abbreviations used in this paper: ASC, antibody-secreting cell; DHB, 2,3-dihydroxybenzoate; PABA, *p*-aminobenzoic acid.

ories, Inc., Detroit MI), 1% (wt/vol) Bacto-tryptone (Difco Laboratories, Inc.), 1.5% Bacto-agar (Difco Laboratories, Inc.), 0.5% NaCl (Sigma Chemical Co., St Louis, MO), 0.2% (wt/vol) casamino acids (Difco Laboratories, Inc.), 0.25% (wt/vol) glucose (Sigma Chemical Co.), 73 mM PABA, 73 mM DHB (Sigma Chemical Co.), and 100 µg/ml ferric ammonium citrate (Sigma Chemical Co.), which maximized the growth of CVD906 relative to ISP1820. Liquid cultures were grown at 37°C with shaking for 16 h in Aro-broth which is Aro-agar without the addition of Bacto-agar. Strains were stored in storage medium consisting of an equal volume mixture of 100% (vol/vol) glycerol and Aro-broth at -85°C.

#### *Growth in human serum*

Pooled normal human sera (Gibco, Grand Island, NY) was tested for *S. typhi*-specific antibody by enzyme-linked immunosorbent assays (ELISA) using *S. typhi* lipopolysaccharide (LPS) or formalin-inactivated *S. typhi* Ty2 cells as antigens (4). Serum substantially free of antibody specific for *S. typhi*, was heat inactivated at 56°C for 1 h. To triplicate 2-ml aliquots of serum, *S. typhi* bacilli, grown in 0.8% (wt/vol) Nutrient broth (Difco, Inc.) at 37°C anaerobically, suspended in 10 µl of *N*-saline (0.9% wt/vol NaCl) were added at a starting viability of 50 colony-forming units (cfu)/ml. Immediately a sample was taken and spread onto Aro-agar and incubated at 37°C for 18 h. The remaining serum/cell suspension was placed at 37°C. Then at 3, 6, 9, and 24 h after inoculation additional samples were taken and viable counts were determined. Results are expressed as the log<sub>10</sub> of the arithmetic mean cfu/ml of each triplicate ± 2 SD versus time in hours.

#### *Growth in U937 cells*

Human histiocytic lymphoma cell line U937 was cultured in RPMI 1640 (Gibco) supplemented with 10% (vol/vol) fetal calf serum (Gibco) as described (23). U937 cells cultured without antibiotics were stimulated with phorbol myristate acetate (PMA, Sigma Chemical Co.) at a final concentration of 6 ng/ml for 3 d before infection and allowed to differentiate into adherent macrophage-like cells (23). Microscopic examination of U937 cells 3 d after PMA induction revealed high levels of intracellular vacuolation. Each well of a 96-well tray contained ~ 10<sup>6</sup> adherent U937 cells. Liquid cultures of *S. typhi* were grown in Aro-broth at 37°C with shaking to about 4 × 10<sup>8</sup> cfu/ml. The bacterial cells were harvested by centrifugation then washed three times in the U937 culture medium. Dilutions of the bacterial cell suspensions were added to U937-containing culture wells such that the multiplicity of infection was two bacteria per U937 cell. Triplicates for each strain for each time point were cultured in parallel. After addition of the bacteria the plates were centrifuged at 250 *g* for 5 min to bring more bacteria in close contact with the U937 cells and enhance the numbers of bacteria phagocytosed (24). After 1 h at 37°C gentamicin (Sigma Chemical Co.) was added at a final concentration of 200 µg/ml to inactivate extracellular bacteria (24). After 1 h at 37°C with this high level of gentamicin, the cells were washed four times in culture medium and then maintained for the remaining time of the experiment with gentamicin at 10 µg/ml and incubated at 37°C/5% CO<sub>2</sub>. Samples were taken at the time of, and 1 (0-h time point), 9 (8 h), and 25 (24 h) h after, the initial addition of gentamicin as described and bacterial viable counts were determined. The results were plotted graphically as the log<sub>10</sub> of the mean of each triplicate ± 2 SD versus time in hours.

