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Characterization of *Salmonella enterica* Derivatives Harboring Defined *aroC* and *Salmonella* Pathogenicity Island 2 Type III Secretion System (*ssaV*) Mutations by Immunization of Healthy Volunteers

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The attenuation and immunogenicity of two novel *Salmonella* vaccine strains, *Salmonella enterica* serovar Typhi (Ty2 Δ*aroC* Δ*ssaV*, designated ZH9) and *S. enterica* serovar Typhimurium (TML Δ*aroC* Δ*ssaV*, designated WT05), were evaluated after their oral administration to volunteers as single escalating doses of 10^7, 10^8, or 10^9 CFU. ZH9 was well tolerated, not detected in blood, nor persistently excreted in stool. Six of nine volunteers elicited anti-serovar Typhi lipopolysaccharide (LPS) immunoglobulin A (IgA) antibody-secreting cell (ASC) responses, with three of three vaccinees receiving 10^8 and two of three receiving 10^9 CFU which elicited high-titer LPS-specific serum IgG. WT05 was also well tolerated with no diarrhea, although the administration of 10^8 and 10^9 CFU resulted in shedding in stools for up to 23 days. Only volunteers immunized with 10^9 CFU of WT05 mounted detectable serovar Typhimurium LPS-specific ASC responses and serum antibody responses were variable. These data indicate that mutations in type III secretion systems may provide a route to the development of live vaccines in humans and highlight significant differences in the potential use of serovars Typhimurium and Typhi.

The *Enterobacteriaceae* are gram-negative bacteria that have adopted a lifestyle that involves periods of survival in both the mammalian host and the environment. Enterobacteria such as *Escherichia coli* harbor many genes that contribute directly to colonization of the intestine. Pathogenic strains of *E. coli* and most *Salmonella enterica* have acquired additional genes that can enhance host-specific virulence, increasing their pathogenic potential. For example, *S. enterica* strains have acquired sets of genes that facilitate the invasion of tissues (14, 15) and the ability to survive and grow within mammalian cells, including immune cells such as macrophages (13, 16). This enhanced ability to target immune cells makes *S. enterica* potentially highly immunogenic. *S. enterica* has evolved into a large number of different serovars that exhibit distinct characteristics, such as host restriction and the ability to induce different disease syndromes, depending on the particular host-pathogen combination. For example, whereas most *S. enterica* serovar Typhimurium isolates cause diarrhea and localized gastroenteritis in humans, *S. enterica* serovar Typhi isolates cause systemic infection leading to typhoid fever (32). The ability to cause different types of disease in the same target species is not well understood but is almost certainly due to the different gene repertoires of the two serovars (36).

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In part because of their ability to target immune cells, *S. enterica* strains have attracted a great deal of interest as potential live oral vaccines and antigen delivery systems (28, 31, 40). Oral or parenteral challenge of mice with virulent *Salmonella* causes a systemic, typhoid fever-like infection, and this model has been exploited to identify genes that contribute to the in vivo survival and replication of *S. enterica*. Since infection of mice with *Salmonella* does not result in quantifiable diarrheal symptoms, other models have been used to identify factors contributing to the induction of gastroenteritis, including ligated ileal-loop models and oral challenge of calves (50). *S. enterica* derivatives harboring one or more attenuating mutations have been evaluated as potential oral vaccines in animal models (5, 34, 41). However, surprisingly few of these *S. enterica* derivatives have been evaluated in species which are targets for real vaccine development, such as humans. Early studies in humans with genetically defined strains focused on serovar Typhi derivatives harboring auxotrophic mutations affecting the purine and aromatic biosynthetic pathways (26, 42–44). Human volunteer studies with serovar Typhi derivatives with either single or double *aro* mutations demonstrated that, although the *aro* mutants were attenuated and highly immunogenic, they gave rise to silent bacteremia, a property which is not desirable. This stimulated a search for additional attenuating mutations which when introduced into an *aro* mutant will yield a vaccine that retains immunogenicity but does not cause bacteremia.

The *Salmonella* pathogenicity island 2 (SPI-2) encodes a

type III secretion system (TTSS) and is essential for the expression of full serovar Typhimurium virulence in mice (39). Serovar Typhimurium strains that harbor mutations in SPI-2 are defective in the systemic phase of infection and show a reduced ability to replicate within macrophages both in vivo and in vitro (4, 19, 35, 38, 48). This replication defect has also been observed in human epithelial cell lines (4). TTSSs are found in a number of gram-negative pathogenic bacteria and are required for the transfer of specific virulence-associated effector proteins across the host cell membrane. The SPI-2 TTSS is activated under intracellular conditions (4, 7) and translocates effector proteins from the *Salmonella*-containing vacuole across the vacuolar membrane into the cytosol of target host cells such as macrophages. The function of a number of the SPI-2 TTSS proteins has been predicted by sequence comparisons with proteins of other TTSSs. SsaV and SsaC are thought to be integral to the inner and outer bacterial cell membranes, respectively, and form part of the secreton, a needle-like organelle that exports proteins across these two membranes. SseB, SseC, and SseD are believed to be substrates of the TTSS that are exported by the secreton onto the cell surface to form a translocon. SseC and SseD are thought to form a translocon pore in the vacuolar membrane which is linked to the secreton by the translocon containing SseB (20, 23, 33). Some of the effector proteins secreted by the SPI-2 TTSS have been identified. SpiC (also designated SsaB) inhibits its cellular trafficking events in macrophages (48). SifA is required to maintain the integrity of the vacuolar membrane (3), and an unknown effector allows *Salmonella* to avoid respiratory burst by inhibiting trafficking of NADPH oxidase subunits to the vacuole (49). SspH2, SseI, SseF, SseG, SifB, and SseJ are also believed to be SPI-2 TTSS effectors, but their function is unknown (18, 30).

Our novel strategy for the development of oral *S. enterica* vaccines was to combine a mutation in *aroC* with an SPI-2 mutation. An *ssaV* mutation was chosen since SsaV is proposed to be integral to the SPI-2 secreton, and it has also been demonstrated that strains harboring mutations in *ssaV* cannot secrete SPI-2 TTSS effectors (18, 23, 33). Defined *aroC* and *ssaV* deletions were introduced into strains serovar Typhi Ty2 and into serovar Typhimurium TML. The rationale for inclusion of the *ssaV* mutation in the serovar Typhi strain is that this mutation may be expected to prevent the systemic spread of serovar Typhi in humans by virtue of reduced replication and survival in macrophages and target cells and therefore to prevent the bacteremia associated with *aro* mutants. The equivalent mutations were introduced into serovar Typhimurium in order to evaluate the potential of the strain as a vector for delivery of heterologous antigens. *aro* mutants of serovar Typhimurium are attenuated in animal models of salmonellosis (5, 41). Again, the rationale for the addition of the *ssaV* mutation was that it may be expected to reduce replication and survival of this strain in host cells, thereby further attenuating this strain and giving additional safety assurance for use in humans.

Here we report, for the first time, the direct comparison in humans of oral immunization with serovar Typhi and serovar Typhimurium derivatives harboring defined deletion mutations in *ssaV* and *aroC*. In this study, immunogenicity and safety were evaluated in healthy volunteers receiving a single escalating dose of bacteria.

MATERIALS AND METHODS

Study design. The study was open label, with sequential, dose-escalating cohorts, and not placebo controlled. The study was conducted on an Exemption from Licenses Order from the UK Medicines Control Agency (number MF8000/11082). Ethical permission was obtained from the St. George's Local Research Ethics Committee. Written informed consent was obtained after submission of a detailed explanation of the protocol. Subjects were excluded if they had a sensitivity to ciprofloxacin, had a known immunodeficiency or were on immunosuppressive therapy, had a history of gallbladder or gastrointestinal disease or some other significant medical condition, had received vaccination against *Salmonella* in the preceding 6 months, had had a *Salmonella* infection within the previous 5 years, or had a anti-*S. enterica* serovar Typhi O-antibody titer in serum as determined by enzyme-linked immunosorbent assay (ELISA) that was >2 standard deviations above the mean of a group of healthy controls. Subjects were also excluded if they had household contacts who were at risk for transmission of vaccine bacteria.

