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Pilot Study of *phoP*/*phoQ*-Deleted *Salmonella enterica* Serovar Typhimurium Expressing *Helicobacter pylori* Urease in Adult Volunteers

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Attenuated *Salmonella enterica* serovar Typhi has been studied as an oral vaccine vector. Despite success with attenuated *S. enterica* serovar Typhimurium vectors in animals, early clinical trials of *S. enterica* serovar Typhi expressing heterologous antigens have shown that few subjects have detectable immune responses to vectored antigens. A previous clinical study of *phoP*/*phoQ*-deleted *S. enterica* serovar Typhi expressing *Helicobacter pylori* urease from a multicopy plasmid showed that none of eight subjects had detectable immune responses to the vectored antigen. In an attempt to further define the variables important for engendering immune responses to vectored antigens in humans, six volunteers were inoculated with 5×10^7 to 8×10^7 CFU of *phoP*/*phoQ*-deleted *S. enterica* serovar Typhimurium expressing the same antigen. Two of the six volunteers had fever; none had diarrhea, bacteremia, or other serious side effects. The volunteers were more durably colonized than in previous studies of *phoP*/*phoQ*-deleted *S. enterica* serovar Typhi. Five of the six volunteers seroconverted to *S. enterica* serovar Typhimurium antigens and had strong evidence of anti-*Salmonella* mucosal immune responses by enzyme-linked immunospot studies. Three of six (three of five who seroconverted to *Salmonella*) had immune responses in the most sensitive assay of urease-specific immunoglobulin production by blood mononuclear cells in vitro. One of these had a fourfold or greater increase in end-point immunoglobulin titer in serum versus urease. Attenuated *S. enterica* serovar Typhimurium appears to be more effective than *S. enterica* serovar Typhi for engendering immune responses to urease. Data suggest that this may be related to a greater stability of antigen-expressing plasmid in *S. enterica* serovar Typhimurium and/or prolonged intestinal colonization. Specific factors unique to nontyphoidal salmonellae may also be important for stimulation of the gastrointestinal immune system.

Attenuated salmonellae have been extensively studied as live bacterial vectors for delivery of heterologous antigens because these intracellular microorganisms stimulate humoral, mucosal, and cellular immune responses in humans and animals. A goal of these studies has been to develop multivalent oral vaccines based upon *Salmonella enterica* serovar Typhi for human use. The existence of the safe live attenuated vaccine strain Ty21a, an effective vaccine against typhoid fever, and the species specificity of *S. enterica* serovar Typhi for humans has focused investigative attention on this serotype. Most human studies of attenuated *S. enterica* serovar Typhi have been based on preclinical data obtained in the murine model of systemic salmonellosis in which BALB/c mice are infected orally with attenuated *Salmonella enterica* serovar Typhimurium. More recently, mice have been infected nasally with *S. enterica* serovar Typhi strains (8). Although murine experiments are useful screening studies, their predictive value in forecasting immunogenicity and safety in clinical trials of attenuated *S. enterica* serovar Typhi strains in humans is uncertain (4, 14).

Several rationally attenuated *S. enterica* serovar Typhi vectors have been evaluated (12, 13, 28–30), but relatively few studies have evaluated *S. enterica* serovar Typhi expressing heterologous antigens in humans (4, 11, 23, 30). We have studied *phoP*/*phoQ*-deleted *S. enterica* serovar Typhi Ty2 (Ty800) and found it to be safe and immunogenic in adult volunteers (12). We have used a stable immunogenic protein of gram-negative bacterial origin of importance to the gastrointestinal tract, *Helicobacter pylori* urease, as a model antigen. Recombinant urease and relevant antibodies are available, and two studies have shown protection of mice from *Helicobacter* infection after immunization with attenuated *S. enterica* serovar Typhimurium expressing urease (3, 10). We previously showed that *phoP*/*phoQ*-deleted *S. enterica* serovar Typhimurium resulted in strong mucosal and humoral immune responses against the vectored urease antigen in mice (4). This prompted an evaluation of the analogous *phoP*/*phoQ*-deleted *S. enterica* serovar Typhi strain (designated Ty1033) in adult volunteers. None of eight adult volunteers who received the analogous *S. enterica* serovar Typhi strain had detectable immune responses to the urease antigen, despite the fact that immune responses to *S. enterica* serovar Typhi antigens were preserved and robust. In evaluating possible reasons for this lack of immunogenicity, we considered the possibility that the successful use of *S. enterica* serovar Typhimurium in animals was due to biological features of this serotype or features common to nontyphoidal salmonellae in general. Specifically, we hypothesized that the more prolonged intestinal phase of nontyphoidal salmonellosis might result in quantitatively or qualitatively different immunological stimulation of the gastrointestinal immune system. We tested this hypothesis here by studying a "murine" *S. enterica* serovar Typhimurium strain expressing *H. pylori* urease in humans. This report shows that 50% of inoculated subjects had detectable immune responses to *H. pylori* urease delivered via a single oral dose of attenuated *S. enterica* serovar Typhimurium. This is the first published study evaluating *S. enterica* serovar Typhimurium as a vector microorganism in humans.