#### *Inocula preparation*

Methods for handling, storage, and inocula preparation of CVD 906 for the clinical trials were strictly in accordance with methods described in an Investigational New Drug Master File reviewed by the Food and Drug Administration. Briefly, a master seed lot of strain CVD 906 was prepared by streaking a single colony isolate of CVD 906 onto an Aro-agar plate and incubating at 35°C for 18 h. Cells were harvested from this plate by means of a sterile swab, suspended in storage medium, and dispensed as 1-ml aliquots in 2-ml vials. Several vials containing the master seed lot were stored at -85°C. Purity was checked by culturing from the frozen master seed lot onto Aro-agar and testing the biotype,

serotype, and growth requirements of several single colony isolates. To prepare the inocula the frozen master seed of CVD 906 was streaked onto Aro-agar and incubated for 18 h at 35°C. About four single colonies were suspended in ice-chilled phosphate-buffered saline (PBS) then placed on ice. A sample of this suspension was serotyped. The optical density at 660 nm (OD<sub>660</sub>) of the remaining suspension was measured and the viability was estimated based on previously determined correlation between the viability and the OD<sub>660</sub> of a given suspension of CVD 906. The suspension then was diluted such that 1 ml of the bacterial suspension contained a single inoculum. Dilutions of the final suspension were spread onto Aro-agar for direct determination of the viable count and purity of the inocula.

#### *Clinical microbiological techniques*

All stool samples taken after vaccination were weighed and graded as described (4). Rectal swabs were taken if a volunteer failed to produce a stool in the previous 24 h. Quantitative and qualitative bacteriological screening methods of stool samples were as described (4). Biotyping and serotyping of CVD 906 and clinical isolates were performed as described (4). Gelatin-encapsulated string devices for collecting duodenal fluid containing bile were used as described on days 6, 9, and 13 after vaccination (4). Blood cultures were taken promptly from volunteers who developed temperatures > 38.2°C. In addition, routine blood cultures (5 ml) were collected on days 5, 8, 12, and 15 after vaccination. Blood culture methods were as described (4).

#### *Volunteers*

The 20 volunteers who participated in the clinical evaluation of strain CVD 906, 9 who ingested 5 × 10<sup>7</sup>, and 11 who ingested 5 × 10<sup>3</sup>, were healthy adults, 18–33 yr of age, with no prior history of typhoid fever or typhoid vaccination. They were recruited from the metropolitan Baltimore community and admitted to the isolation ward of the Center for Vaccine Development for 20 d. Preclinical screening and informed consent was obtained from each volunteer (4).

#### *Vaccination and clinical surveillance*

The candidate vaccine strain CVD 906 was administered by the oral route. The volunteers were allowed to acclimatize in the isolation ward for a 48-h period before vaccination and refrained from eating for 90 min before immunization. To neutralize the gastric acid of each volunteer, 2 g of NaHCO<sub>3</sub> were dissolved in 150 ml of sterile distilled water (pH 8.15), and each volunteer ingested 120 ml of the bicarbonate solution. 1 min later, each volunteer ingested the remaining 30 ml of bicarbonate solution mixed with the appropriate inoculum dose of the candidate vaccine strain CVD 906 (mixed no more than 10 min before vaccination). The viability of CVD 906 was not affected when CVD 906 was suspended in this bicarbonate solution for up to 30 min at room temperature (data not shown). The volunteers remained without food and water for 90 min after ingestion of the inoculum dose.

Clinical surveillance was performed as described (4). The volunteers were interviewed daily after vaccination to determine whether such side effects as fevers, chills, headache, malaise, or diarrhea were experienced. Temperatures, measured orally, were taken at 6-h intervals for 20 d after vaccination. If a volunteer developed culture-confirmed bacteremia or if their temperature remained elevated > 38.2°C for > 48 h, then treatment with oral amoxicillin (a first dose of 1.5 g, then 1 g every 6 h for 14 d) was begun. Ciprofloxacin (500 mg every 12 h for 14 d) was given to all volunteers beginning on day 12 after vaccination.

#### *Immunological methods*

*Sample collecting and processing.* Blood samples for serology were collected before and on days 7, 21, and 28 after vaccination. Approximately 70 ml of jejunal fluid was collected and processed before and 15 d after vaccination by upper intestinal intubation as described (4). Total IgA in the jejunal fluids was measured by ELISA and each specimen was standardized to contain 20 mg of IgA per 100 ml of jejunal fluid (4). PBMC were obtained before and 4, 7, 10, and 14 d after



This volunteer was treated with ciprofloxacin on day 12 after vaccination.

The other febrile reaction began on day 4, when volunteer 13001-1 (87.3-kg male) developed an oral temperature of 38.6°C. The temperature of the volunteer briefly subsided before increasing to a peak of 39.5°C (Fig. 1). This febrile volunteer also experienced a headache, nausea, abdominal discomfort, and cough but showed no evidence of toxemia or significant malaise. Antibiotic therapy was initiated 24 h after onset of this symptom complex after which the volunteer's elevated oral temperature and other symptoms gradually abated (Fig. 1).

At a dose of  $5 \times 10^3$  cfu *S. typhi* CVD 906 did not cause any notable clinical side effects among the 11 vaccinees.

