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Research Article

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Safety, Infectivity, Immunogenicity, and In Vivo Stability of Two Attenuated Auxotrophic Mutant Strains of *Salmonella typhi*, 541Ty and 543Ty, as Live Oral Vaccines in Humans

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Abstract

Two *Salmonella typhi* mutants, 541Ty (Vi+) and 543Ty (Vi−), auxotrophic for *p*-aminobenzoate and adenine, were evaluated as live oral vaccines. 33 volunteers ingested single doses of 10^8, 10^9, or 10^{10} vaccine organisms, while four others received two 2×10^9 organism doses 4 d apart. No adverse reactions were observed. Vaccine was recovered from coprocultures of 29 of 37 vaccinees (78%) and from duodenal string cultures of two; repeated blood cultures were negative. The humoral antibody response to *S. typhi* O, H, Vi, and lysate antigens in serum and intestinal fluid was meager. In contrast, all vaccinees manifested cell-mediated immune responses. After vaccination, 69% of vaccinees overall and 89% of recipients of doses $\geq 10^9$ responded to *S. typhi* particulate or purified O polysaccharide antigens in lymphocyte replication studies but not to antigens of other *Salmonella* or *Escherichia coli*. All individuals, postvaccination, demonstrated a significant plasma-dependent mononuclear cell inhibition of wild *S. typhi*.

Introduction

Typhoid fever remains an important public health problem in school age children and young adults in developing areas of the world and a health risk for travelers from industrialized countries who visit such areas (1). Heat-phenolized and acetone-dried killed whole cell *Salmonella typhi* parenteral vaccines have been shown to provide moderate to excellent efficacy in placebo-controlled field trials in endemic areas; in one study efficacy persisted for at least 7 yr (2–7). However, these parenteral killed vaccines are unsatisfactory public health tools because of the frequency and severity of the adverse reactions that they evoke (2, 4, 6). Approximately 25% of recipients develop fever, malaise, and notable local reactions; often the systemic reactions are severe and debilitating.

Ty21a, an attenuated *S. typhi* strain that lacks the Leloir pathway enzyme UDP galactose-4-epimerase (8), has shown the advantages of live oral vaccines (9–12). In field trials involving ~ 16,000 vaccinated schoolchildren in Egypt (10) and 500,000 in Chile (11, 12), this live oral vaccine has provided moderate to excellent protection while causing no notable adverse reactions. Despite these very encouraging results with Ty21a, there is room for further improvement. For example, Ty21a affords very little protection when given in only a single dose (11, 12). Furthermore, the method by which Ty21a was created, i.e., treatment of virulent *S. typhi* strain Ty21a with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (8), inadvertently gave rise to a vaccine strain with multiple genetic lesions beyond the enzyme activity alterations that were sought.

There is expectation that newer attenuated *S. typhi* mutants prepared by more precise genetic techniques may overcome the drawbacks of the Ty21a strain. Stocker and co-workers (13, 14) have developed two attenuated *S. typhi* strains that are being proposed as prototypes of the next generation of live oral typhoid vaccines. Vaccine strain 541Ty was derived from wild *S. typhi* strain CDC10-80 (phage type A) by transducing deletions in two separate genes, each previously characterized in *S. typhimurium* and affecting a different biosynthetic pathway (13–15). The deletion mutations of gene *aroA* create a requirement for several aromatic compounds, including two, *p*-aminobenzoic acid and 2,3-dihydroxybenzoic acid, which are not mammalian metabolites (13–15). The second deletion mutation, at gene *purA*, causes a specific requirement for adenine (or an assimilable compound such as adenosine) (14–16). These nutritional requirements render *S. typhi* mutant 541Ty unable to maintain growth in mammalian tissues. A third mutation, of the *hisG* gene, leads to a histidine requirement. Although this last mutation does not affect virulence, it provides an additional biochemical marker to differentiate the vaccine strains clearly from wild *S. typhi*. Strain 543Ty is a spontaneously derived mutant of 541Ty that lacks the Vi polysaccharide capsular antigen; in all other ways this Vi-negative mutant is identical to 541Ty. The method of preparation of strains 541Ty and 543Ty and their genetic characteristics are described in detail elsewhere (14). This report summarizes the first clinical studies in man to assess the safety, infectivity, immunogenicity, and genetic stability in vivo of these oral typhoid vaccine strains.

Methods

Volunteers

Volunteers were 37 healthy young adults, 18–33 yr of age, from the metropolitan Baltimore community admitted to the isolation ward of the Center for Vaccine Development for a period of 18 d. The study was explained in detail and witnessed; written consent was obtained. To ensure the informed nature of the consent procedure, volunteers were required to pass a written examination covering all aspects of the study including bacteriology, immunology, risks, benefits, and procedures.

The health of the participants before vaccination was assessed by

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medical history, physical examination, electrocardiogram, chest radiograph, psychological examination, and laboratory tests including complete blood count, blood chemistry analyses, liver function tests, and serologic tests for syphilis, hepatitis B surface antigen, and human immunodeficiency virus (HIV). Before vaccination, stool specimens were examined for bacterial enteropathogens, ova and parasites. After a 48-h period of acclimation on the ward, volunteers (4–10 per dose per vaccine strain) ingested the vaccine. Studies were carried out sequentially with increasing doses being given to three separate cohorts; results of completed studies with lower doses were reviewed before proceeding to a higher dose.

Vaccine

Stock cultures of 541Ty and 543Ty maintained at −70°C in skim milk were thawed and plated onto sheep's blood agar supplemented with adenine (10 μg/ml), hypoxanthine (1 μg/ml), and 2,3-dihydroxybenzoic acid (1 μg/ml) 2 d before vaccination. After incubation at 37°C overnight, 20–30 typical colonies of the vaccine strains were picked, suspended in trypticase soy broth, and inoculated onto trypticase soy agar supplemented as above. After overnight incubation at 37°C, the bacteria were harvested with 3 ml of 0.85% saline and the concentration of bacteria was standardized turbidometrically. Dilutions of the suspensions were made in phosphate-buffered saline (PBS) to achieve the desired concentration of viable organisms per milliliter. The identity of the inoculum was confirmed by slide agglutination with *S. typhi* O, H, and Vi antisera. Replicate pour plate quantitative cultures were made of the inocula before and after vaccination to confirm the inoculum size.

Oral vaccination

2 g of $NaHCO_3$ were dissolved in 150 ml of distilled water after which each volunteer drank 120 ml in order to neutralize gastric acid and enhance survival of the vaccine organisms during passage through the stomach. 1 min later the vaccine inoculum suspended in the remaining 30 ml of bicarbonate solution was ingested. Volunteers neither ate nor drank for 90 min before and after vaccination.

Clinical surveillance

Oral temperatures were taken every 2 h throughout the period of observation. All stools from every volunteer were collected in plastic "cholera seats" and examined, the consistency was graded on a five-point scale as previously described (17), and the volume measured if the stool was loose. Vaccinees were examined daily by the same physicians and interviewed for complaints of nausea, change in appetite, abdominal discomfort, headache, malaise, feverishness, or other symptoms. An oral temperature ≥ 37.8°C was considered fever, whereas 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.

Bacteriology

Every stool passed by the volunteers was cultured to detect excretion of the vaccine strain or revertant *S. typhi.* Stool was inoculated into supplemented selenite F broth, incubated overnight at 37°C and subcultured onto supplemented *Salmonella-Shigella* (S-S)[1] and xylose-lysine-desoxycholate agar. To quantitate shedding of the vaccine strain, 1 g of stool was serially diluted 10-fold in PBS and each dilution was plated onto S-S agar supplemented as above. Suspicious colonies were transferred to supplemented Kligler's triple sugar iron agar slants and confirmed by agglutination with *S. typhi* O, H (and for 541Ty, Vi) antisera (18, 19).

Approximately 20 and 44 h after ingestion of vaccine, and in some instances also on the 6th and 11th days after vaccination, fasting volunteers ingested gelatin-encapsulated string devices (Enterotest, HDC Corp., Mountain View, CA) to collect samples of bile-stained duodenal fluid (20). After 4 h the strings were removed, and the color and pH of the distal 15 cm on the string were recorded. By means of a gloved hand, duodenal fluid was tweezed from the distal portion of the string and cultured both qualitatively and quantitatively as above.

5 ml of blood were collected on days 2, 4, 6, 8, 10, 12, and 14 after vaccination and inoculated into 50 ml of supplemented brain heart infusion broth containing 0.025% sodium polyanetholsulfonate.