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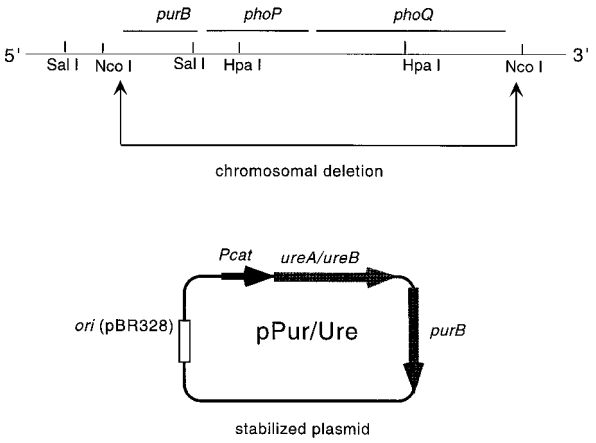

FIG. 1. Chromosomal deletion and plasmid map for *S. enterica* serovar Typhimurium LH1160. The chromosomal deletion (Δ*phoP* Δ*phoQ* Δ*purB*) between endogenous *Nco*I sites is shown. The *purB* and *phoP* genes are contiguous. The urease expression plasmid encodes the urease A and B subunit genes, *ureA* and *ureB* (without other regulatory or structural elements of urease), cloned behind the chloramphenicol acyltransferase promoter (*Pcat*). The *purB* gene encodes adenylosuccinate lyase enzyme, which catalyzes an essential step in the de novo synthesis of AMP, was cloned from *S. enterica* serovar Typhimurium, and is driven from its native promoter. The origin of replication (*ori*) of the plasmid is derived from plasmid pBR328, a moderate-copy-number plasmid.

MATERIALS AND METHODS

Bacteriology. An *S. enterica* serovar Typhimurium strain expressing *H. pylori* urease was derived from *S. enterica* serovar Typhimurium ATCC 14028 as described previously (4). Strain ATCC 14028 is a smooth, wild-type, mouse-virulent strain that is frequently used in the study of *Salmonella* pathogenesis and has a 50% lethal dose of ≤20 CFU in susceptible BALB/c mice inoculated intraperitoneally (13, 21). Inactivation of the *phoP* and *phoQ* virulence regulatory genes via either deletion or transposon insertion results in a profound virulence defect of this strain, with an increase of the murine 50% lethal dose to approximately 7×10^5 CFU. A "balanced lethal" plasmid stabilization system similar conceptually to the well-known *asd*-based plasmid system (22) was used to express *H. pylori* urease. Figure 1 shows the relevant chromosomal deletion and plasmid utilized. Briefly, a single large chromosomal deletion was made in the contiguous *purB*, *phoP*, and *phoQ* genes, and this strain was shown to be a purine auxotroph (LH954). Plasmid Pur/Ure, bearing the *S. enterica* serovar Typhimurium *purB* gene, which expressed enzymatically inactive *H. pylori* urease from a strong constitutive chloramphenicol acyltransferase promoter, was mobilized into this auxotroph by electroporation. The urease A and B subunit genes in this plasmid were PCR amplified from a clinical isolate of *H. pylori*. This "stabilized" plasmid derived from pBR328 complemented the purine auxotrophy in *S. enterica* serovar Typhimurium with a *phoP*/*phoQ*/*purB* deletion. Plasmid Pur/Ure was isolated from the previously evaluated *S. enterica* serovar Typhi strain Ty1033 (4) to ensure that the identical plasmid was evaluated in both serotypes. As demonstrated previously for *S. enterica* serovar Typhi, the plasmid was stably maintained even when the strain was grown in "rich" Luria broth medium, which contains appreciable purines (4). Preclinical murine studies showed that the chromosomal *purB* deletion and introduction of the urease-bearing plasmid even further attenuated *phoP*/*phoQ*-deleted *S. enterica* serovar Typhimurium ATCC 14028. Inocula for clinical studies were grown in Luria broth to stationary phase, harvested by centrifugation, washed twice with sterile normal saline, and standardized spectrophotometically by measurement of the optical density at 600 nm.

The stability of plasmid Pur/Ure was evaluated in vitro in *S. enterica* serovar Typhimurium LH1160 and compared with that of the analogous *S. enterica* serovar Typhi strain Ty1033 previously tested in volunteers. Inocula were grown in Luria broth as was done for strains used in delivery to volunteers. At various time points after inoculation, aliquots were removed, serially diluted, and spread on Luria broth agar plates in duplicate. After a 24-h incubation, the plates were examined for morphology and the numbers of large (~2-mm) and small (~1-mm) colonies were counted. It was previously shown that large colonies were those which had retained plasmid while smaller colonies were those that had lost plasmid (4) and whose growth was limited by the modest amount of exogenous purine in Luria broth agar plates.