#### BACTERIOLOGY

**Excretion and string cultures.** After ingestion of  $5 \times 10^7$  CVD 906 bacilli, five of nine volunteers (55.5%) excreted viable CVD 906 bacilli on the day of vaccination (first 24 h). All nine volunteers excreted from  $\sim 1 \times 10^3$  to  $2 \times 10^6$  viable CVD 906 bacilli per gram of stool during the period 24–48 h (day 1) after vaccination. Seven of nine volunteers (77.8%) excreted  $\sim 1 \times 10^3$  to  $2 \times 10^5$  CVD 906 bacilli per gram of stool 48–72 h (day 2) after vaccination. Only two of nine volunteers (22%) excreted  $\sim 2 \times 10^4$  viable CVD 906 bacilli 72–96 h (day 3) after vaccination. All the remaining daily coprocultures from each volunteer in this cohort were negative until day 10 when one volunteer passed a single positive coproculture containing  $10^4$  viable CVD 906 bacilli per gram of stool but no further positive stool cultures were received from this volunteer. Similarly on day 11 after vaccination another volunteer in this cohort passed a single positive coproculture containing CVD 906 bacilli. Insufficient numbers were obtained to provide quantitative estimates, but as with the other case no further positive cultures were isolated from this volunteer.

None of the volunteers in this cohort experienced diarrhea during the course of the study. All duodenal string cultures from volunteers in the high dose cohort, performed on days 6, 9, and 13 after vaccination, were negative for CVD 906 bacilli.

After ingestion of  $5 \times 10^3$  CVD 906 bacilli, none of the volunteers excreted detectable CVD 906 bacilli, none of the volunteers experienced diarrhea, and no duodenal string cultures was positive in this group.

**Blood cultures.** The volunteer who became febrile on day 4 after vaccination was blood culture positive for CVD 906 bacilli at the time of the febrile episode and remained blood culture positive until day 7 (Fig. 1). Also, four afebrile volunteers produced positive blood cultures on day 7 after vaccination. One of this group of afebrile, blood culture-positive volunteers produced a second positive blood culture containing CVD 906 bacilli on day 10 after vaccination. Blood isolates of CVD 906 from the volunteers were stored at  $-85^\circ\text{C}$ . The blood isolates displayed no measurable changes in aromatic growth factor requirements, serotype, and biotype, as compared to that of the parental strain CVD 906.

#### SEROLOGY

The majority of volunteers vaccinated with  $5 \times 10^7$  cfu of CVD 906 manifested highly significant serum humoral responses specific for *S. typhi* LPS and H-d antigen (Table I). Also Widal-positive sera were found in six of the nine volunteers in this cohort (66.7%; Table I). No significant rises in serum antibody specific for Vi antigen were detected (data not shown).

As evidence of intestinal immunopriming, impressive IgG-secreting as well as IgA-secreting ASC responses specific for either LPS or H-d flagella antigen developed in 89% of this high-dose cohort (Table II). Highly significant elevations of IgM-secreting ASC specific for *S. typhi* LPS antigen and IgM-secreting ASC specific for H-d flagella antigen were found in 78% and 89% of this high-dose group respectively (data not

Table I. Humoral Responses after Vaccination with  $5 \times 10^7$  cfu of Strain CVD 906

Volunteer	Clinical reactions	LPS antibody						H-d antibody						Widal <sup>‡</sup> titers	
		Serum IgG*		Serum IgM*		Serum IgA*		Serum IgG*		Serum IgM*		Serum IgA*			
		Pre	Peak	Pre	Peak	Pre	Peak	Pre	Peak	Pre	Peak	Pre	Peak	Pre	Peak
13001-1	F <sub>4</sub> /B <sup>§</sup>	0.11	1.83 <sup>+</sup>	80	1,280*	40	640*	400	25,600*	<25	3,200*	25	1,600*	<20	320*
-2		0.41	1.41 <sup>+</sup>	80	160	20	40	400	800	<25	1,600*	100	800*	<20	40*
-3	B	0.33	1.56 <sup>+</sup>	40	160*	20	160*	25	1,600*	25	6,400*	25	3,200*	<20	320*
-4	F <sub>1</sub> /B	0.25	1.86 <sup>+</sup>	40	320*	40	640*	50	3,200*	<25	6,400*	25	1,600*	<20	320*
-5		0.08	1.18 <sup>+</sup>	20	320*	40	320*	200	3,200*	<25	200*	50	800*	<20	40*
-6	B	0.45	1.75 <sup>+</sup>	20	40	<20	160*	100	1,600*	<25	200*	50	1,600*	<20	40*
-7		0.38	0.61 <sup>+</sup>	80	160	<20	<20	800	800	<25	25	50	50	<20	<20
-8		0.68	0.84	320	2,560*	40	320*	<25	800*	<25	51,200*	<25	3,200*	<20	1,280*
-9	B	0.38	1.44 <sup>+</sup>	40	320*	<20	160*	800	6,400*	50	800*	50	1,600*	<20	<20
Responders (%)		8 (89)		6 (67)		7 (78)		7 (78)		8 (89)		8 (89)		7 (78)	
Mean increase among responders		1.03		10.0		13.7		42.3		662.0		59.0		33.7	