Vaccines were manufactured according to conditions of Good Manufacturing Practice protocols by Avecia Life Science Molecules (Billingham, United Kingdom) and were derived from a tested and characterized Development Cell Bank, also manufactured according to cGMP protocols. The clinical batches were stored at −75°C in 10% glycerol. On the day prior to vaccination, the number of viable cells (in CFU) contained in a vial of the ZH9 or WT05 vaccine was determined by the spread plate method. On the day of vaccination, the result of the viable count assay was used to determine the dilution required to obtain the required dose in 1 ml. A fresh vial of the vaccine was thawed and diluted to the appropriate concentration in 2% (wt/vol) sodium bicarbonate solution. Then, 1 ml of the diluted vaccine was added to 100 ml of 2% (wt/vol) sodium bicarbonate solution for administration to the volunteers and mixed, and a 2-ml aliquot was removed for a further viable count determination to ensure that the remaining 99 ml of vaccine solution contained the expected inoculum.

Eighteen healthy volunteers (eleven male, seven female) were recruited from among the students at St. George's Hospital Medical School (age range, 18 to 26 years; mean, 20.5 years). After fasting overnight, the volunteers swallowed 100 ml of 2% (wt/vol) sodium bicarbonate solution to buffer gastric acid and then received a single oral dose of either 10^7, 10^8, or 10^9 CFU of either ZH9 or WT05 suspended in 100 ml of the same buffer. Volunteers were resident in the Institute for 72 h to enable isolation and observation (including recording of pulse, temperature, and blood pressure every 4 h) and then followed up daily for 1 week and weekly for 1 month postimmunization. Volunteers were assessed for reactogenicity and other adverse events by physical examination and by the completion of diary cards. Subjects were enrolled to the next dose level after a satisfactory evaluation of reactogenicity at 14 days postimmunization.

Construction of serovar Typhi ZH9. Serovar Typhi (Ty2 Δ*aroC* Δ*ssa*) ZH9 (abbreviated to ZH9) is a derivative of the wild-type serovar Typhi strain, Ty2 (obtained from the National Collection of Typed Cultures at the Public Health Laboratory Service, Colindale, United Kingdom). Ty2 was originally isolated from a patient with typhoid fever and has been used for the derivation of all licensed typhoid vaccines. The Pir-dependent suicide plasmid pCVD442 (11) was utilized to introduce defined *aroC* and *ssaV* deletion mutations into Ty2. A defined 600-bp deletion was introduced into the wild-type serovar Typhi *aroC* gene in plasmid pTAC2 by amplifying the 5′ and 3′ regions of the gene and cloning the resulting PCR products into pUC18 to give plasmid pMIAC23. The 4.8-kb *Hin*dIII fragment containing the mutated *aroC* gene was then isolated from pMIAC23 and cloned into pCVD442, and the resulting plasmid pYCVC21 was introduced into serovar Typhi Ty2 by electroporation. The plasmid was then able to integrate into the Ty2 genome after recombination between the homologous regions on the plasmid and the genome to give ampicillin-resistant transformants. The transformants contained a copy of both the original wild-type *aroC* and the deleted *aro*C gene separated by the vector DNA sequences. Growing the transformants in the absence of ampicillin allowed for a second recombination event to occur which resulted in loss of the pCVD442 vector DNA sequences and one copy of the *aroC* gene, either the wild-type copy or the deleted copy. Serovar Typhi bacteria, which had undergone this second recombination event, were identified as ampicillin-sensitive derivatives that were able to grow in the presence of 5% sucrose (pCVD442 carries the *sacB* gene which when expressed results in a sucrose-sensitive phenotype). Strains that had retained only the deleted *aroC* gene were initially identified as strains that were unable to grow on minimal medium plates in the absence of a supplement of aromatic compounds. The *aroC* genotype and the presence of the required deletion was confirmed by

PCR and sequence analysis of five individual isolates, and one of them, serovar Typhi (Ty2 Δ*aroC*) DTY8, was selected for introduction of the *ssaV* mutation.

The wild-type serovar Typhi *ssaV* gene is contained on a 7.5-kb *Pst*I fragment in plasmid pTYSV21. A defined 1,893-bp deletion was created within the *ssaV* open reading frame on this plasmid by using inverse PCR, and the resulting construct was denoted pYDSV1. The 5.5-kb *Pst*I fragment containing the mutated *ssaV* gene was cloned into pCVD442 to generate plasmid pYDSV214. pYDSV214 was introduced into serovar Typhi (Ty2 Δ*aroC*) DTY8 by electroporation, and the *ssaV* mutation was transferred to the genome by homologous recombination as described above. PCR was used to identify ampicillin-sensitive, sucrose-resistant strains possessing the *ssaV* deletion mutation, and sequence analysis of the resulting PCR products confirmed the presence of the required deletion mutation in five individual isolates. Strain serovar Typhi (Ty2 Δ*aroC* Δ*ssaV*) ZH9 was chosen for further development.

Construction of serovar Typhimurium WT05. Serovar Typhimurium (TML Δ*aroC* Δ*ssaV*) WT05 (abbreviated as WT05) is a derivative of the wild-type serovar Typhimurium strain TML. Serovar Typhimurium TML was originally isolated from a patient suffering from gastroenteritis and was obtained from John Stephens, Birmingham University, Birmingham, United Kingdom. For construction of WT05, plasmid p7-2 (39) was used as the source of *ssaV* DNA. A defined 1,910-bp deletion was introduced into the open reading frame of *ssaV* in p7-2 by inverse PCR to give plasmid pMDSV1, and the 5.6-kb *Pst*I fragment containing the mutated *ssaV* gene was then isolated from pMDSV1 and cloned into pCVD442 to give pMDSV22. pMDSV22 was introduced into serovar Typhimurium TML by using conjugation, and the *ssaV* mutation was transferred to the genome by homologous recombination. The required strain, ZH26, containing the *ssaV* deletion mutation was identified among ampicillin-sensitive, sucrose-resistant isolates by PCR and sequence analysis.

A 600-bp deletion mutation was introduced into the wild-type *aroC* gene on the 5.4-kb *Hin*dIII fragment of plasmid pDAC1 by amplifying the 5′ and 3′ regions of *aroC* from pDAC1 and cloning the resulting PCR products into pUC18. The resulting construct, pMIAC8, contains the mutated *aroC* gene on a 4.8-kb *Hin*dIII fragment. The *Hin*dIII fragment was cloned into pCVD442 to yield plasmid pMCVC16, which was then introduced into serovar Typhimurium ZH26 by electroporation to generate strain WT02. Resolution of plasmid DNA sequences and the wild-type copy of the *aroC* gene from WT02 gave rise to several strains containing the *aroC* deletion. Sequence analysis confirmed the presence of the required deletion in four individual isolates. One of these, serovar Typhimurium (TML Δ*aroC* Δ*ssaV*) WT05, was chosen for further development.

Microbiological, hematological, biochemical, and clinical monitoring. Blood, stool, and midstream urine cultures were collected before immunization on the day of immunization, daily for the first 7 days, and weekly for 28 days after immunization. According to standard United Kingdom Public Health guidelines, patients infected with nontyphoidal *Salmonella* should be considered noninfectious when two separate stool samples are negative on culture (2). The protocol stated that, if subjects had uninterrupted shedding of vaccine bacteria in stools beyond day 28, then oral ciprofloxacin would be administered, and the subject was monitored until two successive stool samples were negative. Some cohorts provided daily stool cultures for an extended time after immunization, until two consecutive samples were negative. The ability of the attenuated strains to grow on standard microbiology media was confirmed in validation studies. Therefore, blood, stool, and urine cultures were performed by the routine pathology service of St. George's Hospital. Fecal samples were inoculated onto desoxycholate citrate agar (DCA) and xylose lysine desoxycholate (XLD) agar, and a small aliquot was emulsified into selenite F (SF) enrichment broth. After overnight incubation at 37°C, one loopful of SF broth was subcultured onto afresh DCA plate to obtain discrete colonies. Equal volumes of urine and double-strength SF broth were incubated at 37°C for 24 h. A loopful of broth was removed and inoculated onto DCA plates. Then, 5 ml of blood was inoculated into aerobic and anaerobic bottles, maintaining a ratio of 1:10, and incubated at 37°C by using the Organon Teknika BacT/Alert blood culture system. Blood cultures were continuously monitored for 5 days, and Gram stain was performed upon all bottles triggering a positive reaction. Those containing gram-negative bacilli were subcultured onto DCA and XLD as described above. Non-lactose-fermenting isolates on DCA and XLD and negative by oxidase test were characterized for biochemical reaction by using the Analytical Profile Index system (bioMérieux). Organisms identified as *Salmonella* were serotyped by using *Salmonella* agglutinating antisera (PROLAB and Central Public Health Laboratory, Colindale, United Kingdom). Urea and electrolytes, liver function tests, C-reactive protein (CRP) full blood count, erythrocyte sedimentation rate were measured in the routine pathology laboratories of St. George's Hospital. Volunteers kept a daily record of symptoms for 14 days, including frequency and consistency of stool, headache, fever, nausea, vomiting, and other constitutional upset.