Clinical study. The clinical study protocol and procedures were reviewed and approved by the Human Research Committee at Massachusetts General Hospital. Volunteers were adults in excellent health who were medically screened as described previously (12). Additionally, these volunteers were HLA-B27 negative and seronegative for *H. pylori* infection as measured by a commercial enzyme-linked immunosorbent assay (ELISA; Wampole, Cranbury, N.J.). Volunteers with a history of typhoid fever vaccination were not excluded. Volunteers were admitted to the General Clinical Research Center and received a single oral dose of 5×10^7 to 8×10^7 CFU of the attenuated vaccine strain on the day of admission (study day 0). Vaccine bacteria were suspended to a specific turbidity and administered in 25 ml of 0.9% saline after the volunteers drank an antacid solution (2 g of $NaHCO_3$ in 125 ml of water). The volunteers were monitored closely in hospital for 10 days. Stools were collected and graded (20), and daily stool cultures were performed by direct plating and after overnight enrichment in selenite broth onto Hektoen Enteric and MacConkey agar plates (12). At least three clones of the vaccine bacterium were isolated from each subject and evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole bacterial cell proteins to determine whether excreted bacteria still produced urease antigen. A single blood sample was obtained daily for culture and with temperature spikes and was incubated and analyzed for 7 days using the Bactec 9240 system. All bacteriology tests were performed by the Clinical Microbiology Laboratory at Massachusetts General Hospital. Heparinized blood samples were obtained for enzyme-linked immunospot (ELISPOT) studies on study days 0, 7, and 10. Volunteers were discharged to home on study day 10 and returned for six weekly outpatient follow-up visits for a clinical check, follow-up stool culture, and collection a blood specimen for serology. Blood was obtained for serology on study days 0, 7, 10, and 14 and weekly thereafter at follow-up visits.

Immunologic assays. All immunologic assays used the same three vaccine-specific antigens, which were applied in 40 mM carbonate buffer (pH 10): *S. enterica* serovar Typhimurium lipopolysaccharide (LPS) (Sigma no. L-6511), *S. enterica* serovar Typhimurium flagella, and purified recombinant *H. pylori* urease (18) (a gift of T. Monath, OraVax, Cambridge, Mass.). Flagella were purified from wild-type *S. enterica* serovar Typhimurium ATCC 14208 using blender shearing, ultracentrifugation, and dialysis as previously described (16). Purified native *H. pylori* urease was not available for immunological assays, but previous animal studies showed immune responses to recombinant protein (4). For all enzyme-linked immunosorbent assay (ELISA) studies, antigens were used at 10 μg/ml (1 μg/well); ELISPOT assays used 10 times this concentration to ensure saturation of membrane-bottomed wells.

ELISPOT studies were performed using freshly isolated peripheral blood mononuclear cells as described previously (12). Vaccine-specific immunoglobulin A (IgA) bearing cells were enumerated at ×25 magnification, and six or more spots per 10^6 peripheral blood mononuclear cells was considered a positive result (12, 28). Analysis of vaccine-specific IgA and IgG released by densely cultured mononuclear cells into the tissue culture medium (6) after 48 h of culture was also performed as previously described (4). Samples from different subjects had markedly different baseline optical density values in these assays, probably reflecting differing exposures to nontyphoidal salmonellae. Because of this variability and the small number of volunteers studied in these new assays to date, a threefold or greater increase in antigen-specific optical density was empirically and arbitrarily chosen as a minimum to define a positive result.

End-point dilution ELISAs were developed for evaluation of serum IgG and IgA directed at vaccine antigens, as previously described (12). The blocking agent was 5% dried milk in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Sera were applied starting at a dilution of 1/10 in blocking agent and serially diluted twofold across the microtiter plates. Affinity-purified goat anti-human antibodies conjugated to alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, Md.) were used at dilutions of 1:5,000 or 1:10,000 with *p*-nitrophenylphosphate (pNPP) substrate to develop plates. The optical density values of wells were read at 405 nm with a Vmax Microtiter Devices microtiter plate reader. End-point dilutions were defined as the serum dilution at which the optical density was ≥0.15 optical density unit, and a fourfold or greater increase in end-point dilution titer was deemed significant. Seroconversion for *S. enterica* serovar Typhimurium was defined as at least a fourfold increase in the end-point titer of serum IgG directed against either LPS or flagellar antigens. ELISA results were evaluated with paired serum samples drawn 14 to 18 days apart from 20 healthy asymptomatic normal volunteers. A commercial kit for serodiagnosis of *H. pylori* infection (Wampole) was also used to study sera. Fisher's exact test was used to calculate *P* values.

Western blotting was performed on bacterial protein lysates using a rabbit polyclonal antiserum directed against native *H. pylori* urease (18) and a goat anti-rabbit IgG conjugated to horseradish peroxidase. Blots were developed with a chemiluminescent substrate (ECL kit; Amersham). For Western blotting of human serum samples, 200 ng of urease A/B was applied to nitrocellulose dots or transferred from gels loaded such that approximately 500 ng was applied per lane.