\* Serum IgG, IgM, and IgA responses were measured by ELISA using purified *S. typhi* LPS or purified *S. typhi* H-d flagella as antigens. *S. typhi* LPS-specific IgG responses are expressed in net OD<sub>405</sub> units: <sup>+</sup> = significant increase—net OD<sub>405</sub> increases of > 0.2 were significant. All other serum responses are expressed as the inverse of the serum ELISA end-point titers: \* = significant increase—rises of more than fourfold are significant. <sup>‡</sup> Widal assays were performed with *S. virginia* (H-d) as the agglutinating antigen and are expressed as the inverse of the end-point titer: \* = significant increase—rises of greater than fourfold are significant. <sup>§</sup> F<sub>1</sub>, febrile reaction day 1; F<sub>4</sub>, febrile reaction day 4; B, blood culture positive.

Table II. ASC and Jejunal Responses after Vaccination with  $5 \times 10^7$  cfu of Strain CVD 906

Volunteer	LPS antibody				H-d antibody				LPS antibody (Jejunal IgA <sup>‡</sup> )	
	IgG ASC*		IgA ASC*		IgG ASC*		IgA ASC*		Pre	Peak
	Pre	Peak	Pre	Peak	Pre	Peak	Pre	Peak		
13001-1	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	<4	8*
-2	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	<4	4
-3	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	<4	16*
-4	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	ND	ND
-5	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	<4	8*
-6	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	ND	ND
-7	0	11*	0	12*	0	12*	0	7*	ND	ND
-8	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	ND	ND
-9	0	>2,000*	0	>2,000*	0	>2,000*	0	>2,000*	<4	<4
Responders (%)	9 (100)		9 (100)		9 (100)		9 (100)		3 (60)	
Mean increase among responders	—		—		—		—		5.3	

\* Peripheral blood IgG and IgA ASC were measured using the ELISPOT assay (25) with either *S. typhi* LPS or purified *S. typhi* H-d, as antigens. The results are expressed as the number of ASC/10<sup>6</sup> PBMC; \* = increases of > 4 ASC/10<sup>6</sup> PBMC are significant. ‡ Jejunal IgA responses to *S. typhi* LPS were measured by ELISA and results are expressed as the inverse of the ELISA end-point titer; \* = rises of greater than fourfold are significant.

shown). Also significant jejunal IgA responses manifested in three of five volunteers of the high-dose cohort (Table II).

The serum humoral immune responses elicited by CVD 906 after volunteers ingested  $5 \times 10^3$  CVD 906 bacilli are shown (Table III). None of this low-dose cohort developed significant rises in serum IgM or IgA specific for *S. typhi* LPS and no significant IgG, IgM, or IgA serum responses specific for H-d flagella antigen were detected by ELISAs (not shown).

Some of the volunteers in the low-dose cohort developed rises in peripheral blood ASC specific for *S. typhi* LPS antigen

and H-d flagella antigen after vaccination with  $5 \times 10^3$  cfu of CVD 906 (Table III).

#### Growth in human serum

Strain CVD 906 grew for about nine generations in pooled human serum whereas parent strain ISP1820 grew through many more generations in pooled human sera (Fig. 2). Adding PABA to human serum at a final concentration of 5.0  $\mu$ M did not significantly increase or decrease the growth rate of, or number of generations achieved by, CVD 906 in pooled hu-

Table III. Humoral and ASC Responses after Vaccination with  $5 \times 10^3$  cfu of Strain CVD 906

Volunteer	LPS antibody				H-d antibody					
	Serum IgG*		IgG ASC <sup>‡</sup>		IgA ASC <sup>‡</sup>		IgG ASC <sup>‡</sup>		IgA ASC <sup>‡</sup>	
	Pre	Peak	Pre	Peak	Pre	Peak	Pre	Peak	Pre	Peak
13002-1	0.27	0.21	0	0	0	0	0	0	0	0
-2	0.81	1.02 <sup>+</sup>	0	2	0	4*	0	0	0	0
-3	0.11	0.19	0	0	0	0	0	0	0	0
-4	0.31	0.31	0	0	0	0	0	0	0	0
-5	0.36	0.55	0	0	0	49*	0	0	0	0
-6	0.34	0.92 <sup>+</sup>	0	41*	0	1,106*	0	0	0	5*
-7	0.78	0.78	0	0	0	0	0	0	0	0
-8	0.78	0.78	0	0	0	0	0	0	0	0
-9	0.51	0.51	0	0	0	0	0	0	0	0
-10	1.41	1.71 <sup>+</sup>	0	0	0	0	0	0	0	2
-11	0.29	0.29	0	0	0	0	0	0	0	0
Responders (%)	3 (27)		1 (9)		3 (27)		0		1 (9)	
Mean increase among responders	0.32		—		—		—		—	