Serology

Sera were collected before and 11, 15, 22, and 29 d after vaccination for measurement of antibodies to the O, H, and Vi antigens of *S. typhi.*

O antibody. IgG antibody to purified *S. typhi* O antigen (Difco Laboratories, Detroit, MI) was measured by enzyme-linked immunosorbent assay (ELISA). *S. typhi* lipopolysaccharide O antigen (100 μl, 10 μg/ml) in carbonate coating buffer (pH 9.6) (21) was applied to alternating wells of polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) for 2 h at 37°C followed by 18 h at 4°C. For each well containing antigen, a corresponding background control well without O antigen was similarly treated. The wells were emptied and washed three times with PBS, pH 7.2, using a Titertek Microplate washer (Flow Laboratories, Inc., McLean, VA). Unbound plastic sites in the wells were blocked with 5% heat-inactivated fetal bovine serum in PBS for 1 h at 37°C, after which the wells were washed three times with PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20, J. T. Baker Chemical Co., Phillipsburg, NJ), hereafter referred to as washing buffer. Sera diluted 1:100 in PBS-Tween containing 1% heat-inactivated fetal bovine serum were incubated in the wells for 1 h at 37°C; the wells were then washed five times with washing buffer. Affinity column-purified (heavy chain-specific) goat anti–human IgG labeled with alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was reacted in all wells for 1 h at 37°C. (The specificity of the anti-IgG and anti-IgA conjugates was documented by reacting them in ELISA against chromatographically purified human IgG and IgA). After washing, as above, *p*-nitrophenyl phosphate in 10% diethanolamine buffer (1 mg/ml) (Kirkegaard & Perry Laboratories, Inc.) was added for 30 min at 37°C, and the reaction was stopped with 3 M NaOH. Optical density was measured at 405 nm with a Titertek Multiscan-MC reader. Net optical density was defined as the optical density of the antigen well minus the optical density of the corresponding background control well. Significant seroconversion was defined as an increase in net optical density of ≥ 0.15. This degree of increase was statistically derived by testing paired sera from 30 Marylanders who received oral cholera or *Escherichia coli* vaccines and represents a value equal to the mean rise in net optical density of these paired sera plus 3 SD. The positive control serum used with each microtiter plate contains a high level of O antibody and represents a pool of high-titer sera from 12 healthy Chileans who manifested strong IgG O antibody responses after immunization with Ty21a vaccine.

IgM antibody to O antigen in sera diluted 1:1,000 was measured in Stockholm by ELISA as previously described (22) using a swine anti–human IgM (heavy chain-specific) conjugate (Orion Diagnostics, Helsinki). A rise in net optical density of ≥ 0.40 was calculated to be significant based on the mean rise plus 3 SD of paired sera of the negative control population.

Intestinal secretory IgA antibody to *S. typhi* O antigen was also measured by ELISA. Before vaccination and 15 to 21 d thereafter, the vaccinees ingested polyvinyl chloride intestinal tubes as previously described to collect jejunal fluid (23). Immediately after collection the fluids were heated to 56°C for 30 min to inactivate proteolytic enzymes. The serum IgA content of the fluids was measured by radial immunodiffusion as previously described (23) and the fluids were lyophilized. Jejunal fluids were reconstituted to a concentration of 20 mg IgA per 100 ml of jejunal fluid, before testing for specific antibody by ELISA.

Alternating wells of microtiter plates were coated as above with *S. typhi* O antigen. Serial twofold dilutions of jejunal fluids in PBS, beginning at 1:4, were applied to the microtiter wells. The ELISA procedure was identical as described for the serum IgG assay except that alkaline phosphatase-conjugated goat anti–human IgA (heavy chain-specific, Kirkegaard & Perry Laboratories, Inc.) was used. Fourfold rises in titer were considered significant.

H antibody. H (flagellar) antibody was measured by the Widal tube

1. *Abbreviations used in this paper:* CFU, colony-forming unit; LPS, lipopolysaccharide; PHA, phytohemagglutinin; PWM, pokeweed mitogen; S-S, *Salmonella-Shigella.*

Table I. Clinical Response of Healthy Adult Volunteers after Ingestion of Various Doses of Aro-, Pur- Auxotrophic Mutant Salmonella typhi Live Oral Vaccine Strains 541Ty or 543Ty

Vaccine	No. of vaccinees	No. of vaccinees with: Diarrhea	Fever*	Abdominal discomfort
541Ty	23	0	0	0
543Ty	14	0	0	0

* Oral temperature ≥37.8°C.

agglutination test as previously described (24) using *Salmonella virginia.* Like *S. typhi,* this serotype has the flagellar antigen d, but shares no somatic or capsular antigens with *S. typhi* (19).

Vi antibody. Vi antibody was measured by passive hemagglutination as previously described (25, 26) using sheep erythrocytes sensitized with highly purified Vi antigen prepared from *Citrobacter freundii* (kindly provided by Dr. John Robbins, National Institute of Child Health and Human Development, Bethesda, MD).

Antibody to S. typhi cell lysate. In order to detect antibody against possible uncharacterized protein antigens, an ELISA was also performed against a lysate of *S. typhi.* The lysate was prepared by treating log-phase organisms of strain 5077 with a Ribi cell fractionator (DuPont-Sorvall, Newtown, CT). Antigen (25 μg in 100 μl) coated on microtiter plates as for the O antibody assay. Serial twofold dilutions of serum were tested, beginning with a dilution of 1:20. The ELISA was performed as for measurement of O antibody in serum. Fourfold or greater rises were considered significant.

Measurement of cell-mediated immunity

Assays to detect the development of cell-mediated immunity after vaccination included measurement of the replication of lymphocytes in vitro in the presence of selected *S. typhi* and control antigens and assessment of the ability of mononuclear cells and plasma, alone and in combination, to inhibit the growth of *S. typhi* in vitro. These assays were carried out using blood specimens collected before, and 22 and 61–96 days after vaccination.

Lymphocyte replication assays. The lymphocyte replication assay employed mononuclear cells isolated from peripheral blood after Ficoll-Hypaque gradient centrifugation (lymphocyte separation medium, Litton Bionetics, Kensington, MD). These cells were cultured in microculture plates at an initial density of 1×10^5 cells per well in a total volume of 0.22 ml of medium RPMI 1640 (M. A. Bioproducts, Walkersville, MD)

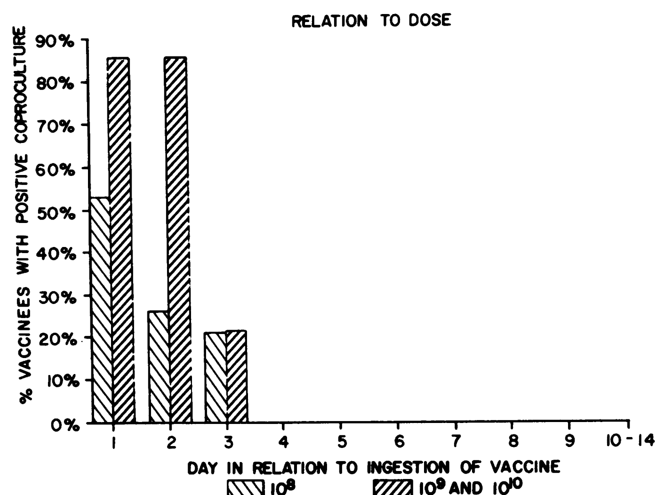

Figure 1. The prevalence and duration of excretion of 541Ty or 543Ty vaccine after ingestion of a single (or first) dose is shown for those who ingested doses of 10^8 or ≥ 10^9 vaccine organisms. The percentage of vaccinees with positive coprocultures on each day was notably higher in those who received larger (≥ 10^9 organisms) doses of vaccine. No vaccine organisms were isolated beyond the third day after ingestion.

containing 10% pooled normal human serum, 2 mM glutamine, and 50 μg/ml gentamicin. In preparing the human serum pool, individual sera of prospective donors were evaluated to determine whether the sera were intrinsically suppressive to phytohemagglutinin (PHA) or pokeweed mitogen (PWM)-induced lymphocyte replication or whether the sera alone stimulated lymphocyte replication in the absence of mitogen or antigen. Sera that were mitogenic or suppressive to lymphocyte replication were discarded.

The mononuclear cells were cultured in medium alone or in medium containing mitogens or antigens. The mitogens, PHA (Difco Laboratories) and PWM (Gibco, Grand Island, NY), were used at 250 μg/culture or at a final dilution of 1:110, respectively. Both inactivated whole bacteria (particulate antigen) and purified O polysaccharides free of endotoxin (27, 28) were employed as antigens. The particulate *S. typhi* antigen consisted of heat-phenolized whole bacteria (Wyeth typhoid vaccine, Wyeth Laboratories, Marietta, PA); control particulate antigens, which were prepared in this laboratory by the same method of heat-phenol treatment, included *S. enteriditis* (SH1262), *S. thompson* (ATCC 8391, American Type Culture Collection, Rockville, MD), and *E. coli* strain HS (29). Briefly, to prepare particulate antigens, bacterial strains were

Table II. Isolation of S. Typhi Vaccine Strains 541Ty and 543Ty from Stool, Duodenal Fluid and Blood Cultures in Relation to Dose of Vaccine Ingested

Vaccine	Inoculum* (no. of doses)	No. of vaccinees	No. (%) of vaccinees with positive coprocultures	Peak geometric‡ mean excretion (range)	No. with positive duodenal string cultures	No. with positive blood cultures
541Ty	10^8 (1)	10	7 (70)	5×10^1 (10^1–5×10^3)	0	0
	10^9 (1)	5	5 (100)	4×10^4 (10^1–2×10^5)	0	0
	10^9 (2)	4	3 (75)	3×10^3 (10^1–9×10^5)	1	0
	10^{10} (1)	4	4 (100)	6×10^4 (10^1–5×10^6)	1	0
543Ty	10^8 (1)	9	5 (56)	3×10^1 (10^1–2×10^3)	0	0
	10^9 (1)	5	5 (100)	10^4 (10^1–6×10^5)	0	0