RESULTS

Preclinical bacteriology. When analyzed on a per CFU basis, inocula of the *S. enterica* serovar Typhimurium strain LH1160 contained an amount of immunoreactive urease which was indistinguishable from that produced by the previously evaluated *S. enterica* serovar Typhi strain Ty1033 by semiquantita-

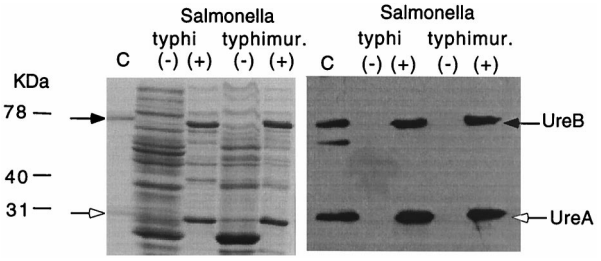

FIG. 2. Cell-associated urease A and B within attenuated *phoP/phoQ*-deleted *Salmonella* vectors. Bacterial strains were grown to early stationary phase (12 h), as for clinical studies. Whole-bacterial-cell protein lysates were made, separated by SDS-PAGE, and either stained with Coomassie blue (left panel) or blotted to nitrocellulose and probed with a polyclonal antibody for *H. pylori* urease (right panel). *S. enterica* serovar Typhi and Typhimurium strains carrying either the urease-expressing stabilized plasmid (+) or the isogenic "empty plasmid" lacking the *ureA* and *ureB* genes (−) are shown side by side. Loading was normalized by CFU determinations such that each lane contains protein from 5×10^7 CFU. As a control, 1 μg of recombinant urease A and B was loaded (lane C). The immunoblot was developed with a chemiluminescent substrate, and an autoradiogram is shown. Urease A and B subunits (arrows) are easily visualized on both the Coomassie-stained gel and immunoblot, and the amounts are indistinguishable between serotypes.

tive Western blotting (Fig. 2). The stabilities of the urease-bearing plasmids within the two strains were markedly different in vitro, however. For both *S. enterica* serovar Typhimurium and Typhi strains, more than 90% of colonies retained the plasmid after 12 to 14 h of culture. At 16 h and later, the percentage of colonies bearing the urease plasmid declined rapidly in the *S. enterica* serovar Typhi culture but more than 90% of *S. enterica* serovar Typhimurium 1160 colonies retained the plasmid until 36 h (Fig. 3). Interestingly, isogenic "empty plasmid" containing the *purB* gene but lacking the urease A/B subunit genes was equally maintained in the two serotypes over the time studied.

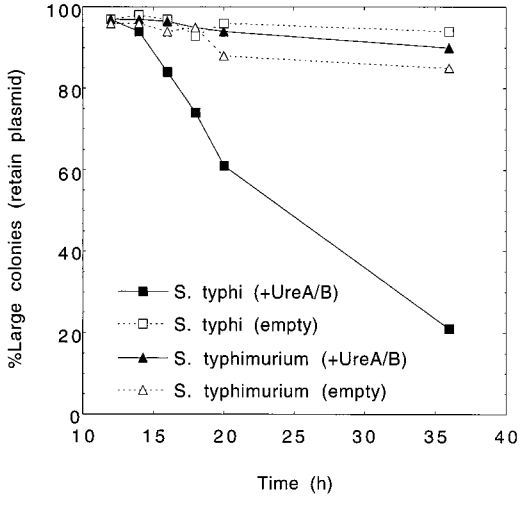

FIG. 3. In vitro stability of stabilized plasmids in *S. enterica* serovar Typhi and Typhimurium strains. Bacterial strains were grown as for the generation of volunteer inocula in Luria broth. Aliquots were removed at the designated times and plated in duplicate for CFU determinations and assessment of colonies size. The percentage of large-morphology colonies (those which retained *purB*-bearing plasmids) is shown. The *S. enterica* serovar Typhimurium LH1160 tested here is denoted by solid triangles, and the previously evaluated *S. enterica* serovar Typhi 1033 is denoted by solid squares. Empty plasmid controls lacking the *ureA* and *ureB* genes are denoted by open symbols. The data suggest that more rapid loss of plasmid in vitro is related to the presence of the *ureA* and *ureB* genes in *S. enterica* serovar Typhi, but not in *S. enterica* serovar Typhimurium.

Clinical responses. The demographics and clinical responses of volunteers are shown in Table 1. Four volunteers felt completely well for the duration of the study. Volunteers 4 and 6 developed acute onset of fever, which was clearly attributable to the investigational vaccination, at 22 and 76 h after vaccination, respectively. Both of these volunteers had anorexia, constitutional symptoms, prominent headache, and minimal abdominal cramping. After the maximum temperature spikes, both subjects defervesced slowly and did not have recurrent high fever. Other symptoms resolved within 36 h after the maximum fever without therapy beyond encouraging oral fluid intake. Neither had vomiting or diarrhea. No volunteer had blood cultures which were positive for the vaccine organism, including additional cultures obtained at the time of the fevers. No volunteer had delayed or recurrent symptoms, nor did any have evidence of postinfectious inflammatory phenomena.