\* Serum IgG responses shown were measured by ELISA using *S. typhi* LPS as antigen and are expressed as net OD<sub>405</sub> units: \* = significant increase—net OD<sub>405</sub> increases of > 0.2 were significant. ‡ Peripheral blood IgG and IgA ASC were measured using the ELISPOT assay (25) with either *S. typhi* LPS or purified *S. typhi* H-d as antigens. The results are expressed as numbers of ASC/10<sup>6</sup> PBMC: \* = significant increase—increases of > 4 ASC/10<sup>6</sup> PBMC were significant.

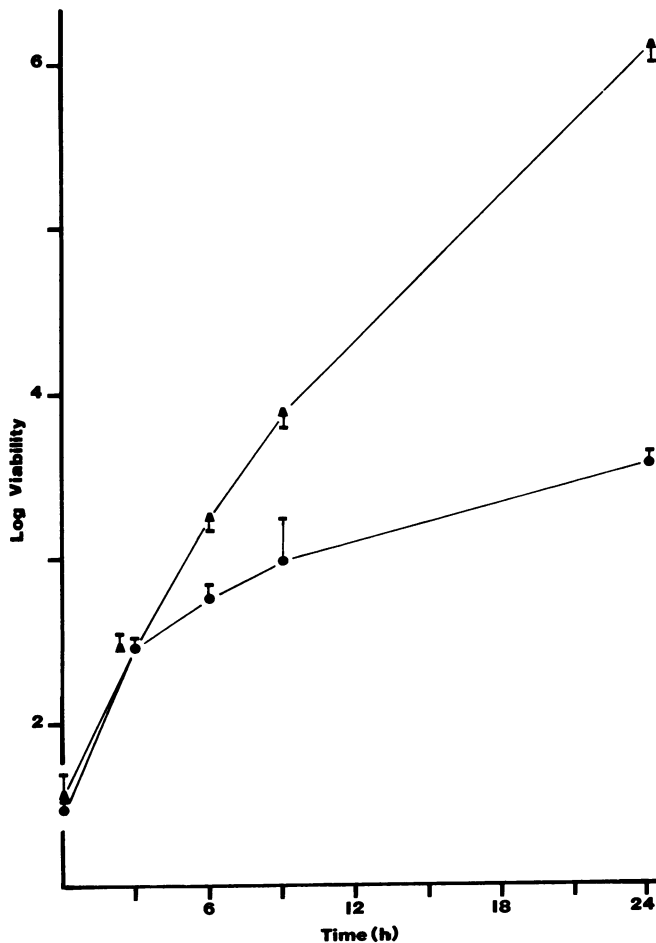


Figure 2. Serum growth curves. The growth curves of *S. typhi* strains ISP1820 ( $\Delta$ ) and CVD 906 ( $\circ$ ) in pooled human sera are shown in this figure. Each point represents the arithmetic mean of  $\log_{10}$  viability/ml of triplicate samples  $\pm 2$  SD at a given time in hours. The data shown are representative of duplicate experiments.

man serum (not shown). The minimum concentration of PABA required to restore maximum growth rate of CVD 906 on minimal media was between 5.0 and 10.0 nM; below this concentration (but above 0.05 nM), CVD 906 replicates but at a markedly reduced rate (data not shown). The minimum concentration of PABA required for growth of the blood isolates of CVD 906, was not measurably altered when compared to CVD 906 (data not shown).

#### Growth in U937 human macrophages

The growth of ISP1820 and CVD 906 in U937 cells is shown (Fig. 3). Of  $\sim 10^6$  viable bacilli of *S. typhi* ISP1820 added to each well containing  $\sim 10^6$  U937 cells, 4.12% remained viable 1 h after addition of gentamicin (i.e., the initial intracellular viable count). A 3.4-fold lower percentage of viable CVD 906 bacilli (1.22%) was recovered 1 h after gentamicin exposure. This lower initial intracellular viable count of CVD 906 was reproduced in two independent experiments. The number of viable ISP1820 and CVD 906 bacilli recovered from U937 cells increases with time but ISP1820 increases at a rate 1.4-fold faster than CVD 906 (Fig. 3). The viability of U937 cells cultured with either ISP1820 or CVD 906 was comparable throughout the experiment.