ELISA and ELISPOT assays for *Salmonella*-specific serum antibody and ASC responses. Blood and saliva samples were collected prior to immunization and then on days, 7, 14, 21, and 28 after immunization. In some cohorts the final two time points were varied, as indicated in the figure and table legends. Saliva and serum were frozen at −70°C until analysis by ELISA. Peripheral blood mononuclear cells (PBMC) were collected from the volunteers, as well as from an unvaccinated negative control (the same person for all assays), and assayed for the presence of antibody-secreting cells (ASCs) by using the enzyme-linked immunospot (ELISPOT) technique. Briefly, PBMC obtained by antecubital fossa venipuncture were isolated by Ficoll-Hypaque centrifugation. Between 1×10^5 and 2.5×10^5 cells in 100 μl of RPMI were added to wells of 96-well Nunc Maxisorb plates, which were either uncoated (control wells) or previously coated overnight at 4°C with 100 μl of 1 μg of either serovar Typhi lipopolysaccharide (LPS; Sigma Chemical L6386), serovar Typhimurium LPS (Sigma Chemical L6511), or serovar Typhi H antigen (purified for use in ELISA in this study)/ml in carbonate coating buffer (pH 9.6). Cells were incubated overnight at 37°C in 5% CO_2 in a vibration-free incubator. After the cells were washed, wells received 100 μl of either goat anti-human immunoglobulin A (IgA; gamma chain specific) alkaline phosphatase-conjugated antibody (ISL 075-1001) diluted 1:1,000 in phosphate-buffered saline (PBS) or goat anti-human IgG (gamma chain specific) alkaline phosphatase-conjugated antibody (ISL 075-1002) diluted 1:5,000 in PBS. After 1 h of incubation at 37°C, wells were washed, and spots were then visualized by the addition of nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) substrate in agarose overlay and enumerated under low-power magnification. To account for nonspecific binding of the cells to the wells of the plate, the mean number of spots in uncoated wells (always <4 spots) was subtracted from the mean number of spots in antigen-coated wells to give a mean number of ASCs. To account for interassay variation and nonspecific immunity (since there was no placebo control group in this preliminary study), the subject who had never been vaccinated against *Salmonella* was bled at each time point and served as a negative control. The mean number of ASCs obtained with this negative control was subtracted from that of the trial participants, although the numbers were extremely low (mean, six IgA and five IgG ASCs to serovar Typhi and one IgA and two IgG ASCs to serovar Typhimurium). The presence of more than four *Salmonella*-specific ASCs per 10^6 PBMC was considered significant. Before ELISPOT assays were performed on cells from the trial volunteers, the validity of the ELISPOT assay was verified by using cells from a person vaccinated with three doses (days 0, 2, and 4) of Ty21a (Vivotif; Medeva UK; 2×10^9 CFU/dose). Cells were assayed for the presence of serovar Typhi LPS-specific IgA and IgG ASCs on days 0, 4, 7, 11, 18, and 25. Peak numbers of IgA ASCs were detected on day 7 and gradually declined over the period of observation. IgG-producing ASCs were also detected but at lower levels than IgA ASCs.

For the ELISA assay of serum, the same reagents and dilutions were used as in the ELISPOT assay. Flagellin was prepared from serovar Typhi strain BRD961 (the Δ*aroC* Δ*aroD* mutant of Ty2). The purity of the preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by performing a *Limulus* amebocyte lysate assay for detection of LPS (Sigma). A single band of ~50 kDa was observed after SDS-PAGE, and LPS was found to be present at <1% (wt/wt). For the IgG subclasses goat anti-human IgG1 (Sigma Chemical 9050-04), IgG2 (Sigma Chemical 9060-04), IgG3 (Sigma Chemical 9210-04), and IgG4 (Sigma Chemical 9200-04) alkaline phosphatase-antibody conjugates were used instead of the anti-IgG conjugate. Antigen-coated and control wells received replicate 100-μl portions of serum diluted 1:50 in PBS. Sequential doubling dilutions were made, and each plate contained dilutions of positive and negative sera from healthy volunteers, patients convalescing from clinical typhoid fever, and subjects vaccinated with the intramuscular whole-cell vaccine and/or live oral serovar Typhi vaccine Vivotif (Medeva). After 1 h of incubation, plates were washed and received alkaline phosphatase- conjugated antibodies as described above for 1 h at 37°C. Anti-LPS or anti-flagellin IgG and IgA activity was detected by incubating wells for 30 min at room temperature with pNPP substrate, and the absorbance was read at 405 nm. Activity is expressed as relative ELISA units by using the parallel-line assay, described previously, in which simultaneously assayed positive controls with known activity are used to determine relative potency. The ELISA units quoted are therefore the dilution of the test serum which gives an optical density of zero relative to the reference sample, which has a predetermined extinction dilution. Values after immunization that were higher than the day 0 mean for all 18 subjects, plus two standard deviations, were considered significant. For the anti-H IgG responses in the subjects receiving 10^9 organisms of ZH9, three subjects had evidence of existing immunity evidenced by raised levels of anti-H IgG. For

TABLE 1. Excretion of vaccine strains in stool and CRP level after immunization

Strain and dose	Subject	Excretion of vaccine strains and CRP[a] at (days after oral vaccination):																										
		−7	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	28
Serovar Typhi ZH9																												
10^7	044	−	−	−	+	−	−	−	−	−							−							−				−
	009	−	−	−	−	−	−	−	−	−							−							−				−
	045	−	−	−	−	−	−	−	−	−							−							−				−
10^8	048	−	−	+	−	−	−	−	−	−							−							−				−
	046	−	−	−	−	−	−	−	−	−							−							−				−
	035	−	−	−	−	−	−	−	−	−							−							−				−
10^9	030	−	−	−	−	−	−	−	−	−							−							−				−
	023	−	−	+	−	+	−	−	−	+							−							−				−
	057	−	BNO	−	BNO	−	−	BNO	BNO	−							−							−				−
Serovar Typhimurium WI05																												
10^7	008	−	−	+	−	+	−	−	−	−							−							−				−
	003	−	−	+	−	−	−	−	+	+							−							−				−
	010	−	−	−	−	+	+	+	+	+							−							−				−
10^8	029	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−		−	−		−	−	−				−
	011	−	−	−	+	+	+	+	−	+	−		−	−	−	−		−						−				−
	050	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−			−				−
10^9	012	−	−	+[b]	+[b]	+		+	+	+	+	+	+		−		−	+	+		−	−	+	−	−	+	−	−	−
	062	−	−	+[b]	+	+[b]	−	−	+	−	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−		
	063	−	−	+[b]	+[b]	+[b]	+	+	+	+	+	+	+	+	−	−	−	+		+		+	+	+	−	−		
10^7	008	<4	<4			<4				<4							<4							<4				37[c]
	003	<4	<4			<4				<4							<4							<4				**11**[c]
	010	<4	<4			<4				<4							**24**							<4				<4[c]
10^8	029	<4	<4			<4				<4							<4							<4				<4
	011	<4	<4			<4				<4							6							<4				<4
	050	<4	<4			57				8							<4							<4				4
10^9	012	<4	<4			45				<4							<4							<4				<4
	062	<4	<4			**11**				<4							<4							<4				<4
	063	<4	<4			**16**				4							<4							<4				<4

[a] A "+" indicates a positive stool culture; a "−" indicates a negative stool culture. BNO, bowels not opened on that day. Number values refer to CRP levels in milligrams/liter. The normal range is 0 to 8 mg/liter; abnormal values are highlighted in boldface. Data are shown only for serovar Typhimurium-immunized subjects since the CRP levels were normal at all time points in serovar Typhi-immunized subjects.

[b] Positive on primary DCA plates as well as enrichment culture.

[c] Measured on day 30.

this assay, the mean plus two standard deviations for the whole group excluding these three subjects is also given.