* No. of viable organisms administered with 2.0 g of $NaHCO_3$. ‡ No. *S. typhi*/gram of stool in persons with positive cultures. If only enrichment cultures were positive, a value of 10^1 organisms/gram stool was assigned since the level of detection of direct plating is ≥ 10^2 organisms/gram.

grown on 6-in agar plates containing veal infusion agar. After overnight incubation at 37°C, confluent growth was harvested from the agar surface using 10 ml of PBS, pH 7.2, containing 0.5% phenol (PBS-phenol), centrifuged, and washed twice in PBS-phenol, and resuspended in 10 ml of PBS-phenol. The washed bacterial suspension was heated to 56°C in a water bath for 30 min, washed two more times as above, resuspended in PBS-phenol to a concentration of 2×10^{10} bacterial cells/ml (quantitated by direct count in a Petroff-Hausser chamber), and stored at 4°C. Before use in lymphocyte replication assays, the heat-phenolized bacterial antigens were washed three times and resuspended in isotonic saline to a concentration of 5×10^7 bacterial cells/ml; 1×10^6 bacterial cells were used per culture.

The O polysaccharide antigens were prepared from *S. typhi*, *S. enteriditis*, *S. thompson*, and *S. anatum* as previously described (27, 28). Briefly, lipopolysaccharide (LPS) was extracted from the various *Salmonella* by the hot water-phenol method and then partially delipidated

Table III. Serologic Response of Volunteers after Oral Vaccination with Auxotrophic S. typhi Mutant Strains 541Ty or 543Ty

Vaccinee	Serum IgG O antibody*		Serum IgM O antibody‡		Serum H antibody§		Serum Vi antibody§		Serum antibody to *S. typhi* lysate: IgG§		Serum antibody to *S. typhi* lysate: IgA§		Intestinal serum IgA O antibody§	
	Pre-‖	Peak¶	Pre-	Peak	Pre-	Peak	Pre-	Peak	Pre-	Peak	Pre-	Peak	Pre-	Peak
Cohort I														
7001-1	0.15	0.24	0.57	0.53	<20	<20	80	160	1,280	1,280	80	40	<4	4
-2	0.02	0.03	0.66	0.86	<20	<20	<20	<20	640	320	20	40	NA**	NA
-3	0.14	0.14	0.14	0.40	<20	<20	<20	<20	5,120	5,120	80	80	<4	<4
-4	0.01	0.02	0.11	0.22	<20	<20	<20	<20	320	320	40	40	NA	4
-5	0.00	0.02	0.33	0.65	<20	<20	<20	<20	2,560	5,120	40	160‡‡	<4	<4
-6	0.09	0.03	0.28	0.33	<20	<20	<20	<20	1,280	1,280	80	160	<4	4
-7	0.27	0.36	0.18	0.40	<20	<20	<20	<20	320	1,280‡‡	320	160	NA	4
-8	0.06	0.10	0.44	0.50	<20	40‡‡	<20	<20	1,280	5,120‡‡	320	640	NA	NA
-9	0.52	0.44	1.82	2.19	<20	<20	40	80	2,560	5,120	160	320	<4	4
-10	0.07	0.08	0.97	1.36	160	160	<20	<20	5,120	5,120	80	320‡‡	4	8
-11	0.13	0.13	0.21	0.84‡‡	<20	<20	<20	<20	640	1,280	640	320	8	8
-12	0.40	0.66‡‡	0.31	0.41	<20	<20	80	160	2,560	2,560	320	640	NA	4
-13	0.43	0.29	0.89	1.00	<20	<20	40	40	640	640	320	320	<4	4
-14	0.09	0.14	0.47	0.41	<20	<20	<20	<20	1,280	5,120	320	640	NA	NA
-15	0.07	0.13	0.30	0.50	<20	<20	<20	<20	640	1,280	80	160	NA	NA
-16	0.76	0.59	1.04	0.79	<20	<20	<20	<20	5,120	5,120	80	160	<4	4
-17	0.00	0.03	0.63	0.79	<20	<20	<20	<20	NA	NA	NA	NA	<4	<4
-18	0.00	0.06	0.29	0.27	<20	<20	<20	<20	2,560	5,120	40	80	<4	4
-19	0.58	0.78‡‡	0.60	0.82	<20	<20	640	320	5,120	5,120	320	320	8	4
Cohort II														
7002-1	0.04	0.15	0.32	0.41	<20	<20	20	20	5,120	10,240	80	80	4	8
-2	0.01	0.25‡‡	0.49	0.78	<20	<20	<20	<20	160	320	20	20	4	4
-4	0.00	0.10	0.26	0.47	20	320‡‡	20	40	640	640	160	40	<4	<4
-5	0.00	0.01	0.20	0.45	<20	<20	20	40	640	1,280	160	80	<4	<4
-6	0.12	0.12	0.34	1.00‡‡	<20	<20	<20	<20	5,120	2,560	160	160	4	4
-8	0.09	0.12	0.23	0.42	<20	<20	<20	<20	320	320	160	160	32	32
-9	0.10	0.07	0.21	0.66‡‡	<20	<20	<20	<20	2,560	2,560	160	320	4	64‡‡
-10	0.91	1.04	1.44	0.89	40	40	<20	<20	2,560	5,120	160	160	NA	<4
-11	0.05	0.08	0.42	0.58	<20	40	20	<20	1,280	2,560	80	80	16	16
-12	NA	0.05	NA	NA	NA	<20	NA	<20	2,560	2,560	160	320	NA	NA
Cohort III														
7003-1	0.02	0.16	0.81	0.96	<20	160‡‡	<20	<20	1,280	2,560	80	160	<4	<4
-4	0.09	0.13	0.34	0.56	<20	40‡‡	<20	<20	5,120	5,120	320	320	<4	<4
-5	0.08	0.36‡‡	0.37	0.62	<20	<20	<20	<20	640	1,280	160	320	NA	NA
-6	0.21	0.21	0.89	0.51	<20	<20	<20	20	1,280	2,560	320	320	NA	4
-8	1.04	0.92	0.42	0.40	<20	160	<20	20	640	1,280	80	320	NA	4
-9	0.72	0.71	0.33	0.38	<20	<20	<20	<20	640	640	320	160	4	4
-10	0.00	0.00	0.11	0.16	<20	<20	<20	<20	1,280	640	20	40	NA	NA
-12	0.00	0.06	0.29	0.29	<20	<20	<20	<20	1,280	1,280	160	320	<4	<4

* A rise in net optical density ≥ 0.15 of the postvaccination over the prevaccination specimen is significant. ‡ A rise in net optical density ≥ 0.40 of the postvaccination over the prevaccination specimen is significant. § Fourfold or greater rise in titer is significant. ‖ Prevaccination titer. ¶ Peak postvaccination titer. ** Specimen not available or test not done. ‡‡ Significant rise over prevaccination titer.

Table IV. Replication Responses of Pre- and Postvaccination Lymphocytes to Culture Medium Alone or Mitogens