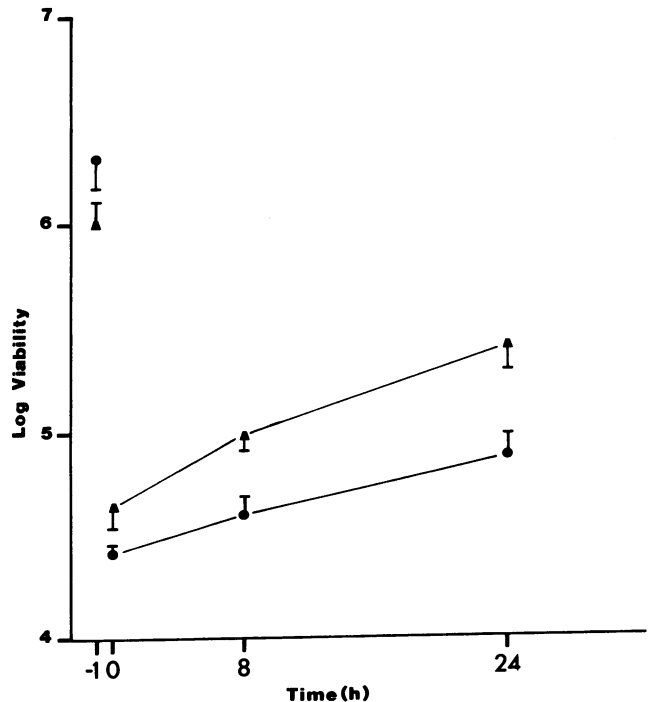


Figure 3. U937 macrophage growth curves. This figure shows the growth characteristics of ISP1820 ( $\Delta$ ) and CVD 906 ( $\circ$ ) after ingestion by PMA-induced U937 human macrophage-like cells. Gentamicin (200  $\mu\text{g}/\text{ml}$ ) was added just after the -1-h time point to kill extracellular bacteria. At the 0 time point the high level gentamicin was washed away and for the remainder of the experiment the concentration of gentamicin was maintained at 10  $\mu\text{g}/\text{ml}$ . Each point represents the arithmetic mean of triplicate samples expressed as  $\log_{10}$  viability of bacteria per well  $\pm 2$  SD at a given time in hours. The data shown are representative of triplicate experiments.

## Discussion

The shortfall of other attenuated *S. typhi* strains 27V, Ty21a, and 541Ty (*aroA purA*) is their variable or poor immunogenicity (4, 5, 28, 29). However, these strains are remarkably safe such that even after volunteers ingest as many as  $10^{10}$  viable 27V, Ty21a, or 541Ty bacilli no significant side reactions are observed (4, 28, 29). Clearly, new candidate typhoid vaccines will be judged against this background and will not be considered advantageous over these existing strains unless they achieve similar safety in humans but with greatly enhanced immunogenicity.

A single dose of  $5 \times 10^3$  CVD 906 bacilli meets this criterion. In placebo-controlled field trials the rate of IgG seroconversion to *S. typhi* LPS among Chileans correlates with protection (28). If this correlate holds true for individuals immunized with CVD 906, then we expect that a single dose of  $5 \times 10^3$  CVD 906 bacilli might induce  $\sim 25\%$  protection.

In a strict sense it is difficult to accurately determine the measure of attenuation of CVD 906 in humans, without data from volunteer studies that document the virulence of wild-type parental strain ISP1820. Some measure of the attenuating effect of *aro* can be deduced by comparing the clinical response of volunteers who ingested CVD 906 with the clinical response of volunteers who ingested virulent *S. typhi* Quail's strain which has a well-documented virulence in humans (29-31).

Without bicarbonate as buffer, Quail strain caused full-blown typhoid fever, defined as a temperature  $\geq 39.5^{\circ}\text{C}$  for a period of  $> 36$  h, in 0% of volunteers who ingested  $10^3$  viable bacilli, in 28% of volunteers who ingested  $10^5$  viable bacilli, in 50% of volunteers who ingested  $10^7$  viable bacilli, and in 95% of volunteers who ingested  $10^9$  viable bacilli (30). In subsequent volunteer challenge studies performed in an identical manner, ingestion of  $10^5$  viable Quail bacilli caused typhoid fever in 50% of unimmunized recipients (29, 31). Thus Quail strain when given without bicarbonate as buffer causes full-blown typhoid fever in 50% of volunteers who ingest between  $10^5$  and  $10^7$  viable Quail bacilli. It is assumed that the rate of attack in volunteers after ingestion of  $10^5$  to  $10^7$  Quail bacilli would increase to as much as 90–100% if bicarbonate buffer preceded challenge. If the virulence of *S. typhi* Quail strains in humans is similar to that of ISP1820, then we would expect full-blown typhoid fever in  $\sim 50\%$  of volunteers who ingest  $5 \times 10^7$  viable ISP1820 bacilli without bicarbonate and as many as 90–100% of volunteers when the same dose is given with bicarbonate. However, the clinical response of CVD 906 is notably ameliorated when compared to that of wild-type *S. typhi* infection in humans. Only one of the volunteers (11%) who ingested  $5 \times 10^7$  CVD 906 bacilli with bicarbonate may have gone on to meet the definition of full-blown typhoid fever described by Hornick et al. (30), if antibiotic had not been used. Furthermore, asymptomatic bacteremia occurs at a high frequency after ingestion of candidate vaccine strain CVD 906 whereas wild-type *S. typhi* infections rarely cause asymptomatic bacteremias in volunteers (32). We conclude that CVD 906 is considerably attenuated. A more precise measurement of the attenuating effect of the *aro* mutation will require the evaluation of a double *aro* mutant of a wild-type *S. typhi* strain that has a well-documented virulence in humans.