RESULTS

Immunization with serovar Typhi ZH9. ZH9 was administered to volunteers as an escalating dose regimen. Blood and urine cultures were negative in all subjects. No serious adverse events were noted with any dose, although two subjects receiving 10^8 organisms reported symptoms: one (subject 046) reported moderate abdominal discomfort between days 5 to 8 postimmunization, and a second (subject 048) experienced an intermittent mild headache between days 2 and 4 and passage of four unformed motions on day 3. One volunteer receiving 10^9 organisms (subject 057) reported passage of a single unformed stool associated with a single episode of nausea and vomiting 11 days after immunization. No volunteers reported fever. Shedding of ZH9 in the stool was detected in only one subject from each cohort. This occurred soon after vaccination, and lasted 1 day only (day 1 for subject 048 and day 2 for subject 044), except for one subject (subject 023) receiving 10^9 organisms, who shed on days 1, 3, and 7 (Table 1). No subjects received ciprofloxacin therapy. All inflammatory markers, including CRP, white blood cell count, and erythrocyte sedimentation rates were normal throughout the study.

None of the subjects receiving the lowest dose of 10^7 organisms mounted a detectable serum IgG or IgA response to LPS or flagellin (Fig. 1), although one (subject 044) had a high IgG and a low IgA anti-flagellin antibody level at the commencement of the study, which did not change after immunization. Two of the three subjects receiving the middle dose of 10^8 organisms mounted extremely brisk serum IgG responses to LPS, resulting in high activity by day 7 postimmunization (Fig. 1A). These two subjects also manifested gastrointestinal symptoms. The third subject in this cohort mounted a smaller and slower IgG anti-LPS response. All three subjects in this group also elicited significant IgA responses to LPS (Fig. 1C). The anti-H responses (both IgG and IgA) in this cohort followed a time course similar to that for the anti-LPS responses (Fig. 1B and D); however, the marked discordance in the anti-H and anti-LPS activities seen in some of the subjects (especially subject 044) makes a cross-reaction between LPS contamination of the H antigen used in the ELISA unlikely. The serum IgG responses to LPS in the cohort receiving the highest dose of 10^9 organisms were lower than the responses of the cohort receiving 10^8 organisms (Fig. 1A). One subject, subject 057, responded from day 7 (and was the subject reporting gastrointestinal symptoms on day 11 after immunization); a second (subject 023) responded from day 14 after an initial lag; and

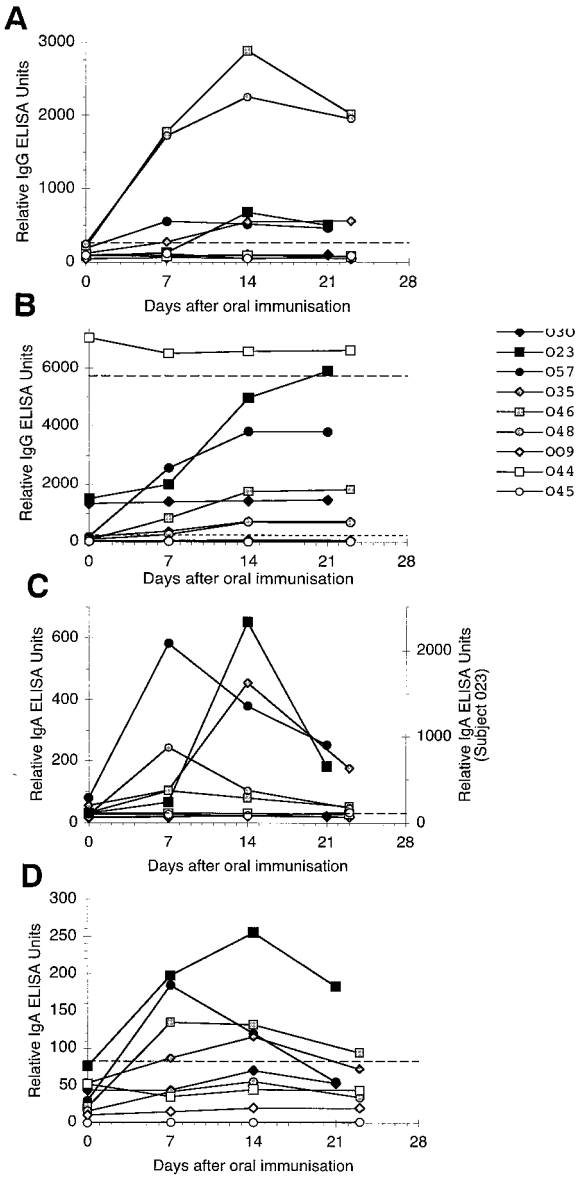

FIG. 1. Serum anti-serovar Typhi IgG and IgA responses. The serum IgG responses to serovar Typhi O antigen (A) and H antigen (B) and the IgA responses to O antigen (C) and H antigen (D) were measured by ELISA before and after immunization with various doses of serovar Typhi (10^7, open symbols; 10^8, shaded symbols; 10^9, solid symbols). The values given are the relative potencies compared to a standard serum and are expressed in nominal ELISA units. In panel C, subject 023 is plotted on the righthand axis. The broken line indicates whole group mean at day 0 plus two standard deviations; the dashed line in panel B indicates the group mean, excluding subjects 030, 023, and 044, plus two standard deviations.

the third (030) did not respond at all. The anti-H responses (both IgG and IgA) followed the same pattern of response, except that subjects 023 and 030 started with a higher preimmunization anti-H IgG activity (Fig. 1B and D). Subject 023, however, had very significant IgA response after vaccination.

Of the three nonresponders, subject 045 probably did not mount an immune response because of the low dose of bacteria administered. The delayed or absent responses in subjects

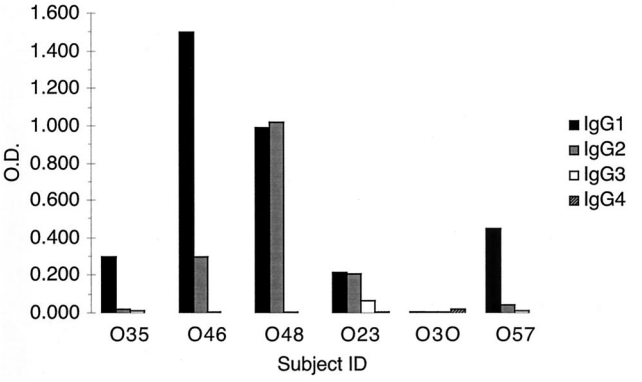

FIG. 2. IgG subclass of serum anti-serovar Typhi O antigen response. The subclass profile of the IgG response to serovar Typhi O antigen was measured in subjects 14 days after they received 10^8 or 10^9 serovar Typhi by ELISA with mouse monoclonal antibodies to human subclasses. The values are expressed as the optical density of serum diluted 1 in 50.

030 and 023 who had high starting levels of anti-H IgG may indicate the effect of prior immunity on the ability of the vaccine strain to induce an immune response. Subjects were excluded if they had received a *Salmonella* vaccine in the 6 months prior to the study or salmonellosis in the 5 years prior to the study. On taking a travel history, both subjects recalled receiving a parenteral *Salmonella* vaccine 3 to 5 years earlier. The low anti-H preimmunization IgA levels in these subjects would correlate with the known short half-life of the IgA responses. Subjects 045 and 030 did not shed bacteria in the stool and had no immune responses to either antigen, whereas subject 023 shed on several occasions and, apart from a delay in the response, had very high IgA responses to O and H antigens, presumably as a result of this mucosal priming.

All volunteers with a measurable serum IgG response to LPS on day 14 had principally IgG1 antibodies, although two subjects had roughly equivalent IgG2 responses (Fig. 2). This pattern is in keeping with mucosal responses to other antigens delivered via the gut.

Although the lowest dose was unable to induce a systemic immune response (IgG or IgA), it appeared able to induce localized mucosal immunity manifested by trafficking anti-LPS IgA ASCs after immunization, peaking on day 7 (Table 2). This dissociation between mucosal and systemic immune responses is intriguing and suggests that the attenuation may prevent systemic invasion, while allowing interaction with the mucosal immune system. The magnitude of the mucosal IgA LPS-specific ASC response did not appear to be dose dependent since higher doses (10^8 and 10^9 CFU) did not result in more frequent (two of three subjects in each cohort) or higher IgA ASC responses. This is in keeping with the stool culture results which showed only transient colonization in only one of three subjects from each cohort (all of whom manifested significant IgA ASC responses). IgG anti-LPS ASC responses were low in all subjects at all doses, in keeping with a preferential IgA switching in gut-associated lymphoid tissue.