Shedding. All volunteers had vaccine bacteria detected in stool cultures after vaccination. Three volunteers cleared the bacterium quickly (within 4 days, as previously seen with the analogous *S. enterica* serovar Typhi strain), but three were more durably colonized. Volunteers 3 and 6 received oral levofloxacin, 500 mg daily for 3 days (study days 9 to 11), per the approved protocol because stool culture results available on study day 9 showed lactose-negative colonies on preliminary enrichment broth cultures, which were suspicious for ongoing shedding of the vaccine bacterium. These two subjects were clinically well at the time; the goal of the antibiotic therapy was to hasten clearance of the recombinant organism. Volunteer 6 was culture positive for the vaccine organism daily until day 10 and negative thereafter, after the levofloxacin administration. Completed workup of enrichment cultures from volunteer 3 showed that the last day of a positive fecal culture was study day 7 and that the "suspicion" of ongoing shedding was not correct. This volunteer may therefore have cleared the vaccine independently by day 7, but because antibiotics were given, this cannot be definitively stated. All volunteers had negative stool cultures on day 14 and at five weekly visits thereafter, including those who received the antibiotics. All volunteers excreted clones which expressed the urease antigen, as visualized by SDS-PAGE analysis of subcultured, whole-bacterial-cell protein lysates (overall, approximately 80% of the colonies evaluated [data not shown]). The stool culture process uses overnight selective enrichment broth, and the colonies isolated may be siblings (4, 12). Therefore, detailed determination of the exact percentages of excreted colonies which retained plasmid will not yield easily interpretable data, and such studies were not performed.

Immune responses to *S. enterica* serovar Typhimurium. Five of the six volunteers had strong evidence of mucosal immune responses by ELISPOT and seroconversion to *S. enterica* serovar Typhimurium by ELISA (Table 2). Increases in the number of vaccine-specific IgA-bearing cells by ELISPOT are widely believed to be a sensitive surrogate marker of mucosal immune responses to live oral bacterial vaccines (5, 6, 17, 28–30). Volunteer 2 was a nonresponder and had no detectable ELISPOT responses to *S. enterica* serovar Typhimurium and no IgG seroconversion. ELISPOT responses to *Salmonella* antigens were unequivocal and vigorous (Table 2) in five of the six subjects. ELISPOT studies were performed on days 0, 7, and 10 because previous studies have shown that the cell numbers are usually maximal on day 7 after receipt of live attenuated *S. enterica* serovar Typhi vaccines (4, 12, 17). In volunteers 3 and 6 (two of those who shed vaccine for a relatively prolonged period and received antibiotics), spot numbers directed against flagella (but not LPS) were greater on day 10 than on day 7. We have not previously observed this pattern in our

TABLE 1. Volunteer demographics and clinical responses

Volunteer no.	Age (yr)/sex	Prior typhoid vaccine	T_{max} (°C) (time)[a]	Shedding[b]	Symptoms
1	24/M	No	98.7	Day 8	None
2	40/M	Yes	99.6	Day 2	None
3	23/M	No	98.3	Day 7[c]	None
4	30/M	No	101.9 (22h)	Day 3	Headache, constitutional symptoms
5	21/F	Yes	98.8	Day 4	None
6	19/F	No	102.2 (76h)	Day 10[c]	Headache, constitutional symptoms

[a] Maximum oral temperature (hours after vaccine of occurrence).
[b] Last day on which a stool culture was positive for *S. enterica* serovar Typhimurium.
[c] Subjects received levofloxacin to hasten the elimination of vaccine bacteria (see the text).

studies of *S. enterica* serovar Typhi vaccines. This timing suggests that these subjects may have had a later peak, perhaps on day 8 or 9, which was "missed" by the day 0, 7, and 10 sampling schedule. A delayed peak in IgA immune responses in serum was observed in a subset of a large number of subjects receiving *S. enterica* serovar Typhi Ty21a studied by Forrest (6). Alternatively, this may represent different kinetics of colonization and stimulation of the intestinal immune system by *S. enterica* serovar Typhimurium in some subjects or different kinetics of responses to proteins from those of responses to LPS.

Serum titers of IgG against LPS and flagella were measured on samples obtained on study day 0 before vaccination and on days 7, 10, and 14 and weekly thereafter (Table 2). The titers reported are the peak values after vaccination (usually day 10 or 14). Five of the six subjects seroconverted. As a comparison, 1 of 20 normal paired sera had a fourfold increase in flagellum titer and 0 of 20 had a fourfold or greater increase in LPS titer (5 of 6 versus 0 of 20, $P = 0.0001$). The baseline titers varied widely among both groups. Overall, baseline anti-flagellum titers were higher than LPS titers in both groups (vaccinees and the normal paired sera). Many individuals will have been previously exposed to *S. enterica* serovar Typhimurium, the second most frequently isolated serotype of *Salmonella* in the United States. Titers in the 20 unvaccinated volunteers varied from 1:10 to 1:5,120 in the LPS ELISA and from 1:10 to 1:1,280 in the flagellum ELISA. Although we were unable to locate any individuals with documented acute *S. enterica* serovar Typhimurium infection in whom we could study "natural" seroconversion, we found one individual with a history of gastroenteritis associated with documented, prolonged *S. enterica* serovar Typhimurium excretion. This person had baseline titers of 1:1,280 and 1:5,120 in the LPS and flagellum assays, respectively. Increases in the levels of IgA in serum directed against *S. enterica* serovar Typhimurium LPS were also measured using an end-point dilution titer ELISA. All six volunteers had fourfold or greater increases in IgA levels in serum directed against LPS, including the volunteer who had no detectable ELISPOT or IgG serological immune responses to *Salmonella* (range, 8-fold increase [volunteer 2] to 64-fold increase [volunteer 5] [data not shown]). None of the 20 paired normal sera had fourfold or greater titer increases in this assay. Vaccine-specific serum IgA is easily detected after oral *S. enterica* serovar Typhi vaccination, although it is not the best predictor of development of secreted mucosal IgA (6).