Dragunsky et al. (33) reported that *S. typhi* strains Ty21a and 541Ty (*aroA purA*) with documented safety in humans are incapable of intracellular growth in U937 monocytes, whereas a *galE* Vi-negative strain, EX462, that retains some virulence for humans underwent limited growth in these monocytes. Thus, the capacity to grow in U937 monocytes correlates with the documented clinical tolerance of these strains in volunteers. We demonstrated that CVD 906 retains some capacity to grow inside phorbol myristate acetate-induced U937 human macrophage-like cells. Intracellular growth by CVD 906, wherein CVD 906 mainly resides during vaccination, might in part contribute to the occasional adverse clinical reactions in humans.

To account for the attenuating effect of the *aro* mutation seen in mice, more importance was placed on the PABA dependence of these mutants (34). Milhous et al. (35) reported that only low levels of PABA (1.0–10.0 nM) are present in human serum. Also, intracellular levels are probably lower or equally low as the serum PABA concentration in that mammals acquire the essential vitamin folate from their diet and mammalian cells do not possess the biochemical pathways or require the synthesis and utilization of PABA for folate production (34). Because the concentration of PABA normally present in mammalian tissue is minute, it has been assumed that, once within mammalian tissue, *Salmonella aro* mutants rapidly deplete their intracellular pool of PABA and cease to grow, resulting in the observed attenuation of these strains in mice (11, 20). In support of this thesis, Hoiseth (36) found that the virulence of an *S. typhimurium aro* mutant in mice was signifi-

cantly increased if the mice were maintained on water supplemented with 73 mM PABA, presumably as a result of increasing the serum PABA concentration.

We found that the growth rate of *aro* mutant CVD 906 was not significantly decreased in minimal medium supplemented with as little as 5.0–10.0 nM PABA. Additionally, CVD 906 is capable of replicating (at reduced growth rates) in minimal media supplemented with minute concentrations of PABA (between 0.05 and 5.0 nM). Thus, the concentration of PABA required to supplement the growth of CVD 906 is within the range reported in human serum (35), and human serum PABA concentration may be sufficient to supplement the PABA requirement of CVD 906. Indeed, although CVD 906 does not replicate for more than a single generation in the complete absence of exogenous PABA, this candidate vaccine strain replicates for about nine generations in pooled human serum. Furthermore, addition of PABA to human serum did not increase the number of generations achieved by CVD 906 in human serum, suggesting that other factors limit the growth of CVD 906 in serum before exhaustion of PABA in this medium. As such the limited capacity of CVD 906 to replicate in human serum appears to be a consequence of the absence of other growth factors such as an iron chelator, or *p*-hydroxybenzoate.

The importance of the known siderophore defect in *S. typhi aro* mutants, an inability to synthesize enterochelin, in the attenuation of these mutants was thought to be minimal based on evidence from animal models (34). Benjamin et al. (37) showed that *S. typhimurium ent* mutants, which cannot synthesize enterochelin (an iron chelating catechol), retained full virulence in mice, and concluded that enterochelin is not a virulence factor of *S. typhimurium* in mice. If in fact the inability to synthesize a siderophore such as enterochelin is the dominant growth-limiting factor in vivo that causes the attenuation of CVD 906 in humans, then this might indicate a difference between the pathogenesis of *S. typhi* in humans and the pathogenesis of *S. typhimurium* in mice. We are currently further investigating this question and how *S. typhi aro* mutants replicate intracellularly, where different growth conditions prevail.