	Mean counts per minute per culture[*]			Net counts per minute per culture for cultures stimulated with:							
	No antigen			PHA				PWM			
Vaccinee[‡]	−1[§]	22	61[‖]	−1	22	61	R[¶]	−1	22	61	R
Cohort I											
1	518	366	ND	59,183	93,965	ND		13,035	34,492	ND	
2	548	364	ND	127,159	80,920	ND		19,834	23,154	ND	
3	288	166	ND	133,524	67,476	ND		39,279	41,527	ND	
4	178	149	ND	132,442	108,504	ND		38,013	25,976	ND	
5	207	519	ND	73,495	87,903	ND		30,003	14,389	ND	
6	681	298	ND	138,658	88,934	ND		29,263	24,912	ND	
7	562	275	ND	49,793	56,335	ND		16,734	28,320	ND	
8	215	208	ND	135,441	64,086	ND		31,349	25,798	ND	
9	326	416	ND	146,112	70,870	ND		41,386	46,156	ND	
10	367	497	ND	74,651	38,478	ND		29,768	41,640	ND	
11	545	298	ND	73,499	71,442	ND		20,073	31,514	ND	
12	385	457	ND	110,898	86,512	ND		19,279	20,375	ND	
13	156	369	ND	62,794	70,598	ND		36,189	28,874	ND	
14	301	935	ND	101,806	86,822	ND		28,735	28,619	ND	
15	407	314	ND	72,637	96,644	ND		13,503	19,786	ND	
16	523	364	ND	81,065	82,095	ND		24,541	20,974	ND	
17	275	224	ND	99,507	59,634	ND		3,872 ![**]	4,343 !	ND	!
18	654	269	ND	134,561	65,989	ND		33,042	28,831	ND	
Mean	396	360		100,401	76,512			25,994	27,204		
SD	166	178		32,386	16,895			10,313	10,016		
No. with significant difference[‡‡]				0	0		0	1	1		1
% with significant difference				0	0		0	6	6		6
Cohort II											
1	253	490	592	77,604	129,766	113,527		26,273	25,040	31,461	
2	480	142	822	123,382	161,851 !	106,286	!	31,945	10,734	32,480	
4	723	420	363	131,469	124,448	79,352		25,589	43,960	22,701	
5	574	503	1,283	34,154	42,203	35,816		35,306	30,487	15,478	
6	1,057	566	824	85,656	107,818	71,718		67,004 !	67,640 !	30,313	!
8	726	637	1,418	100,638	124,926	94,230		55,479	54,457	37,249	
9	301	160	1,416	92,283	99,230	84,245		34,922	39,800	25,606	
10	206	185	379	67,642	101,748	113,551		31,284	35,811	18,013	
11	287	283	688	73,479	93,858	77,706		36,119	35,557	26,363	
12	671	241		53,757	124,046			21,483	23,119		
Mean	528	363	865	84,006	110,989	86,270		36,540	36,661	26,629	
SD	273	182	416	29,760	31,170	24,581		14,110	16,228	7,063	
No. with significant difference				0	1	0	1	1	1	0	1
% with significant difference				0	10	0	10	10	10	0	10
Cohort III											
1		219	550		74,156	79,950			15,800	17,832	
4	795	259	748	136,986	140,482	79,045		23,529	19,434	23,768	
5	383	600		86,956	131,930			20,980	12,398		
6	546	170	332	75,898	44,248 !	75,845	!	24,246	24,677	25,518	
8		146	512		62,693	107,471			28,291	34,693	
9	498	242	528	81,742	127,404	116,775		41,391	30,622	31,197	
10	574	128	193	104,036	99,026	107,958		20,061	20,708	15,099	
12	538	205	514	98,345	90,895	107,136		11,028	11,397	16,067	
Mean	556	246	482	97,327	96,354	96,311		23,539	20,416	23,453	
SD	135	150	176	22,039	34,959	17,228		9,943	7,094	7,601	

Table IV. (Continued)

	PHA				PWM			
	−1	22	61	R[¶]	−1	22	61	R
No. with significant difference	0	1	0	1	0	0	0	0
% with significant difference	0	12	0	12	0	0	0	0
For all cohorts								
No. with significant difference/no. of vaccinees	0/34	2/36	0/16	2/36	2/34	2/36	0/16	2/36
Percent with significant difference	0	6	0	6	6	6	0	6

ND, not done. * Mean counts per minute of triplicate cultures. ‡ The number of vaccinees tested differs from the total number of volunteers because of failure to obtain some postvaccination samples and occasional technical problems with individual assays. § Interval in days in relation to vaccination. ‖ Individuals in cohort III were evaluated at day 93 after vaccination. ¶ Responders (see below) on either day 22 or 61 postvaccination. ** ! denotes responders, defined as those individuals whose average net counts per minute per culture for triplicate cultures was more than 2 SD above or below the prevaccination mean counts per minute per culture of the cohort. ‡‡ Total number of individuals meeting the definition of responder.

by hydrolyzing phosphate bonds and fatty acid-ester linkages in the lipid moiety of the LPS by treatment with NaOH (0.15 M, 100°C, 2 h). After centrifugation, the pH was brought to 3.5 and free fatty acids were removed by successive extractions with chloroform. The pH was then adjusted to 7.0, and the material was dialyzed against distilled water and lyophilized. O polysaccharides were then prepared by hydrolysis of the partially delipidated LPS with weak acetic acid (1%, 100°C, 1 h). The reaction mixture was extracted with chloroform after which the water phase was dialyzed extensively against distilled water and lyophilized. The various O polysaccharides were at least 99.9% pure from contaminating protein or lipid.

After 3 d of incubation in the presence of PHA, 5 d with PWM, or 7 d with antigen, tritiated thymidine (0.5 μCi per culture) was added to the cultures. On the day after addition of thymidine, an automated harvesting device (PHD Cell Harvestor, Cambridge, MA) was used to collect mononuclear cells onto glass filters. Standard liquid scintillation procedures were employed to quantitate the amount of thymidine incorporated.

For each vaccinee's specimens, at each time point, the average counts per minute per culture for a triplicate set of cultures without antigen was subtracted from the average of every triplicate with antigen or mitogen and the resultant value referred to as the net counts per minute per culture. For each antigen used in the lymphocyte replication assay, a cohort mean±standard deviation was calculated from the prevaccination net counts per minute per culture of the individuals in the cohort (i.e., group of volunteers vaccinated at the same time) and this prevaccination mean was used as the basis for identification of "responders." Thereafter, a responder was defined as an individual whose triplicate mononuclear cell cultures incubated with antigen gave a net counts per minute per culture that exceeded by 2 SD the mean net counts per minute per culture for that antigen in the individual's cohort before vaccination.

S. typhi growth inhibition assay. The *Salmonella* growth inhibition assay was modeled after that of Nencioni et al. (30). Except for the periods of incubaton, all procedures were carried out at 4°C with chilled (4°C) reagents. Frozen seed stock of Vi-positive *S. typhi* 5077 (a representative wild-type strain isolated from the blood of a child with typhoid fever in Santiago, Chile) was thawed, inoculated into antibiotic medium 3 (Difco Laboratories), and incubated with agitation for 6 h at 37°C, and the concentration of bacteria was determined photometrically. The *S. typhi* were washed, centrifuged, and resuspended in RPMI 1640 supplemented with 0.25 mM Hepes and bicarbonate at 4°C. Thereupon the *S. typhi* (10^4 colony-forming units [CFU]) were deposited by centrifugation on the bottom of sterile 12 × 75-mm polypropylene tubes. To separate tubes containing bacteria, either the culture medium alone, 2×10^6 mononuclear cells (obtained identically as described for lymphocyte replication studies), a 1:2,000 dilution of (heat-inactivated) plasma, or a mixture of mononuclear cells and plasma from the same vaccinee, were added. The tubes were so prepared in triplicate. At this point, 0.5 ml of the culture medium, supplemented with 32% heat-inactivated fetal calf serum (Gibco), was added to each tube. The tubes were incubated for 60 min in a water bath at 37°C. Thereupon, to determine the inhibitory effects of plasma or mononuclear cells, alone or in combination, each tube was vigorously shaken and serial 10-fold dilutions were inoculated onto trypticase soy agar and the number of CFU that grew after overnight incubation at 37°C were counted; these quantitative cultures from each tube were made in duplicate and the mean was computed.

To simplify analysis of the data, the mean number of CFU in the tubes containing cells, plasma, or the combination were compared with the corresponding tubes with culture medium alone and expressed as a percent "growth inhibition", i.e., 100 − (100) (CFU of experimental tube/CFU of control tube). Three cohorts of vaccinees were given one of the vaccines in varying doses (see Results). The mean percent growth inhibition (± standard deviation) was calculated for each cohort for plasma alone, cells alone and the combination in the specimens collected before vaccination. Results of the growth inhibition assays of each individual before and after vaccination were compared with the mean growth inhibition of that individual's cohort before vaccination. In this manner responders were identified, defined as any individual whose *S. typhi* growth inhibition assay value exceeded by 2 SD the mean of his cohort prevaccination.

Results

Clinical response

Three separate vaccination studies were carried out over several months with three cohorts of volunteers wherein various doses of the two vaccine strains were administered. In the first study, a cohort of 19 individuals ingested a single 10^8 organism dose of strain 541Ty or 543Ty. A single 10^9 organism dose of one or the other strain was given to 10 volunteers in the second study. In the final study four volunteers ingested a single 10^{10} dose of 541Ty, whereas four others ingested 10^9 organisms on two oc-

Table V. Lymphocyte Replication Responses of Volunteers to Various Particulate Antigens before and after Oral Vaccination with Auxotrophic Salmonella typhi Strains 541Ty or 543Ty