Immune responses to *H. pylori* urease. ELISPOT responses directed against the vectored antigen were detected only in volunteer 6, who had 140 IgA-secreting cells specific for recombinant urease on day 7 after vaccination and no urease-specific cells on days 0 and 10. In an attempt to generate even more sensitive immunoassays, we and others have evaluated the in vitro production of vaccine-specific Ig by peripheral blood mononuclear cells cultured at high density (4–6). This assay of in vitro Ig release evaluates soluble Ig in tissue culture medium from mononuclear cells grown at high density (10^7 cells/ml) for 48 h; ELISPOT studies typically evaluate 10^6 cells. An assay of soluble Ig may offer greater reproducibility and objectivity and perhaps enhanced sensitivity compared with ELISPOT studies (4). Cells from all five individuals who seroconverted to *S. enterica* serovar Typhimurium antigens had large increases in vaccine-specific Ig levels directed against *S. enterica* serovar Typhi LPS and flagella (≥3- to 10-fold increases in optical density [Fig. 4B and C]), and three of the volunteers (volunteers 1, 3, and 6) had less vigorous but obvious increases in IgG levels directed against *H. pylori* urease (≥threefold over baseline values on day 0 [Fig. 4A]). Volunteer 5 had a small increase in optical density in the assay detecting anti-urease IgG on day 7, which was not threefold over baseline, a reasonable but arbitrary threshold value for defining a positive result. Volunteers 1, 3, and 6 had two- to threefold increases in IgA directed against urease in cell supernatants. All five subjects who seroconverted for *Salmonella*

TABLE 2. ELISPOT and serological responses to *S. enterica* serovar Typhimurium antigens

Volunteer no.	IgA ASC per 10^6 PBMC[a]		Serum IgG titer versus LPS		Serum IgG titer versus Flagella	
	Versus LPS	Versus Flagella	Preimmune	Postvaccine	Preimmune	Postvaccine
1	812	612	1:10	1:160[c]	1:80	1:640[c]
2	0	0	1:40	1:80 (NS)[d]	1:160	1:320 (NS)
3	170	105[b]	1:20	1:80[c]	1:160	1:160 (NS)
4	268	118	1:320	1:1280[c]	1:320	1:640 (NS)
5	600	788	1:80	1:640[c]	1:160	1:320 (NS)
6	250	218[b]	1:40	1:160[c]	1:10	1:320[c]

[a] More than 6 antibody-secreting cells/10^6 PBMC cells is a positive result.
[b] Peak values for these determinations occurred on day 10.
[c] Significant increase (4-fold or greater increase in titer).
[d] NS, not significant.

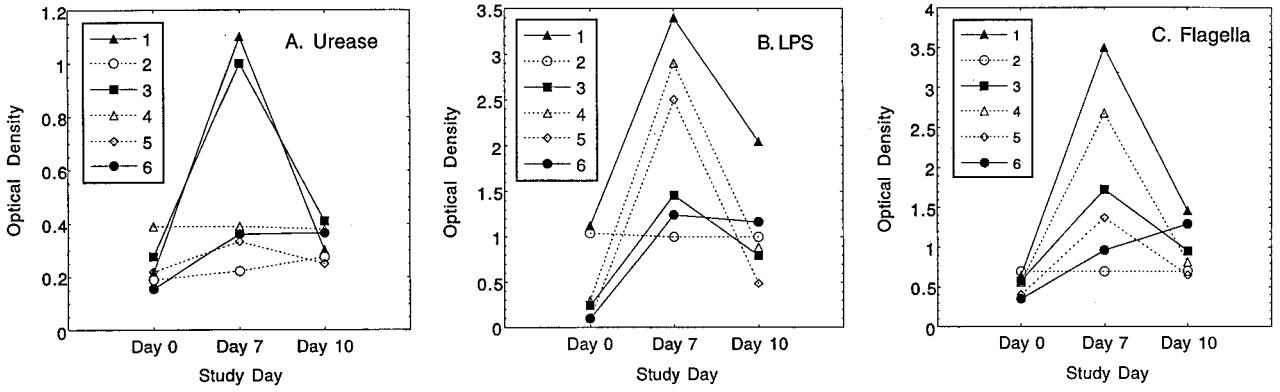

FIG. 4. Assay of vaccine-specific IgG produced by mononuclear cells in vitro. Mononuclear cells were isolated on the study day noted and cultured for 48 h. Culture supernatants were applied in duplicate to ELISA plates coated with either recombinant urease (A) or *S. enterica* serovar Typhimurium LPS (B) or flagella (C). The plates were developed with a peroxidase-labelled goat anti-human antibody directed against human IgG. A greater than threefold increase in optical density over the baseline day 0 value was considered a positive result. Samples from volunteers who had a threefold or greater increase in optical density in the urease-contained wells are denoted by solid symbols and solid lines (volunteers 1, 3, and 6). Samples from volunteers who did not have an increase IgG directed against urease are represented by open symbols and dotted lines. All volunteers except volunteer 2 had much larger increases in optical density in LPS and flagellum wells, and the *y*-axis scales differ for the panels, reflecting this finding. PBMC isolated from healthy, unimmunized *H. pylori*-seronegative volunteers had "flat" profiles similar to those of volunteer 2 (data not plotted).