CVD 906 underwent limited growth in individual sera derived from each of the volunteers, suggesting that growth-supplementing metabolites were present in the sera. Hence, growth in human serum per se does not completely explain the occurrence of occasional febrile responses after vaccination. The individuals who developed febrile adverse reactions to CVD 906 might have lower natural immunity (38) or greater sensitivity to *S. typhi* LPS-induced febrile responses (39, 40) or be influenced by genetic factors that otherwise increased their sensitivity to *S. typhi* (38). Alternatively, the reactions caused by CVD 906 might be the result of special properties of this strain, whereas other *S. typhi aro* mutants may behave differently in volunteers. Possible further attenuation might derive from mutations that reduce growth rate in vitro (such as *recA* or *cya-crp*) (41, 42), or mutations that diminish macrophage resistance (e.g., *phoPQ*, *pag*, *prg*, *htrA*, or *groEL*) (43–47). Increasing the phagocytic index of CVD 906 by introducing mutations that block Vi antigen synthesis (48, 49), might also produce a further attenuated typhoid vaccine candidate. Vi-negative strains have the added advantage of being more sensitive to the bactericidal action of complement thus reducing their growth potential in the extracellular compartment (3, 50).



As a caveat, the clinical experience with CVD 906 once again highlights the limitations of animal models used for assessing *Salmonella* strains as noted earlier (3). In calves, a transient increase in rectal temperature of 1.2°C on average, was recorded ~ 1 d after oral vaccination with ~ 1.5 × 10<sup>11</sup> cfu of *S. typhimurium aro* mutant strain SL3261 (16); the calves regained normal body temperature and remained healthy without antibiotic or antipyretic treatment. On the other hand, in mice *S. typhimurium aro* mutant strain SL3261 is regarded as being safely attenuated (12). Because death is used for the mouse typhoid model, in place of, not body temperature as the criterion of virulence, it is possible that a transient febrile response occurs after oral vaccination of mice with *aro* mutants of *S. typhimurium*. In this perspective, the calf *S. typhimurium* model was more predictive of the outcome in humans. The mouse typhoid model might be improved, however, by including the measurement of body temperature along with lethality, thereby including experimental criteria more relevant for human vaccine development. Also, *in vitro* assays such as were performed here provide helpful preclinical data by which attenuated *S. typhi* strains can be evaluated.

In previous clinical and field studies with attenuated *S. typhi* vaccine strain Ty21a, the serum IgG O antibody response (measured by ELISA) was found to correlate with the protective efficacy of different immunization regimens as determined in large-scale field trials of vaccine efficacy (28). For this reason, the IgG-ELISA O antibody response has been used as a practical, helpful, and economical measure of the relative immunogenicity of candidate live oral typhoid vaccines. In this context, CVD 906 is impressively immunogenic when compared to previous live typhoid vaccines (4, 28, 29). To further characterize the specificity of the *S. typhi* LPS responses, sera from the volunteers who ingested 5 × 10<sup>7</sup> CVD 906 bacilli were assayed for rises in IgG, IgM, and IgA specific for hot phenol-purified *S. thompson* LPS (O-antigen 6, 7; not shown). Two of the nine individuals manifested approximately fourfold rises in serum IgG specific for *S. thompson* LPS but there was no elevation of IgM and IgA titers specific for *S. thompson* LPS. Therefore, the LPS responses shown here are highly specific for *S. typhi* O-antigen.

There were no significant rises in serum antibodies to the Vi antigen. This lack of responsiveness of the Vi component of live typhoid vaccines has been observed (51). Also, responses to Vi antigen are uncommon after acute typhoid fever; rather, when high titers of antibody to Vi antigen are seen they are usually associated with the chronic typhoid carrier state (52). In contrast, the Vi antigen, when purified and inoculated parenterally, is highly immunogenic (53), indicating that the poor response to Vi antigen after vaccination with Vi-positive live typhoid vaccine strains might be the result of low level or transient expression of this antigen *in vivo*.

Live oral attenuated *S. typhi* strains have potential as carriers of antigen from bacterial pathogens such as enterotoxigenic *Escherichia coli* (7) or *Vibrio cholerae* (54), or antigens from viral pathogens such as human immunodeficiency virus (T. Fouts, G. A. Lewis, and D. M. Hone, manuscript in preparation), which attach to, or enter the human host at intestinal mucosal sites. It follows that such live oral *S. typhi* vector vaccines must elicit effective intestinal secretory IgA responses. The ASC assay data presented here show that CVD 906 elicits high levels of intestinal priming of B cells (27, 55, 56). In fact, the number of ASC detected by ELISPOT (> 2,000 ASC/10<sup>6</sup>

PBMC) after immunization with 5 × 10<sup>7</sup> CVD 906 bacilli is an order of magnitude higher than the number normally seen after vaccination with multiple doses of live oral typhoid vaccine Ty21a (typically up to 100 ASC/10<sup>6</sup> PBMC) (55, 56).

Although CVD 906 is reactogenic in humans at high doses, CVD 906 retains the important property of immunogenicity. We believe that only modest attenuation of this strain is required to produce a safe and highly immunogenic live oral typhoid vaccine and antigen carrier strain.

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