	Mean counts per minute per culture control			Counts per minute per culture* for cultures stimulated with particulate antigen prepared from:															
	No antigen			*S. typhi*				*S. enteritidis*				*S. thompson*				*E. coli*			
	−1[§]	22	61[‖]	−1	22	61	R[¶]	−1	22	61	R	−1	22	61	R	−1	22	61	R
Cohort I																			
1	232	477	365	814	1,601	8,582 !**	!	−34	432	924		219	728	1,192		−9	−155	72	
2	585	788	890	742	2,060	−306		590	201	−345		1,109	812	−7		940	425	289	
3	649	556	748	3,861	1,510	889		548	−172	−36		1,290	322	431		1,857	48	64	
4	647	293	227	2,632	4,372	566		1,948	195	885		804	220	178		1,724	479	566	
5	527	192	642	1,299	210	28		427	−8	85		2,141	−28	112		529	−11	11	
6	574	755	448	5,190	6,222 !	8,919 !	!	5,865 !	853	1,659		6,115 !	1,318	1,898		2,718 !	263	1,828	
7	835	455	202	141	641	−4		−555	−151	−99		−288	−62	493		−506	−76	147	
8	542	336	635	2,434	905	314		610	−136	143		2,167	185	152		710	−18	389	
9	422	456	643	5,371 !	8,445 !	8,948 !	!	1,605	3,200	−194		3,858	2,779	589		1,559	2,507	221	
10	754	710	837	538	1,912	−474		776	−164	−314		405	221	−383		−138	497	−524	
11	381	542	301	−105	1,816	2,844		−211	−258	100		−126	−66	375		254	222	564	
12	730	396	372	1,311	836	189		816	135	117		917	281	110		596	−86	87	
13	569	578	503	2,533	8,344 !	221	!	229	1,094	247		594	735	309		873	1,251	318	
14	1,315	561	404	2,558	8,009 !	4,597	!	592	127	1,064		2,334	912	2,768		1,673	896	727	
15	389	377	540	972	1,198	−130		646	−32	−1		903	247	−236		−161	−242	−135	
16	1,021	498	582	1,078	4,590	4,955		29	555	1,120		−76	749	1,305		−726	80	1,811	
17	909	250	176	−745	1,862	128		−450	−41	−46		−450	61	−34		−170	19	−46	
18	644	272	742	2,654	2,499	6,374 !	!	724	495	0		872	944	611		121	154	1,548	
Mean	651	472	514	1,849	3,168	2,591		786	351	295		1,266	575	548		658	347	441	
SD	255	172	217	1,709	2,791	3,502		1,414	803	573		1,627	682	792		938	659	660	
No. of responders				1	4	4	6	1	0	0	0	1	0	0	0	1	0	0	0
Percent responders				6	22	22	33	6	0	0	0	6	0	0	0	6	0	0	0
Cohort II																			
1	1,030	536	470	450	22,672 !	4,046 !	!	1,116	984	1,022		724	402	1,075		972	1,633	77	
2	1,210	211	1,281	2,039	622	10,947 !	!	155	734	1,180		−802	580	1,708		1,727	360	5,511 !	!
4	1,001	865	551	212	4,255 !	1,108	!	2,295	1,159	51		742	1,169	258		2,488	2,169	600	

5	842	420	1,263	2,028	439	1,003		2,829	582	220		2,353	175	−325		340	62	880	
6	763	680	719	459	12,907 !	4,738 !	!	1,544	2,223	1,617		1,061	2,014	1,311		725	1,298	2,335	
8	499	783	1,824	762	4,680 !	14,024 !	!	14	1,912	345		1,666	2,188	6,069 !	!	419	1,674	1,435	
9	513	477	1,252	550	4,943 !	2,110	!	1,348	48	−402		−178	588	0		−211	45	2,048	
10	707	177	804	2,598	12	4,709 !	!	1,160	79	212		1,348	−85	1		252	243	1,240	
11	514	1,066	1,270	2,246	17,235 !	6,375 !	!	1,432	3,597 !	1,858	!	1,575	1,510	1,575		2,131	1,512	2,121	
12	1,346	700		322	13,571 !		!	609	1,727			437	1,942			−539	868		
Mean	843	592	1,048	1,167	8,134	5,451		1,250	1,305	678		893	1,048	1,297		830	986	1,805	
SD	300	283	439	937	7,925	4,436		874	1,090	770		925	827	1,941		999	772	1,578	
No. of responders				0	7	6	9	0	1	0	1	0	0	1	1	0	0	1	1
Percent responders				0	70	66	90	0	10	0	10	0	0	11	10	0	0	11	10
Cohort III																			
1		688	699		3,269	5,000 !	!		−17	969			792	391			956	1,783	
4	159	1,076	300	2,411	10,544 !	7,007 !	!	211	1,262	816		521	1,119	147		740	2,047	381	
5	263	1,341		1,928	9,166 !		!	1,560	1,746			1,337	1,497			1,926	1,050		
6	413	534	581	660	6,131 !	6,158 !	!	1,012	109	324		744	1,757	1,549		353	450	−194	
7	2,084	692	1,572	−994	18,245 !	11,347 !	!	3,642	1,579	−255		3,750	2,691	−378		477	322	753	
9		520	950		2,526	2,706 !			73	16			708	166			161	−278	
10	1,814	417	320	−295	15,005 !	6,331 !	!	1,083	512	−28		17	213	−27		−784	1,038	530	
12	540	756	854	2,392	5,794 !	5,883 !	!	6	1,250	15		−147	341	196		429	1,091	261	
Mean	879	753	754	1,017	8,835	6,347		1,252	814	265		1,037	1,140	292		524	889	462	
SD	843	310	437	1,453	5,568	2,606		1,306	724	463		1,431	821	604		868	595	690	
No. of responders				0	6	6	7	0	0	0	0	0	0	0	0	0	0	0	0
Percent responders				0	75	85	87	0	0	0	0	0	0	0	0	0	0	0	0
For all cohorts																			
No. of responders/no. of vaccinees				1/34	17/36	16/34	22/36	1/34	1/36	0/34	1/36	1/34	0/36	1/34	1/36	1/34	0/36	1/34	1/36
Percent responders				3	47	67	61	4	3	0	3	4	0	4	3	4	0	4	3

ND, not done. * Mean value of triplicate cultures. ‡ The number of vaccinees tested differs from the total number of volunteers because of failure to obtain some postvaccination samples and occasional technical problems with individual assays. § Interval in days in relation to vaccination. ‖ Individuals in cohort III were evaluated at day 93 after vaccination. ¶ Responders (see below) on either day 22 or 61 postvaccination. ** ! denotes responders, defined as those individuals whose average net counts per minute per culture for triplicate cultures exceeded by 2 SD the prevaccination mean counts per minute per culture of the cohort.

Table VI. Lymphocyte Replication Responses of Volunteers to Various Polysaccharide Antigens before and after Oral Vaccination with Auxotrophic Salmonella typhi Strains 541Ty or 543Ty

	Counts per minute per cultures[*] for cultures stimulated with O polysaccharide antigen prepared from:															
	S. typhi				*S. enteritidis*				*S. thompson*				*S. anatum*			
Vaccinee[‡]	−1[§]	22	61[‖]	R[¶]	−1	22	61	R	−1	22	61	R	−1	22	61	R
Cohort I																
1	281	−116	932		−12	0	387		ND	ND	ND		579	204	630	
2	1,536	12	249		370	−213	−367		ND	ND	ND		662	357	89	
3	838	1,784 !**	661	!	−23	321	239		ND	ND	ND		1,431	747	215	
4	1,415	12	106		−108	−78	−24		ND	ND	ND		934	52	262	
5	55	18	110		621	18	−173		ND	ND	ND		391	20	243	
6	589	815	4,613 !	!	106	51	98		ND	ND	ND		1,670	1,403	2,036	
7	−229	124	280		773	−170	−81		ND	ND	ND		93	120	50	
8	574	5	−86		335	−61	−247		ND	ND	ND		1,773	145	2,359 !	!
9	1,406	629	2,154 !	!	39	50	−119		ND	ND	ND		949	988	389	
10	227	272	411		−147	266	723		ND	ND	ND		1,107	115	−120	
11	−5	−19	−57		88	−88	51		ND	ND	ND		39	−215	244	
12	30	91	2,521 !	!	81	162	234		ND	ND	ND		296	717	1,639	
13	−15	2,762 !	1,453	!	−88	32	1,130 !	!	ND	ND	ND		1,180	503	1,773	
14	321	2,588 !	1,425	!	477	111	370		ND	ND	ND		2,326	881	1,282	
15	134	352	794		116	166	744		ND	ND	ND		20	600	721	
16	−150	−108	2,878 !	!	−191	−23	278		ND	ND	ND		887	1,353	1,598	
17	−679	263	144		686	248	250		ND	ND	ND		586	394	434	
18	618	1,131	1,515		−147	367	406		ND	ND	ND		965	878	1,090	
Mean	386	590	1,117		165	64	217		ND	ND	ND		883	515	830	
SD	604	904	1,257		305	165	381		ND	ND	ND		634	464	769	
No. of responders	0	3	4	7	0	0	1	1				0	0	1	1	
Percent responders	0	17	22	39	0	0	6	6				0	0	6	6	
Cohort II																
1	−596	6,137 !	1,869 !	!	−591	−257	69		−178	−125	490		ND	ND	ND	
2	−380	1,481 !	4,741 !	!	−352	−4	233		192	148	606		ND	ND	ND	
4	−189	1,000	1,140		−231	−301	152		147	−278	784		ND	ND	ND	
5	1,246	228	−12		−384	−49	68		−564	187	857 !		ND	ND	ND	
6	465	6,430 !	2,544 !	!	198	−305	1,186 !		725	658	526	!	ND	ND	ND	
8	−140	6,718 !	2,674 !		−283	−575	−141	!	−200	−66	−245		ND	ND	ND	
9	−231	295	484	!	−386	455 !	−384		−140	304	−204		ND	ND	ND	
10	591	−38	460		−444	−52	−145	!	44	12	−293		ND	ND	ND	
11	795	3,824 !	2,579 !		−224	−582	−22		127	227			ND	ND	ND	
12	−558	115		!	−931	79			418	507			ND	ND	ND	
Mean	100	2,619	1,831		−363	−159	113		57	157	307		ND	ND	ND	
SD	630	2,858	1,492		287	313	443		358	287	451		ND	ND	ND	
No. of responders	0	5	5	5	0	1	1	2	0	0	1	1				
Percent responders	0	50	50	50	0	10	0	20	0	0	10	10				
Cohort III																
1		601	−122			−301	93			−131	291		ND	ND	ND	
4	76	7,180 !	1,054 !	!	−58	−357	46		1,819	386	582		ND	ND	ND	
5	317	1,562 !		!	−17	−242			1,218	−104			ND	ND	ND	
6	−86	553	1,117 !	!	73	46	−30		48	−202	−47		ND	ND	ND	
8		223	433			−321	−276			−89	−450		ND	ND	ND	
9	345	1,536 !	2,403 !	!	−1,065	637	−24		−691	132	−351		ND	ND	ND	
10	−658	1,446 !	1,199 !	!	−1,400	−86	−91		−1,321	−220	428		ND	ND	ND	
12	550	1,112 !	168	!	−315	−215	−120		−37	−328	554		ND	ND	ND	
Mean	91	1,777	893		−464	−105	−57		173	−70	144					
SD	429	2,241	838		618	328	121		1,170	227	428					
No. of responders	0	5	4	6	0	0	0	0	0	0	0	0				
Percent responders	0	62	50	75	0	0	0	0	0	0	0	0				
For all cohorts																
No. of responders/no. of vaccinees	0/34	13/36	13/34	18/36	0/34	1/36	2/34	3/36	0/16	0/18	1/16	1/18	0/18	0/18	1/18	1/18
Percent responders	0	36	38	50	0	3	6	8	0	0	6	6	0	0	6	6