Immunization with serovar Typhimurium WT05. No serious adverse events were noted at any dose levels. No subjects reported symptoms of postinfective reactive arthropathy, which

TABLE 2. *Salmonella* O-antigen IgA and IgG ASC responses[a]

Cohort	Dose	Subject	Antigen-specific ASCs/10^6 PBMC at: (days after immunization):									
			IgA					IgG				
			0	7	14	23[b]	30[c]	0	7	14	23[b]	30[c]
Serovar Typhi ZH9												
1	10^7	009	0	96	0	12	1	0	0	0	0	0
		044	0	24	6	0	0	0	28	0	0	0
		045	0	0	0	3	0	0	0	0	0	1
3	10^8	035	1	4	0	1	0	0	1	0	4	0
		046	0	0	14	2	4	3	3	0	0	0
		048	3	24	0	1	0	1	0	0	0	0
5	10^9	023	0	32	0	1	1	0	7	0	1	0
		030	0	4	0	0	0	0	4	1	0	0
		057	0	12	11	0	2	0	4	4	0	0
Serovar Typhimurium WT05												
2	10^7	003	15	13	0	0	0	0	0	0	0	0
		008	12	0	1	0	0	0	0	0	0	0
		010	0	5	0	0	1	0	0	0	0	0
4	10^8	011	3	3	0	2	NT	11	0	1	1	NT
		029	0	1	2	2	0	0	0	0	5	0
		050	0	0	0	0	0	0	0	0	0	1
6	10^9	012	1	131	86	6	0	0	103	88	26	8
		062	0	21	5	0	0	0	23	25	11	0
		063	0	27	9	16	3	0	56	21	9	17

[a] Values are antigen-specific ASCs expressed per 10^6 PBMC plated. NT, not tested.
[b] Measured on day 23 for cohorts 1 to 3.
[c] Measured on day 28 for cohorts 4 and 6 and day 24 for cohort 5.

would be expected to manifest within 30 days of infection (27). Volunteers were asked to report any adverse events after the study and to date no reports of any arthropathy have been received. Subjects were not tested for HLA-B27 haplotype, which has been associated with postinfective reactive arthritis, although a recent study specific to serovar Typhimurium did not find susceptibility to reactive arthritis was increased in HLA-B27-positive individuals (29). Blood cultures were negative in all subjects at all time points. Only one subject (subject 050) reported any symptoms, i.e., headache and malaise associated with a fever of 38.7°C, commencing 36 h after receiving 10^8 organisms and lasting 6 h. Stool culture was negative on the day of the symptoms but became positive the next day (Table 1). The CRP was elevated on day 3 (57 mg/liter [normal = ≤8]) but returned to normal by day 7. The white cell count and other biochemical and hematological parameters were normal throughout. Although the remaining volunteers were asymptomatic, intriguingly, those receiving 10^9 bacteria had evidence of an acute-phase response with elevated CRP values on day 3 that fell to normal by day 7 (Table 1). Among the subjects receiving 10^7 bacteria, elevated CRP levels were detected on one occasion on days 14 or 28. Since these days coincided with collection of serum we were able retrospectively to determine the interleukin-6 (IL-6) concentration in serum (measured by ELISA), and this was found to be elevated in subjects 008 and 010 (23.6 and 70 pg/ml, respectively). White cell counts were slightly raised on day 14 in two subjects receiving 10^9 bacteria.

The pattern of stool excretion of the serovar Typhimurium strain was strikingly different from that of serovar Typhi (Table 1). All volunteers receiving the lowest dose shed the organism for at least 2 days, although shedding had stopped by day 14. The protocol was amended for subsequent cohorts to enable better characterization of the duration of shedding. All subjects receiving 10^8 organisms shed from day 2 and up to day 14; those receiving 10^9 organisms shed from day 2 up to day 23. Although bacterial counts were not measured, the degree of shedding was thought to be high since the vaccine organisms grew on the primary DCA culture plates, without requiring enrichment, in all subjects receiving 10^9 organisms in the first 2 to 3 days after immunization (Table 1). All subjects had at least two separate stool samples that were culture negative by day 28, and therefore no subjects required ciprofloxacin therapy as per the protocol.

The systemic anti-LPS responses after immunization with serovar Typhimurium were more varied than those seen after immunization with serovar Typhi, with some subjects responding from all dose levels (Fig. 3). Subject 050 had a strikingly high IgG and IgA response; this was the only subject to report symptoms. Again, there was generally no correlation between the mucosal ASC (Table 2) and antibody responses in serum (for example, subject 050 did not mount any IgA or IgG ASC response), although there was a tendency for subjects to mount both a serum IgG and IgA response. Indeed, despite prolonged shedding of vaccine organisms in the stool, no significant IgA or IgG ASC responses were seen in subjects receiving 10^7 or 10^8 organisms, although some did mount a serum antibody response. In contrast, all subjects receiving 10^9 organisms had a significant IgA ASC response, which is perhaps not surprising given the degree of bacterial stool colonization and evidence of an acute-phase response. These subjects also had a significant IgG ASC response to LPS, but only one had a significant serum IgG and IgA response.

DISCUSSION

We compared here the safety and immunogenicity of serovar Typhi and serovar Typhimurium Δ*aroC* Δ*ssaV* derivatives

TABLE 3. Vaccine-related symptoms, bacterial stool shedding, and immune responses

Parameter	Results for vaccine:																	
	Serovar Typhi ZH9 at dose of:									Serovar Typhimurium WT05 at dose of:								
	10^7 in subject:			10^8 in subject:			10^9 in subject:			10^7 in subject:			10^8 in subject:			10^9 in subject:		
	009	044	045	035	046	048	023	030	057	003	008	010	011	029	050	012	062	063
Symptoms (day no)[a]	–	–	–	–	A (4–6)	H (2–4), L (3)	–	–	L, N/V (11)						H, P (11)			
Stool excretion (day no.)[b]	–	2	–	–	–	1	1–7	–	–	1–7	1–3	3–7	2–7	2–12	2–14	1–23	1–16	1–21
ASCs[c]																		
IgA	**96**	**24**	0	3	**14**	**21**	**32**	4	**12**	0	0	5	0	2	0	**130**	**21**	**27**
IgG	0	**28**	0	1	0	0	**7**	4	4	0	0	0	0	0	0	**103**	**25**	**56**
Anti-O																		
IgA[d]	22	31	35	**455**	104	**245**	**2,327**	24	**583**	**162**	**95**	**1,059**	33	**2,391**	**1,520**	**1,037**	**347**	**631**
IgG[e]	58	89	114	**564**	**2,884**	**2,252**	**681**	101	**554**	**1,644**	172	305	59	**825**	**7,023**	**904**	198	56
Anti-H																		
IgA[f]	28	**89**	2	**116**	**135**	55	**255**	70	**184**									
IgG[g]	86	**6,744**	35	*811*	*2,163*	*811*	**5,916**	*1,541*	*3,827*									

[a] Symptoms: A, abdominal discomfort; H, headache; L, loose stool; N/V, nausea and vomiting; *P*, pyrexia. –, no symptoms.
[b] The range from the first to the last day of positive stool culture is given where applicable.
[c] Peak increase in antigen-specific ASCs expressed per 10^6 PBMC plated after day 0 value at any time postimmunization. Boldface type indicates a value >4 ASCs increased above the day 0 value.
[d] Peak anti-O IgA level in serum expressed as relative ELISA units at any time postimmunization. Boldface type indicates a value greater than the group mean day 0 activity plus two standard deviations (111 and 85 ELISA units for ZH9 and WT05 groups, respectively).
[e] Peak anti-O IgG level in serum expressed as relative ELISA units at any time postimmunization. Boldface type indicates a value greater than the group mean day 0 activity plus two standard deviations (268 and 341 ELISA units for the ZH9 and WT05 groups, respectively).
[f] Peak anti-H IgA level in serum expressed as relative ELISA units at any time postimmunization. Boldface type indicates a value greater than the group mean day 0 activity plus two standard deviations (83 ELISA units).
[g] Peak anti-H IgG level in serum expressed as relative ELISA units at any time postimmunization. Boldface type indicates a value greater than the group mean day 0 activity plus two standard deviations (5725 ELISA units). The value was 240 ELISA units if subjects 044, 030, and 023 are excluded; values above this level are in italics.

after oral immunization of healthy volunteers. Both ZH9 and WT05 were well tolerated by the volunteers. There was no evidence for systemic spread of ZH9, since bacteria were not detected in the blood at any time. Serovar Typhi Ty2 *aro* derivatives reproducibly cause bacteremia at the doses utilized in this study (21, 44, 45) and, although a serovar Typhi Δ*aro* strain was not utilized in this study, since it was ethically inappropriate, we feel these preliminary data are sufficient to suggest that the *ssaV* mutation was modulating the virulence of this strain, thus reducing the bloodstream survival of ZH9 and eliminating postimmunization bacteremia, which has been identified as a key safety feature in potential live oral typhoid vaccines. This would be in keeping with data from animal models demonstrating that a functional SPI-2 system is required for intracellular survival and establishment of the early bloodstream phase of serovar Typhi infection. The introduction of an *htrA* mutation into a Ty2 *aro* mutant (to yield strain CVD 908-htrA) and into a serovar Typhi CDC10-80 *aro* mutant (to yield strain PBCC222) was also found to abolish bacteremia while retaining immunogenicity (9, 45, 46).