antigens had greater than threefold increases in the levels of IgA directed against LPS (data not shown). We have found that IgG is more easily detected than IgA in this assay, perhaps because of the greater avidity of the secondary antibodies detecting IgG. Although the groups were not studied simultaneously, it is useful to note that in our previous study (4), none of eight volunteers who received the analogous *S. enterica* serovar Typhi strain expressing urease had immune responses in these assays (3 of 6 versus 0 of 8; $P = 0.055$).

Serum samples were examined by ELISA for IgG and IgA directed against recombinant urease. Volunteers 1 and 6 had eightfold and fourfold increases, respectively, in IgA titers in serum directed against recombinant *H. pylori* urease. Only volunteer 6 met the criteria of a fourfold increase in end-point titer in IgG directed against urease (1:20 to 1:80). None of 20 paired normal sera had a fourfold increase in IgA or IgG titers against urease in these assays. The ELISA serological results were reproducible, but we were not able to consistently confirm these results by Western blotting. Samples from an individual with chronic *H. pylori* infection and known positive serum IgG and IgA directed against urease were used as a positive control in urease immunological studies and had positive Western blots at >1:1,000 dilutions of serum and IgG ELISA titers of >1:5,120. None of the volunteers seroconverted for *Helicobacter* infection by the Wampole commercial clinical ELISA, which uses a sonicated *H. pylori* extract as antigen. Because of the variability of saliva samples and disappointing results encountered previously in assessing secretory IgA directed against immunodominant *Salmonella* antigens (4), we did not evaluate salivary responses in this study.

DISCUSSION

This study represents the first report of attenuated *S. enterica* serovar Typhimurium used as a vaccine vector in humans. Two of six subjects had unacceptable fever and constitutional symptoms, but no bacteremias or other serious adverse events occurred, and, interestingly, no diarrhea occurred. As expected, some subjects were more durably colonized than was previously seen in our studies of *phoP*/*phoQ*-deleted *S. enterica* serovar Typhi, in which 3 log units greater CFU were given (10^{10} CFU) (4). The duration of colonization did not obviously correlate with symptoms. Two subjects received antibiotics for "prolonged" colonization in order that they not leave hospital excreting recombinant organisms at high levels. Unfortunately, this limited our ability to define the true duration of colonization and perhaps also the magnitude of the immune responses had they been allowed to clear the organism independently. Clinically, administration of antibiotics has been associated in some studies with prolonged duration of positive stool cultures or even symptomatic relapse in subjects with nontyphoidal *Salmonella* gastroenteritis (2, 24, 25). This was not observed in our study, perhaps in part because a 3-day course of an antibiotic with little effect on the anaerobic intestinal flora was used. Additionally, the *S. enterica* serovar Typhimurium strain used lacks *phoP*/*phoQ* transcriptional regulation, which is probably important for optimal adaptation and persistence within complex intraluminal and intracellular environments.

We concluded that five of six subjects had mucosal immune responses and seroconversion for *S. enterica* serovar Typhimurium antigens. This was notable because a low dose was given and because subjects were not selected in any way to be *Salmonella* naive. In our prior *S. enterica* serovar Typhi studies, we found end-point dilution ELISAs to be the most rigorous studies for documentation of seroconversion and not the most sensitive (4, 12, 13), and so we believe that the present results provide convincing evidence of systemic immune responses. Although not compared head-to-head, the magnitude of responses to *Salmonella* antigens here are comparable to those in our previous studies of *phoP*/*phoQ*-deleted *S. enterica* serovar Typhi (4, 12). We also show that a single oral dose of *S. enterica* serovar Typhimurium expressing *H. pylori* urease resulted in detectable immune responses to the vectored antigen in our most sensitive assay, in vitro production of IgG by peripheral blood mononuclear cells after vaccination. Three of six total volunteers (three of five volunteers who seroconverted to *S. enterica* serovar Typhimurium) had responses to urease in this assay. Although the responses were modest, they were confirmed by small but reproducible serological responses to urease in two of three responders (volunteers 1 and 6). Volunteer 6 (the individual with both prolonged colonization and fever) had positive responses in all urease immunoassays: detectable

IgA-secreting cells, in vitro production of IgA and IgG, and seroconversion. Surprisingly, although this individual was the only one in whom urease-specific IgA-secreting cells were detected, she was not the one with the largest detectable Ig production in vitro. The detection of vaccine-specific Ig in vitro is very sensitive, but how these assays relate to ELISPOT data and other more established measures of immunity or correlate with protection is unknown and will require further study. The data are useful because they are in marked contrast to those of our previous study. There, none of eight volunteers who received up to 3 log CFU higher doses of the analogous *S. enterica* serovar Typhi strain had even a glimmer of an immune response in the same immunological assays (4). The statistical comparison of responders for the *S. enterica* serovar Typhi and Typhimurium experiments ($P = 0.055$) lacks statistical rigor because the groups were sequentially studied and not randomized or contemporaneous. Nevertheless, in conjunction with the dose differences, the findings suggest that additional direct comparisons of *Salmonella* vaccine serotypes in humans may be of value.