ND, not done. * Mean value of triplicate cultures. ‡ The number of vaccinees tested differs from the total number of volunteers because of failure to obtain some postvaccination samples and occasional technical problems with individual assays. § Interval in days in relation to vaccination. ‖ Individuals in cohort III were evaluated at day 93 after vaccination. ¶ Responders (see below) on either day 22 or 61 postvaccination. ** ! denotes responders, who are those individuals whose average net counts per minute per culture for triplicate cultures exceeded by 2 SD the prevaccination mean counts per minute per culture of the cohort.

casions, 4 d apart. The vaccine strains were very well tolerated, with none of the vaccinees exhibiting notable adverse reactions during the 15 d of clinical observation after vaccination (Table I). Clinical signs and symptoms of enteric fever, such as fever, diarrhea, or abdominal discomfort were not encountered. No volunteer's temperature exceeded 37.7°C. The vaccinees were not given antibiotics and no complaints of adverse reactions were reported at follow-up visits 7 and 14 d after discharge from the research isolation ward.

Bacteriology

Bacteriologic results are summarized in Table II and Fig. 1. The rate of positive coprocultures, the geometric mean titer of vaccine organisms excreted per gram of stool, and the duration of shedding increased as the vaccine dose increased. All positive coprocultures occurred within the first 96 h after ingestion of vaccine. S-S agar proved to be superior for isolation of the vaccine strains. Isolates recovered from individuals who ingested 541Ty were Vi-positive, whereas isolates recovered from recipients of strain 543Ty were Vi-negative.

Two individuals who ingested strain 541Ty, including one who received 10^{10} and another who received two 10^9 organism doses, had recovery of the vaccine organism from bile-stained duodenal string cultures. In both instances the vaccine strain was recovered within 48 h of ingestion of the vaccine. In one of these individuals the vaccine strain was not recovered in coprocultures. Of the 259 blood cultures systematically collected from the vaccinees, beginning 24 h after ingestion of the vaccine and continuing every other day for 2 wk, none were positive.

Serology

Results of the serum antibody assays are summarized in Table III. By serologic tests the vaccines did not appear to stimulate a potent humoral antibody response, either of circulating or local intestinal antibody. A small proportion of vaccinees manifested rises in serum or intestinal fluid antibody to *S. typhi* O antigen or serum antibody to H or lysate antigen. None of the 37 vaccinees manifested significant rises in serum Vi antibody.

Cell-mediated immune responses

In contrast to the humoral antibody responses, which were meager, the assays for cell-mediated immunity demonstrated a prominent and specific response that correlated with the dose of vaccine ingested (see Tables V–VII).

Lymphocyte replication assays. Vaccination did not significantly alter the basal rate of lymphocyte replication in vitro, in comparison with prevaccination values (Table IV). Similarly, the nonspecific mitogenic response of lymphocytes to PHA and PWM was not significantly affected by vaccination (Table IV).

The prevaccination, mean cohort values±standard deviation for lymphocyte reactivity in the presence of heat-phenolized bacterial antigen and O polysaccharide antigens are shown in Tables V and VI. In specimens collected before vaccination from 34 individuals, lymphocyte reactivity was similarly rare after incubation with *S. typhi* (1 of 34), *S. enteritidis* (1 of 34), *S. thompson* (1 of 34), or *E. coli* (1 of 34) particulate antigens (Table V) or following incubation with O polysaccharide antigens of *S. typhi* (0 of 34), *S. enteritidis* (0 of 34), *S. thompson* (0 of 16), or *S. anatum* (0 of 18) (Table VI). In contrast, both particulate preparation and purified O polysaccharide of *S. typhi* stimulated significant reactivity of the lymphocytes of most vaccinees in specimens collected 22 and 61–96 d after vaccination (Tables V and VI). These responses were specific, because there was not a corresponding increase in reactivity of lymphocytes to the control antigens from *E. coli* and other *Salmonella*, including serologically closely related *S. enteritidis* (Tables V and VI). The rate of responders detected after incubation of postvaccination lymphocytes with *S. typhi* particulate antigen (22 of 36) is significantly different than the rate encountered following incubation of postvaccination lymphocytes with *S. enteritidis* (1 of 36), *S. thompson* (1 of 36), or *E. coli* (1 of 36) particulate antigens ($P < 0.0000001$ for each comparison, Fishers exact test, two tails) (Table V). The difference in response of postvaccination lymphocytes to the O polysaccharide antigens was equally notable. Postvaccination lymphocytes of 18 of 36 vaccinees responded to *S. typhi* O polysaccharide but only 3 of 36 to *S. enteritidis* and 2 of 36 to *S. thompson* or *S. anatum* O polysaccharides ($P < 0.0002$ for each comparison) (Table VI). There was no difference between the two vaccines in immunogenicity.

S. typhi growth inhibition assay. In specimens collected before vaccination there was virtually no inhibitory effect observed when plasma alone, mononuclear cells alone, or the combination were incubated with *S. typhi* (Table VII).

In contrast, the most sensitive assay to detect immune response to these live oral vaccines turned out to be the *S. typhi* growth inhibition assay using plasma and mononuclear cells collected before vaccination and 22 and 61 d thereafter. With only two exceptions, postvaccination plasma alone had no inhibitory activity against a representative wild type *S. typhi* strain (Table VII). In contrast, mononuclear cells from 18 of 35 individuals after vaccination, in the absence of plasma, did significantly inhibit the growth of pathogenic *S. typhi*, particularly with mononuclear cells derived from recipients of higher doses of vaccine (Table VII) ($P < 0.000001$). However, the most prominent inhibition of *S. typhi* growth was detected when an individual's postvaccination plasma was used in combination with his mononuclear cells in the assay. Under these conditions, a plasma-associated mononuclear cell immune response, manifested as inhibition of the growth of pathogenic *S. typhi*, was detected in 100% of the vaccinees. The effect of the combination of mononuclear cells plus plasma (36 of 36 responders) was significantly greater than the inhibitory effect of cells alone (18 of 36 responders, $P < 0.000001$).

Discussion

Considerable evidence supports the contention that live attenuated *Salmonella* vaccines stimulate superior immunity to that achieved with killed whole cell parenteral vaccines (9, 31–41). Only live *S. typhimurium* vaccines can protect hypersusceptible C3H/HeJ mice from an otherwise lethal challenge with virulent *S. typhimurium* in the "mouse typhoid" model (38–41). Similarly in calves, where *S. typhimurium* often causes a generalized infection of the reticuloendothelial system (and in this feature resembles *S. typhi* infection in humans), in addition to gastroenteritis, an Aro− auxotrophic mutant employed as a live oral vaccine provided significantly greater protection than a killed whole-cell parenteral vaccine (35). By using an experimental model of typhoid fever in volunteers in the late 1960s and early 1970s to assess the efficacy of typhoid vaccines (9, 36, 37, 42), it was found that a streptomycin-dependent *S. typhi* vaccine strain (36, 37) and Ty21a (9) vaccine given as freshly harvested organisms provided higher levels of protection than parenteral killed whole-cell typhoid vaccines (42). The great advantage of

Table VII. Salmonella typhi Growth Inhibition Responses of Volunteers after Oral Vaccination with Auxotrophic Salmonella typhi Strains 541Ty or 543Ty