The absence of fever after administration of ZH9 is in accordance with results obtained with strains CVD 908-htrA and PBCC22 that produced fever only in a low proportion of individuals receiving a higher dose of vaccine than was used in this study (9, 45). Fever has been attributed to an endotoxic response to the amount of LPS delivered at these high dose levels.

ZH9 elicited a mucosal immune response as indicated by the presence of anti-LPS IgA ASCs in the bloodstream. There were 67% responders with a mean of 33 ASCs/10^6 PBMC. The generation of LPS IgA ASCs did not appear to be dose dependent. It is difficult to make a true comparison between results obtained in this study and those of other studies, since the numbers involved in this study are small and also the methodologies used may not be directly comparable between different laboratories; however, this degree of response far exceeds that reported with a single dose of the liquid formulation of Ty21a, the currently licensed oral typhoid vaccine (50% response, GMN three ASCs), and is comparable to that obtained with three doses of 2×10^9 CFU of Ty21a formulated as enteric coated capsules (90% responders, GMN 23 ASCs) (25). The percentage of responders and the magnitude of the response to ZH9 was found to be lower than that obtained over a similar dose range for CVD 908-htrA. There is some indication that the production of ASCs correlates with the degree of colonization of the intestines by ZH9; all three volunteers who shed ZH9 also produced ASCs. It could be surmised from these data that ZH9 has a lower tendency to colonize the intestines than CVD 908-htrA. Indeed, although ZH9 did not cause diarrhea, diarrheal symptoms were recorded by a small proportion of volunteers receiving CVD 908-htrA (45, 46).

LPS IgG serum antibody response to ZH9 showed some degree of dose dependency, with none of three of low dose but five of six (83%) of individuals receiving the two higher dose

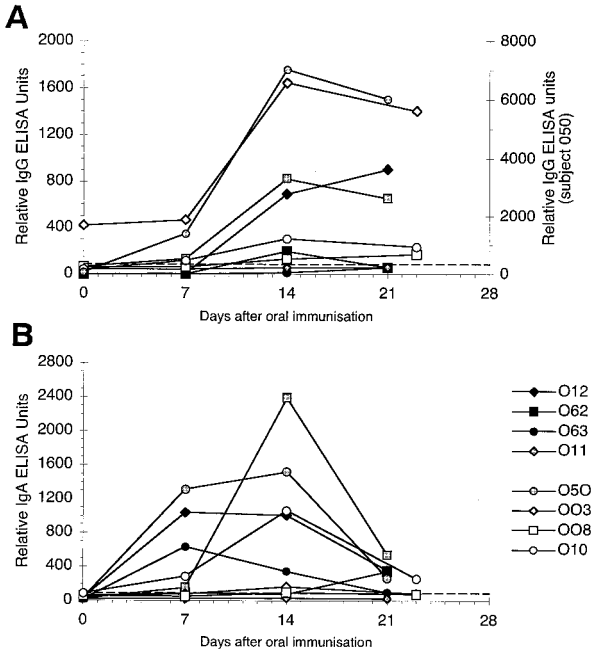

FIG. 3. Serum anti-serovar Typhimurium IgG and IgA responses. The serum IgG (A) and IgA (B) response to serovar Typhimurium O antigen was measured by ELISA before and after immunization with various doses of serovar Typhimurium (10^7, open symbols; 10^8, shaded symbols; 10^9, solid symbols). The values given are the relative potencies compared to that in a standard serum and are expressed in nominal ELISA units. In panel A, subject 050 is plotted on the righthand axis. Broken line indicates group mean at day 0 plus two standard deviations.

levels responding. This far exceeds the rate of responders obtained with a single dose of Ty21a, is greater than that obtained with a lyophilized formulation of CVD 908-htrA or PBCC222 (9, 46), but is lower than that recorded when volunteers were immunized with freshly harvested CVD 908-htrA (45). Unfortunately, comparison of the magnitude of responses obtained with the vaccine strains is not possible due to methodological differences in the assays employed in these studies.

Serovar Typhimurium TML was selected as the background strain for the construction of WT05 since it was originally isolated from a case of human diarrhea (17). Although serovar Typhimurium does have a broad host range, we felt that it was more appropriate to use a human isolate as a human vaccine candidate than an isolate from another species. In addition, *aro* derivatives of TML had been shown to be attenuated in the rabbit ileal loop models of human gastroenteritis (12). A close correlation has been demonstrated between fluid secretion in rabbit ileal loops and virulence for humans of diarrhea-inducing serovar Typhimurium. In this model (and also a bovine ileal loop model [our unpublished results]), TML wild-type strain causes significant accumulation of fluid in the gut lumen. In one study (12), the fluid was bloodstained and contained high levels of polymorphonucleocytes, a finding indicative of an inflammatory response. Fluid accumulation was associated with damaged and shortened villi. In contrast, an Δ*aroA* TML mutant produced inflammation and some slight histological changes but did not cause the fluid accumulation that is associated with an ability to cause diarrhea in humans. It was concluded that this strain was invasive but nondiarrheagenic. In this study it was also reported that the wild-type strain SL1344 behaved in the same way as wild-type TML and that the introduction of an SPI-2 mutation (Δ*ssrA*) into SL1344 did not result in any degree of attenuation. It was concluded that SPI-2 is not required for induction of fluid accumulation in this model. In keeping with this was the observation that that, although a serovar Typhimurium *aroA* mutant was severely attenuated in a calf model of gastroenteritis, an SPI-2 mutant (Δ*spiB*, alternative designation Δ*ssaD*), attenuated in the mouse model of typhoid fever, caused diarrhea and morbidity comparable to those caused by the wild-type strain (47).

In the current study in humans the double *aro*–SPI-2 mutant WT05 did not cause diarrhea. By reference to the murine models (12, 47), it appears likely that the *aroC* mutation is the major contributor to this characteristic of WT05, and the role, if any, of the *ssaV* mutation is unclear. The basis of attenuation of *aro* mutants in both the murine and calf models of infection is believed to be that they are nutritionally dependent on substrates (*para*-amino benzoic acid and 2,3-dihydroxybenzoic acid) that are not available in sufficient quantity on the host tissues to allow replication. Since SPI-2 is required for intracellular survival and replication, it could be predicted that this mutation could only influence steps in gastroenteritis that depend on serovar Typhimurium replicating in enterocytes or the infiltrating polymorphonuclear leukocytes. It is possible that the addition of a SPI-2 mutation may lead to further debilitation of *aro* mutants residing in these host cells. Comparison of single *aro* and SPI-2 mutants with a double *aro*–SPI-2 mutant in a model of gastroenteritis may shed light on this.

Although volunteers receiving WT05 displayed no detectable signs of fluid loss or diarrhea, they were heavily colonized by this strain, as indicated by prolonged shedding, and at the highest dose levels induced a significant acute-phase response, indicative of inflammation, and immune response evidenced by circulating anti-LPS ASCs. Again, the presence of inflammation is consistent with the behavior of *aro* mutants in the rabbit ileal loop model (12). Similar observations were made after administration of a *phoP*/*phoQ* derivative of serovar Typhimurium to humans (1). After a single dose of 5 to 8×10^8 CFU, volunteers were durably colonized (last day of shedding ranged from days 2 to 10), but none had diarrhea or bacteremia. The dose of this strain was slightly lower than the high dose of WT05, and the rate of mucosal priming as indicated by the presence of circulating anti-LPS ASCs was comparable to that achieved by WT05. However, the degree of mucosal priming, indicated by the numbers of ASCs, appeared to be slightly higher for the *phoP*/*phoQ* strain. In addition, the numbers of volunteers giving an anti-LPS serum IgG response was greater for the *phoP*/*phoQ* derivative than for WT05 at a similar dose level (1). That study is the only previous report of a serovar Typhimurium strain being evaluated as a vector in humans and the only previous opportunity to compare serovar Typhi (10) and serovar Typhimurium strains with the same mutations after administration to humans.