We conclude that at least for this combination of antigen, expression system, and mechanisms of attenuation, *S. enterica* serovar Typhimurium is a "more immunogenic" vector than the previously studied *S. enterica* serovar Typhi. The strains used were both attenuated by virtue of the same defined chromosomal deletion and carried the identical "stabilized" plasmid based on complementation of a chromosomal purine auxotrophy. Although it is beyond the scope of this pilot study to definitively show why *S. enterica* serovar Typhimurium is more effective, several hypotheses may be advanced. First, the plasmid studied was markedly more stable in *S. enterica* serovar Typhimurium than in serovar Typhi. The urease-encoding plasmid included genes from pBR328 (a ColE1 replicon), *S. enterica* serovar Typhimurium (the *purB* gene), and *H. pylori* (the *ureA* and *ureB* genes). Greater plasmid stability with *S. enterica* serovar Typhimurium may reflect differences in segregation and replication of foreign plasmid DNA or enhanced "tolerance" of the overexpressed urease subunits in this serovar. In vitro studies showed that *S. enterica* serovar Typhimurium was more tolerant of the plasmid containing the *H. pylori ureA* and *ureB* genes but that both serotypes maintained empty plasmids equally. This suggests that plasmid loss is related to *H. pylori* DNA itself or, more likely, to expression of the urease proteins. High-level expression of heterologous antigens may adversely affect bacterial vectors, but the serotype specificity of this phenomenon has not been well studied. Evaluation of other antigen genes and proteins would help determine whether this phenomenon is generalizable or antigen specific. Regardless of the mechanism, enhanced plasmid stability could result in prolonged antigen presentation and greater immunogenicity.

Enhanced colonization of the gastrointestinal system could also result in greater and longer presentation of antigens carried by an oral bacterial vector. Although the numbers were small, the subjects in this study had more durable colonization than was found in our prior studies of Ty800 and derivatives thereof (usually 3 days or less), and those with immune responses to urease were the three with the longest shedding. A comprehensive review from the Centers for Disease Control and Prevention found that the median duration of shedding of nontyphoidal salmonellae after gastroenteritis was approximately 5 weeks (1). Although not as well studied, *S. enterica* serovar Typhi appears to have a briefer duration of intestinal colonization as measured by older human challenge studies (15), live-vaccine studies (12, 13, 28–30), and the modest percentage of patients with typhoid fever with positive stool cultures at diagnosis (9). Nickerson and Curtiss showed that the presence of an intact *S. enterica* serovar Typhimurium *rpoS* gene contributes to colonization of the gastrointestinal lymphoid system in mice (26), and that might account in part for the more prolonged colonization of nontyphoidal serotypes in humans. Many strains of Ty2 (27), including that from which our *S. enterica* serovar Typhi vaccine strain Ty1033 was derived, are *rpoS* null (4). In one study in which an attenuated *S. enterica* serovar Typhi strain derived from a recent Chilean clinical isolate (probably *rpoS* positive) was compared to an identically attenuated derivative of Ty2 (probably *rpoS* negative), there was no obvious major difference in shedding or immunogenicity patterns (29), suggesting that this locus may not be critical in *S. enterica* serovar Typhi.

Lastly, it is possible that specific features of nontyphoidal salmonellae contribute to the immunogenicity of this serotype. Proteins secreted by the type III secretion system interact with host epithelial and antigen-processing cells and may be immunomodulatory (for reviews, see references 7 and 19). In preliminary experiments of bacterial culture supernatants, we have found that *S. enterica* serovar Typhi secretes many less proteins than do most *S. enterica* serovar Paratyphi strains and nontyphoidal strains.

In summary, for this combination of attenuating mutation, antigen, and plasmid-based expression system, *S. enterica* serovar Typhimurium appeared to be a more effective vector microorganism than *S. enterica* serovar Typhi for engendering immune responses to the vectored foreign antigen urease. The data suggest that this may be related to enhanced plasmid stability and greater colonization of the intestine by a nontyphoidal serotype. A more highly attenuated *S. enterica* serovar Typhimurium strain given at larger doses could result in fewer adverse events and in a more consistent, vigorous and clinically relevant immune responses to urease. The presence of animal and environmental reservoirs for nontyphoidal strains and the possibility of postinfectious inflammatory arthropathies (particularly in individuals who are HLA-B27 positive) are obvious barriers which may limit clinical development of nontyphoidal *Salmonella* vectors. Despite this, our results are immunologically provocative and raise interesting questions for future human and animal experiments. Additional clinical studies of *S. enterica* serovar Typhimurium expressing other plasmid-borne or chromosomally integrated heterologous antigens will help clarify the relative importance of serotype and other variables.

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