	Contents of cultures														
	Control *S. typhi* only			*S. typhi* plus:											
				Plasma				MN cells				MN cells + plasma			
Vaccinee*	−1‡	22	61§	−1	22	61	R‖	−1	22	61	R	−1	22	61	R
Cohort I															
1	1.4¶	1.5	2.8	7.8**	2.3	−8.7		20.5	36.8 !‡‡	3.3	!	16.9	43.8 !	35.9 !	!
2	1.3	1.6	2.7	−4.9	3.9	1.1		6.5	19.4	21.9		4.0	41.1 !	56.0 !	!
3	1.3	1.4	2.8	−4.2	−4.4	−3.2		9.0	20.9			5.9	28.5 !	57.3 !	!
4	1.4	1.6	3.9	8.4	5.2	15.3		17.6	21.3	29.5		15.1	35.7 !	35.7 !	!
5	1.3	1.5	3.0	12.7	2.9	17.0		3.0	37.6 !	41.9 !	!	15.1	45.1 !	58.0 !	!
6	1.2	1.5	3.5	−0.4	−12.7	6.8		4.1	23.0	3.4		5.5	12.7	50.8 !	!
7	1.3	1.9		−30.8	15.6			35.0 !	40.5 !		!	−16.3	50.2 !		!
8	1.3	1.5	4.6	2.5	−3.7	26.6 !	!	−0.8	−1.2	48.0 !	!	5.7	16.6	66.8 !	!
9	1.1	1.5	4.9	5.2	−5.3	21.2		1.6	17.2	21.8		−2.4	31.4 !	66.7 !	!
10	1.4	1.6	2.8	−2.8	−3.2	18.1		10.6	−2.3	29.8		10.1	35.1 !	51.1 !	!
11	1.2	1.5	2.5	9.9	−15.2	4.7		0.3	11.0	20.0		2.9	34.8 !	41.2 !	!
12	1.4	1.6	2.8	4.4	−3.9	13.0		8.8	39.9 !	6.5	!	10.7	52.2 !	27.2 !	!
13	1.5	1.5	3.6	7.3	1.8	14.9		20.9	26.8	23.9		16.6	35.1 !	54.5 !	!
14	1.1	1.6	3.0	−2.6	5.1	0.0		8.6	−1.1	28.7		4.4	32.0 !	49.5 !	!
15			2.9			11.2				31.6				60.2 !	!
16	3.2	1.8		−32.0	19.3			28.4	35.5 !		!	−11.5	42.1 !		!
17	1.1	1.4	3.0	−11.8	−1.9	−17.5		−3.8	−1.9	21.3		−3.8	22.4	38.8 !	!
18	1.1	1.5	2.5	−17.8	7.9	−9.3		7.6	11.6	30.9		−11.2	48.8 !	47.6 !	!
Mean	1.4	1.6	3.2	−2.9	0.8	7.0		10.5	19.7	24.2		4.0	35.7	49.8	
SD	0.5	0.1	0.7	13.3	8.8	12.4		10.7	15.3	12.8		10.2	11.3	11.5	
No. of responders				0	0	1	1	1	5	2	6	0	14	16	18
Percent responders				0	0	6	6	6	29	13	33	0	82	100	100
Cohort II															
1	1.8	2.5		−0.7	−0.1			0.3	15.9			−7.6	45.6 !		!
2	2.0	2.4	1.4	−8.0	−11.4	−7.3		9.3	9.8	12.5		13.5	45.6 !	42.6 !	!
4	2.1	2.6	1.4	6.7	−2.4	0.4		11.7	33.6 !	24.4 !	!	15.6	47.6 !	50.2 !	!
5	1.8	2.0	1.5	−14.9	−0.1	1.0		11.5	45.0 !	9.0	!	2.3	43.2 !	58.7 !	!
6	2.0	2.3	1.7	−3.0	3.5	−1.6		14.0	17.9	13.0		11.1	43.3 !	49.0 !	!
8	1.7	2.2	1.6	−7.6	−18.3	−4.1		−4.0	14.8	40.6 !	!	2.7	35.0 !	52.2 !	!
9	1.6	2.2	1.6	9.2	−5.7	4.8		−11.5	38.2 !	25.6 !	!	0.9	46.7 !	60.6 !	!
10	1.6	2.0	1.6	−19.9	−10.7	20.9 !	!	−10.3	34.2 !	36.6 !	!	−12.2	46.9 !	40.9 !	!
11	1.8	2.8	1.5	−19.9	4.1	1.6		4.9	34.0 !	15.7	!	4.4	45.8 !	56.0 !	!
12	1.9	2.3	1.9	−4.2	7.7	7.5		11.6	21.5	43.0 !	!	−2.8	37.4 !	68.1 !	!
Mean	1.8	2.3	1.6	−6.2	−3.3	2.6		3.8	26.5	24.5		2.8	43.7	53.1	
SD	0.2	0.3	0.2	10.0	8.1	8.2		9.6	11.9	13.0		8.9	4.2	8.7	
No. of responders				0	0	1	1	0	5	5	7	0	10	9	10
Percent responders				0	0	11	11	0	50	56	70	0	100	100	100
Cohort III															
1	1.5	1.6	ND	0.8	4.1	ND		18.4	16.9	ND		7.0	48.3 !	ND	!
4	1.2	1.9	ND	−19.7	12.6	ND		−5.8	30.3 !	ND	!	5.6	51.6 !	ND	!
5	1.2	1.6	ND	−14.2	−5.7	ND		5.4	17.1	ND		6.8	48.1 !	ND	!
6	1.0	1.7	ND	−15.1	−17.8	ND		5.4	33.0 !	ND	!	1.0	51.2 !	ND	!
8	1.7	1.6	ND	−9.3	−4.5	ND		6.4	24.7 !	ND	!	6.6	47.3 !	ND	!
9	1.2	1.8	ND	36.2 !	−3.3	ND		10.3	27.9 !	ND	!	4.4	62.3 !	ND	!
10	1.1	1.6	ND	−3.6	6.7	ND		9.0	32.9 !	ND	!	10.9	47.9 !	ND	!
12	1.3	1.5	ND	−3.2	16.5	ND		16.1	22.6	ND		8.8	52.2 !	ND	!
Mean	1.3	1.7		−3.5	1.1			8.2	25.7			6.4	51.1		
SD	0.2	0.1		17.5	11.1			7.4	6.5			2.9	4.9		

Table VII. (Continued)

Contents of cultures	S. typhi plus:											
	Plasma				MN cells				MN cells + plasma			
	−1	22	61	R[‖]	−1	22	61	R	−1	22	61	R
No. of responders	1	0		0	0	5		5	0	8		8
Percent responders	13	0		0	0	63		63	0	100		100
For all cohorts												
No. of responders/no. of vaccinees	1/35	0/35	2/25	2/35	1/35	15/35	7/24	18/35	0/35	32/35	25/25	36/36
% responders	3	0	8	6	3	43	29	51	0	91	100	100

ND, not done. * The number of vaccinees tested differs from the total number of volunteers because of failure to obtain some postvaccination samples and occasional technical problems with individual assays. ‡ Interval in days in relation to vaccination. § Individuals in cohort III were evaluated at day 93 after vaccination. ‖ Responders (see below) on either day 22 or 61 postvaccination. ¶ Mean number of colony-forming units × 10,000 for triplicate cultures. ** Percent of control cultures calculated as: 100 − (100)(mean CFU of experimental tubes/mean CFU of control tubes). ‡‡ ! denotes responders, defined as those individuals whose average percent of control value exceeded by 2 SD the prevaccination mean for the cohort.

the attenuated *S. typhi* strains used heretofore in humans is that these strains elicit protective immunity without causing notable adverse reactions (9–12, 36, 37), in stark contrast to the highly reactogenic parenteral killed whole-cell vaccines.

The widely used *galE* mutant attenuated *S. typhi* vaccine strain, Ty21a, is empirically safe and protective (9–21). Nevertheless, it suffers from several theoretical drawbacks, most notable of which are the lack of knowledge of the genetic lesions responsible for changes in the activity of Leloir pathway enzymes and the inadvertent occurrences of other phenotypic changes consequent to the nonspecific method of mutagenesis employed to derive the vaccine strain. The perception of these drawbacks in Ty21a stems from the general progress in vaccine development biotechnology that has occurred since the *galE* mutants were prepared in the early 1970s. The suggestion that these deficiencies exist in no way diminishes recognition for the pathfinder role that Ty21a has played in demonstrating the superiority and advantages of live oral typhoid vaccines over parenteral killed vaccines (12).

In the 1980s, modern methods of genetic manipulation have been applied to develop attenuated *S. typhi* mutants with known and precise attenuating genetic lesions (13, 14). The first candidate strains to be created in this manner are the Aro−, Pur−, auxotrophic mutants, 541Ty and 543Ty, of Stocker and coworkers in ongoing work. Accordingly, studies were undertaken in man with these two auxotrophic mutants of *S. typhi* to assess their infectivity, safety, and immunogenicity. The genetic lesions responsible for attenuation in these vaccine strains are deletions in specific genes causing nutritional requirements and not affecting other portions of the genome. As reported herein, these Aro−, Pur− strains elicited no notable adverse reactions among 37 young adults, even when given with buffer in doses as high as 2×10^{10} organisms (Table I). These doses are an impressive measure of the innocuity of the vaccines, since with wild *S. typhi* 10^5 organisms cause typhoid fever in ∼ 50% of recipients and 10^9 lead to illness in 90%–100% of individuals (9, 36, 37, 42).