The marked differences in the clinical and immunological events observed in human typhoid fever or gastroenteritis caused by serovar Typhi and nontyphoidal salmonellae are well known. These phenotypic differences have also been charac-

terized recently at the genetic level. A significant observation made in this study is the large difference in the shedding patterns between ZH9 and WT05 in the volunteers. ZH9 did not become established in the human intestine at a detectable level, and shedding was only transient. In comparison, WT05 was shed consistently for many days after administration. This is similar to the comparative behavior of the *phoP*/*phoQ* derivatives of serovar Typhi and serovar Typhimurium (1, 10). These observations could be explained in part by the different lifestyles of the two *S. enterica* serovars. Serovar Typhimurium is adapted to cause localized infection in the intestine, and luminal growth is thought to be an important component of pathogenicity. In contrast, serovar Typhi causes systemic infection and may have compromised its ability to grow in the intestine. In clinical typhoid fever, chronic carriers shed large numbers of bacteria, but these may be replicating in the gall bladder rather than in the gut. Significantly, during the recent sequencing of the serovar Typhi and serovar Typhimurium genomes, serovar Typhi was found to harbor over 200 pseudogenes not present in serovar Typhimurium (36). Some of these pseudogenes may be derived from genes required for normal intestinal colonization. This suggests that it may be possible to further attenuate live oral vaccine strains by mutating genes that contribute to shedding. Indeed, some shedding-associated genes have already been described in *S. enterica* (22). This may facilitate the safe use of live vaccines by preventing the inappropriate shedding of genetically manipulated bacteria.

Ty2 was chosen as the parent strain of ZH9 as an additional safety measure. Mutant derivatives of Ty2 have been found to be less virulent than derivatives of other wild-type serovar Typhi strains containing identical mutations (21, 45), and although not demonstrated conclusively, this has been attributed to the presence of an *rpoS* mutation in the Ty2 parent strain (6, 37). The safety and immunogenicity of the *Salmonella* strains used here was established in preclinical murine studies with serovar Typhimurium. Two transposon SP12 serovar Typhimurium mutants showed a reduction in virulence of at least 5 orders of magnitude after oral and intraperitoneal delivery compared to the parental strain (39). This profound attenuation of virulence by both routes of inoculation demonstrates that SP12 is required for events in the infective process after epithelial cell penetration in mice. In a second study the in vivo kinetics of SPI-2 transposon mutant *ssa*J::mTn*5* (P11D10) was determined. Whereas all mice receiving wild-type strain died, at which time the bacterial load in the spleens and livers exceeded 10^8, mice receiving P11D10 largely cleared bacteria from livers and spleens by 16 weeks (38). These data showed that an SP12 mutant can be transported from the peritoneal cavity to spleen and liver, suggesting some limited growth in these organs but that the SP12 secretion defect effectively prevents further accumulation of bacterial cells in these organs, leading to their eventual clearance. A further study found that PD11D10 delivered orally was present in Peyer's patches at levels 2 to 3 logs lower than in the wild type, was virtually undetectable in mesenteric lymph nodes, and was not recovered from the spleen and liver (8). Comparison of WT05 with a serovar Typhimurium *aro* mutant in the mouse typhoid model demonstrated that the double mutant WT05 colonized mouse tissues at a lower level and than the single *aro* mutant and was cleared more rapidly from the tissues. In addition to the studies described above with derivatives of SL1344, studies with derivatives of TML or ZH9 showed that, whereas the single *aro* mutant was able to kill immunocompromised mice, the double mutant was completely attenuated and did not result in any deaths (unpublished data). In these studies, comparison of ZH9 with the single *aro* mutant serovar Typhi (Ty2 Δ*aroC*) DTY8 in human macrophage-like cells demonstrated that, while the *aroC* mutation reduced replication of serovar Typhi, the addition of the *ssaV* mutation completely prevented replication. These data demonstrate that in each strain the presence of the *ssaV* mutation leads to more severe attenuation than that observed with the *aro* mutation alone. In addition to published studies, we conducted studies in immunodeficient mice, prior to seeking regulatory approval for the present study, and the results of those studies were made available to the UK Medicines Control Agency. No differences in PD11D10 numbers were detected in the spleens and livers of anti-IL-12-treated mice 3 days after intravenous inoculation compared to control mice. Liver and spleen counts in controls and anti-IL-12-treated mice remained similar throughout the course of the experiment and did not exceed the initial inoculum levels at any time. In separate experiments, no differences in PD11D10 numbers were detected in spleens and livers of gamma interferon knockout (IFN-γ KO) mice and controls 1 day after intravenous challenge with SPI-2 mutants. Higher bacterial numbers were present in the livers of the IFN-γ KO mice compared to control animals on days 7, 12, 16, and 22 of the infection, but by day 22 the numbers of organisms in livers and spleens was reduced to ca. 10^4, indicating that the organisms were being cleared. In contrast, *aro* mutants lead to a lethal infection in this model. These unpublished experiments demonstrated that SPI-2 mutants of *Salmonella* were unable to kill this class of immunocompromised animals, whereas *aro* mutants do. The combination of an SPI-2 mutation such as *ssa*V with an *aro* mutation in a *Salmonella* vaccine candidate was therefore thought to provide additional safety assurance for use of these strains in humans. Finally, an independent, single-dose toxicity study was performed in mice with 10^9 CFU of both vaccine strains, which resulted in no evident clinical or histopathological evidence of disease (unpublished data).

The objective of the present study was to characterize ZH9 and WT05 as novel vaccine candidates. To our knowledge, this is one of few side-by-side comparisons in humans of bacterial isolates representative of two *S. enterica* serovars capable of causing gastroenteritis or typhoid fever (1, 10) (Table 3). We believe it is also the first report on the properties of *S. enterica* derivatives harboring mutations in type III secretion systems (SPI-2) in humans, and may provide a general method for attenuating pathogens that harbor these systems. The data provide some intriguing insights into the properties of these *S. enterica* serovars that have important implications for the development of *Salmonella* vaccines and live oral vaccines in general.

Both ZH9 and WT05 were immunogenic. ZH9 was consistently the more immunogenic in terms of both ability to generate anti-*Salmonella* ASC responses and ability to generate anti-*Salmonella* serum antibodies. There was evidence of mucosal priming without systemic responses, suggesting a degree of mucosal compartmentalization in the immune response to

gut colonization. There was also a suggestion that prior immunity may modulate the response to these live vaccines as described in murine studies (24). Overall, the immunogenicity and lack of reactogenicity is encouraging for the potential of both strains as future *Salmonella* vaccines and as carriers for the delivery of heterologous antigens to the immune system. The more information we glean from human studies, the more likely we are to be able to design optimal vehicles for antigen delivery.