Vaccine organisms were recovered from 30 to 37 vaccinees, mostly in stool cultures (29 of 37). The number of positive cultures and the geometric mean titer of organisms per gram of stool were maximum within the first 48 h after vaccination, greatly diminishing thereafter; no positive cultures occurred beyond 96 h after ingestion of vaccine. It is believed that attenuated *S. typhi* vaccine strains such as 541Ty, 543Ty, and Ty21a rapidly translocate from the intestinal lumen to the mesenteric lymph nodes from which they enter the lymphatic circulation, thoracic duct, and blood circulation, and finally reach the reticuloendothelial system where they are ingested by fixed macrophages (43, 44). In these steps the attenuated *Salmonella* resemble the pathogenesis of infection with pathogenic *S. typhi* or *S. typhimurium* (in animals). However, whereas pathogenic *S. typhi* and *S. typhimurium* survive and proliferate within the macrophages of the reticuloendothelial system for many weeks, attenuated *Salmonella,* which are impaired in their ability to proliferate and survive, remain viable within the macrophages for much shorter periods (31, 35). Ideally, the period of intracellular survival is sufficiently long to stimulate immunity (35, 38, 40, 41, 44, 45), particularly cell-mediated responses (38, 40, 44, 45), but not long enough to lead to clinical illness. Studies with Aro− *S. typhimurium* vaccine in calves (44), and with a *galE* mutant of *S. typhimurium* in mice (31), have documented these events by autopsy of animals at varying intervals after vaccination and by examining organs of the reticuloendothelial system histologically and bacteriologically. It is presumed that these events also occur in humans after ingestion of attenuated *S. typhi* strains 541Ty, 543Ty, and Ty21a but this is extremely difficult to document. Repeated blood cultures after vaccination of volunteers with Aro− *S. typhi* in this study or with Ty21a in previous studies (12) failed to recover *S. typhi.* This does not exclude that a silent primary bacteremia occurred; rather it implies a very short bacteremia with few organisms and draws attention to the limitations of our sampling methods to detect the event. Even in full-blown typhoid fever, the concentration of bacteria in blood is very low, circa 10^1 organisms per milliliter (46–48). Thus, it is probable that we simply failed with our blood culture techniques to detect the event. Aspiration and culture of bone marrow ∼ 1–3 d after ingestion of vaccine should provide greater sensitivity to dem-

onstrate the dissemination of the vaccine organisms to the organs of the reticuloendothelial system (49–55). However, for ethical reasons this was considered too invasive a procedure to incorporate in this phase 1 vaccine evaluation.

During the primary bacteremia that occurs with pathogenic (and presumably also with attenuated) *Salmonella*, the organisms also reach the gall bladder (49–55) where they persist during the acute infection; in 2%–5% of typhoid fever patients (usually those with previous gall bladder pathology) a chronic carrier state ensues (56–59). Isolation of *S. typhi* from the bile by means of bile-stained duodenal string cultures is a sensitive method to recover the organism from suspect cases of typhoid fever, thereby confirming the diagnosis (20, 60–64). As shown in Table II, the attenuated vaccine strain was recovered from duodenal string cultures of two recipients of higher doses of strain 541Ty. One possible explanation for these positive cultures is that the attenuated *Salmonella* temporarily colonized the proximal small intestine. In view of the potency of the peristaltic defense mechanism (43), this is considered unlikely. Rather, these isolations more probably represent the arrival of the vaccine organisms in the gall bladder after dissemination.

The humoral antibody response to *S. typhi* O, H, and lysate antigens in recipients of the Aro− strains was quite meager (Table III), nor did either vaccine stimulate Vi antibodies. In general, the serologic response of North Americans after ingestion of earlier attenuated *S. typhi* vaccines, including streptomycin-dependent and *galE* mutants, was also relatively meager (9, 36, 37, 65). It is difficult to compare the serologic response in recipients of Aro− strains with individuals who received Ty21a or streptomycin-dependent vaccines because of differences in formulation, dosage, and the tests used to monitor the serologic response.

Most data support the concept that the critical immunity elicited by live oral *Salmonella* vaccines is cell-mediated (31–35, 38, 40, 41, 44, 45, 66–69). Until recently, the antigens responsible for the specificity of the cellular immune response against *Salmonella* were not known. Studies in volunteers with Ty21a demonstrated that a smooth lipopolysaccharide O antigen was necessary in order for Ty21a to be immunogenic and protective (9). Lindberg and co-workers (27, 28, 35, 44) working with both pathogenic and attenuated Aro− *S. typhimurium* demonstrated, by means of lymphocyte stimulation assays and delayed hypersensitivity skin tests, that a specific cellular immune response was directed toward the O polysaccharide. Based on these observations in animals, we utilized various O polysaccharides, in addition to killed bacteria, as antigens in lymphocyte replication assays. O polysaccharides were cleaved from lipid A and core sugars to provide highly specific antigens free from nonspecific mitogenic activity. The lymphocyte replication assays with killed whole bacteria demonstrated an immune response in most vaccinees which was shown to be highly specific for *S. typhi* (Table V); bacterial cells of other Enterobacteriaceae, including closely related *S. enteritidis*, did not stimulate the lymphocytes collected postvaccination. Stimulation studies with the O polysaccharides demonstrated that a major component of the cellular immune response was directed at the O antigen component of the LPS. The specificity of the cellular immune response involving the O polysaccharides was quite astonishing (Table VI). Heretofore, it had been accepted that the O antigens of *S. typhi* and *S. enteritidis* bioserotype enteriditis were identical (19, 70). LPS prepared from these two *Salmonella* are virtually interchangeable when used as antigens in ELISA (26), demonstrating considerable immunologic relatedness. Yet in the lymphocyte replication assays, the postvaccination lymphocytes clearly differentiated *S. typhi* from *S. enteritidis*, whether whole bacteria or purified O polysaccharide were used. This implies that while common epitopes exist on these two O polysaccharides that bind antibodies in ELISA, there also exist epitopes within the *S. typhi* O polysaccharide which are specific for *S. typhi*. It is conceivable that, in addition to immunity focussed against the O polysaccharides, there may also be an immune response directed at (as yet uncharacterized) protein antigens on the bacterial surface (71).

Recognition of the importance of the O antigen was the major reason for development of strain 543Ty, the Vi-negative variant of 541Ty. Vi polysaccharide is a linear homopolymer of alpha 1 → 4,2 deoxy-2-*N*-acetylgalacturonic acid forming a capsule that covers the O antigen of *S. typhi* (72, 73). It was not known if the presence of Vi on 541Ty might dampen the immune response to the O antigen; for this reason the Aro−, Pur− variants with and without Vi were tested in parallel. There was no evidence that the immune response to 541Ty was in any way diminished in comparison with 543Ty. Nor was there any evidence of a serologic response to Vi in recipients of either strain. Although Vi is a recognized virulence property of *S. typhi* (73), only approximately one-third of patients with acute typhoid fever manifest detectable antibody titers of Vi antibody (26). In contrast, ~ 80% of chronic *S. typhi* carriers have very elevated titers of Vi antibody (26), making this an excellent screening test for the carrier state even in endemic areas if highly purified Vi is used as antigen in the serologic test (26). Very potent immunity can occur in the absence of Vi. For example, Ty21a lacks Vi (8, 9) yet provides significant protection against typhoid fever due to Vi-positive strains of *S. typhi*.

The most sensitive assay to measure immune response to the Aro−, Pur− vaccine strains involved studies of the inhibition of *S. typhi* in vitro using mononuclear cells, plasma, or both in combination. It was found that, although postvaccination mononuclear cells by themselves exhibited inhibitory activity against *S. typhi*, the addition of postvaccination plasma significantly enhanced the inhibition (Table VII); in contrast, plasma by itself had virtually no inhibitory activity. These results parallel similar findings by Italian investigators (45) showing the development of antibody-dependent cell-mediated inhibition of pathogenic *S. typhi* in persons vaccinated with Ty21a. These investigators show preliminary evidence that T lymphocytes are critical cells involved in the inhibition of the *Salmonella* and that IgA antibody may also be involved (45). Although considerable work has yet to be carried out to investigate the operative components in plasma, the cell types and products involved and the precise effect on the *Salmonella*, the "growth inhibition" assays of Tagliabue et al. (45) and ourselves have proved to be extremely useful in measuring immune responses to oral attenuated *S. typhi* vaccines.

Aro-, Pur-auxotrophic mutants of *S. typhi* used as live oral vaccines in these initial phase 1 studies in humans have been found to be safe, infective, and immunogenic, particularly in stimulating cell-mediated immunity. Because no evidence was found to suggest that the presence of the Vi polysaccharide diminishes immunogenicity, further trials will proceed with strain 541Ty. Stimulated by the highly encouraging results reported herein, expanded phase 2 clinical trials will be carried out in outpatient volunteers with vaccine strain 541Ty to evaluate immune response after variations in dosage, immunization sched-

ules, and formulations. It is anticipated that these further studies will prepare the way for a definitive field trial of vaccine efficacy.

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