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REFERENCES

1. **Angelakopoulos, H., and E. L. Hohmann.** 2000. Pilot study of *phoP*/*phoQ*-deleted *Salmonella enterica* serovar Typhimurium expressing *Helicobacter pylori* urease in adult volunteers. Infect. Immun. **68:**2135–2141.
2. **Anonymous.** 1988. Public health (infectious diseases) regulations. The Stationary Office, London, United Kingdom.
3. **Beuzón, C., G. Banks, J. Deiwick, M. Hensel, and D. Holden.** 2002. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. EMBO J. **19:**3235–3249.
4. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow.** 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. **30:**175–188.
5. **Cooper, G. L., L. M. Venables, R. A. Nicholas, G. A. Cullen, and C. E. Hormaeche.** 1992. Vaccination of chickens with chicken-derived *Salmonella enteritidis* phage type 4 *aroA* live oral *Salmonella* vaccines. Vaccine **10:**247–254.
6. **Coynault, C., V. Robbe-Saule, and F. Norel.** 1996. Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS (sigma S) regulon. Mol. Microbiol. **22:**149–160.
7. **Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel.** 1999. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. Mol. Microbiol. **31:**1759–1773.
8. **Deiwick, J., T. Nikolaus, J. E. Shea, C. Gleeson, D. W. Holden, and M. Hensel.** 1998. Mutations in *Salmonella* pathogenicity island 2 (SPI2) genes affecting transcription of SPI1 genes and resistance to antimicrobial agents. J. Bacteriol. **180:**4775–4780.
9. **Dilts, D. A., I. Riesenfeld-Orn, J. P. Fulginiti, E. Ekwall, C. Granert, J. Nonenmacher, R. N. Brey, S. J. Cryz, K. Karlsson, K. Bergman, T. Thompson, B. Hu, A. H. Bruckner, and A. A. Lindberg.** 2000. Phase I clinical trials of *aroA aroD* and *aroA aroD htrA* attenuated *S. typhi* vaccines: effect of formulation on safety and immunogenicity. Vaccine **18:**1473–1484.
10. **DiPetrillo, M. D., T. Tibbetts, H. Kleanthous, K. P. Killeen, and E. L. Hohmann.** 1999. Safety and immunogenicity of *phoP*/*phoQ*-deleted *Salmonella typhi* expressing *Helicobacter pylori* urease in adult volunteers. Vaccine **18:**449–459.
11. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
12. **Everest, P., J. Ketley, S. Hardy, G. Douce, S. Khan, J. Shea, D. Holden, D. Maskell, and G. Dougan.** 1999. Evaluation of *Salmonella typhimurium* mutants in a model of experimental gastroenteritis. Infect. Immun. **67:**2815–2821.
13. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
14. **Galan, J. E.** 2000. Alternative strategies for becoming an insider: lessons from the bacterial world. Cell **103:**363–366.
15. **Galan, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
16. **Garcia-del Portillo, F., and B. B. Finlay.** 1995. Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors. J. Cell Biol. **129:**81–97.
17. **Giannella, R. A., S. B. Formal, G. J. Dammin, and H. Collins.** 1973. Pathogenesis of salmonellosis: studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. J. Clin. Investig. **52:**441–453.
18. **Hansen-Wester, I., B. Stecher, and M. Hensel.** 2002. Type III secretion of *Salmonella enterica* serovar typhimurium translocated effectors and SseFG. Infect. Immun. **70:**1403–1409.
19. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. **30:**163–174.
20. **Holden, D.** 2002. Trafficking of the salmonella vacuole in macrophages. Traffic **3:**161–169.
21. **Hone, D. M., C. O. Tacket, A. M. Harris, B. Kay, G. Losonsky, and M. M. Levine.** 1992. Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. J. Clin. Investig. **90:**412–420.
22. **Kingsley, R. A., K. van Amsterdam, N. Kramer, and A. J. Baumler.** 2000. The *shdA* gene is restricted to serotypes of *Salmonella enterica* subspecies I and contributes to efficient and prolonged fecal shedding. Infect. Immun. **68:**2720–2727.
23. **Klein, J. R., and B. D. Jones.** 2001. *Salmonella* pathogenicity island 2-encoded proteins SseC and SseD are essential for virulence and are substrates of the type III secretion system. Infect. Immun. **69:**737–743.
24. **Kohler, J. J., L. B. Pathangey, S. R. Gillespie, and T. A. Brown.** 2000. Effect of preexisting immunity to *Salmonella* on the immune response to recombinant *Salmonella enterica* serovar typhimurium expressing a *Porphyromonas gingivalis* hemagglutinin. Infect. Immun. **68:**3116–3120.
25. **Levine, M. M.** 1994. Typhoid fever vaccines, p. 507–633. *In* S. A. Plotkin, and E. A. Mortimer (ed.), Vaccines. W. B. Saunders Co., Philadelphia, Pa.
26. **Levine, M. M., J. Galen, E. Barry, F. Noriega, S. Chatfield, M. Sztein, G. Dougan, and C. Tacket.** 1996. Attenuated *Salmonella* as live oral vaccines against typhoid fever and as live vectors. J. Biotechnol. **44:**193–196.
27. **Maki-Ikola, O., and K. Granfors.** 1992. *Salmonella*-triggered reactive arthritis. Scand. J. Rheumatol. **21:**265–270.
28. **Mastroeni, P., J. A. Chabalgoity, S. J. Dunstan, D. J. Maskell, and G. Dougan.** 2001. *Salmonella*: immune responses and vaccines Vet. J. **161:**132–164.
29. **McColl, G. J., M. B. Diviney, R. F. Holdsworth, P. D. McNair, J. Carnie, W. Hart, and J. McCluskey.** 2000. HLA-B27 expression and reactive arthritis susceptibility in two patient cohorts infected with *Salmonella typhimurium.* Aust. N. Z. J. Med. **30:**28–32.
30. **Miao, E. A., J. A. Freeman, and S. I. Miller.** 2002. Transcription of the SsrAB regulon is repressed by alkaline pH and is independent of PhoPQ and magnesium concentration. J. Bacteriol. **184:**1493–1497.
31. **Mollenkopf, H., G. Dietrich, and S. H. Kaufmann.** 2001. Intracellular bacteria as targets and carriers for vaccination. Biol. Chem. **382:**521–532.
32. **Neidhardt, F. C., R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.).** 1996. *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
33. **Nikolaus, T., J. Deiwick, C. Rappl, J. A. Freeman, W. Schroder, S. I. Miller, and M. Hensel.** 2001. SseBCD proteins are secreted by the type III secretion system of *Salmonella* pathogenicity island 2 and function as a translocon. J. Bacteriol. **183:**6036–6045.
34. **O'Callaghan, D., D. Maskell, F. Y. Liew, C. S. Easmon, and G. Dougan.** 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attention, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56:**419–423.
35. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman.** 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc. Natl. Acad. Sci. USA **93:**7800–7804.
36. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848–852.
37. **Robbe-Saule, V., and F. Norel.** 1999. The *rpoS* mutant allele of *Salmonella typhi* Ty2 is identical to that of the live typhoid vaccine Ty21a. FEMS Microbiol. Lett. **170:**141–143.
38. **Shea, J. E., C. R. Beuzon, C. Gleeson, R. Mundy, and D. W. Holden.** 1999. Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. Infect. Immun. **67:**213–219.
39. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium.* Proc. Natl. Acad. Sci. USA **93:**2593–2597.
40. **Simmons, C. P., S. Clare, and G. Dougan.** 2001. Understanding mucosal responsiveness: lessons from enteric bacterial pathogens. Semin. Immunol. **13:**201–209.
41. **Stocker, B. A., S. K. Hoiseth, and B. P. Smith.** 1983. Aromatic-dependent

"*Salmonella* sp." as live vaccine in mice and calves. Dev. Biol. Stand. **53:**47–54.
42. **Tacket, C. O., J. Clemens, and J. B. Kaper.** 1992. Cholera vaccines. Bio/Technology **20:**53–68.
43. **Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine.** 1992. Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δ*cya* Δ*crp Salmonella typhi* strains in adult volunteers. Infect. Immun. **60:**536–541.
44. **Tacket, C. O., D. M. Hone, G. A. Losonsky, L. Guers, R. Edelman, and M. M. Levine.** 1992. Clinical acceptability and immunogenicity of CVD 908 *Salmonella typhi* vaccine strain. Vaccine **10:**443–446.
45. **Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. N. Chatfield, and M. M. Levine.** 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. Infect. Immun. **65:**452–456.
46. **Tacket, C. O., M. B. Sztein, S. S. Wasserman, G. Losonsky, K. L. Kotloff, T. L. Wyant, J. P. Nataro, R. Edelman, J. Perry, P. Bedford, D. Brown, S. Chatfield, G. Dougan, and M. M. Levine.** 2000. Phase 2 clinical trial of attenuated *Salmonella enterica* serovar typhi oral live vector vaccine CVD 908-*htrA* in U.S. volunteers. Infect. Immun. **68:**1196–1201**.**
47. **Tsolis, R. M., L. G. Adams, T. A. Ficht, and A. J. Baumler.** 1999. Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. Infect. Immun. **67:**4879–4885.
48. **Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman.** 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. EMBO J. **18:**3924–3933.
49. **Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni, and F. C. Fang.** 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science **287:**1655–1658.
50. **Wallis, T. S., and E. E. Galyov.** 2000. Molecular basis of *Salmonella*-induced enteritis. Mol. Microbiol. **36:**997–1005.

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