Attenuated *Salmonella enterica* Serovar Typhi Expressing Urease Effectively Immunizes Mice against *Helicobacter pylori* Challenge as Part of a Heterologous Mucosal Priming-Parenteral Boosting Vaccination Regimen

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Recombinant vaccine strains of *Salmonella enterica* serovar Typhi capable of expressing *Helicobacter pylori* urease were generated by transforming strains CVD908 and CVD908-*htrA* with a plasmid harboring the *ureAB* genes under the control of an in vivo-inducible promoter. The plasmid did not interfere with the ability of either strain to replicate and persist in human monocytic cells or with their transient colonization of mouse lungs. When administered to mice intranasally, both recombinant strains elicited antiurease immune responses skewed towards a Th1 phenotype. Vaccinated mice exhibited strong immunoglobulin G2a (IgG2a)-biased antiurease antibody responses as well as splenocyte populations capable of proliferation and gamma interferon (IFN γ) secretion in response to urease stimulation. Boosting of mice with subcutaneous injection of urease plus alum enhanced immune responses and led them to a more balanced Th1/Th2 phenotype. Following parenteral boost, IgG1 and IgG2a antiurease antibody titers were raised significantly, and strong urease-specific splenocyte proliferative responses, accompanied by IFN γ as well as interleukin-4 (IL-4), IL-5, and IL-10 secretion, were detected. Neither immunization with urease-expressing *S. enterica* serovar Typhi alone nor immunization with urease plus alum alone conferred protection against challenge with a mouse-adapted strain of *H. pylori*; however, a vaccination protocol combining both immunization regimens was protective. This is the first report of effective vaccination against *H. pylori* with a combined mucosal prime-parenteral boost regimen in which serovar Typhi vaccine strains are used as antigen carriers. The significance of these findings with regard to development of a human vaccine against *H. pylori* and modulation of immune responses by heterologous prime-boost immunization regimens is discussed.

Helicobacter pylori, one of a few microorganisms known to colonize the stomach, has been associated with the development of chronic gastritis and peptic ulcers and is considered a risk factor for the development of gastric adenocarcinoma and malignant mucosa-associated lymphoid tissue lymphoma (26). Although combined antibiotic therapy is available to treat this infection, the development of an effective prophylactic or therapeutic vaccine will be of enormous benefit. In developing countries, the high incidence of gastric cancer, high reinfection rates (47), and antibiotic resistance (30) and the relatively high cost of antibiotic treatment make vaccination an especially attractive intervention.

The enzyme urease is considered a prime candidate for inclusion in a vaccine formulation against *H. pylori*. Urease, a cytosolic and surface-exposed nickel metallo-enzyme, is one of the most abundantly expressed proteins in *H. pylori* and consequently one of the best characterized. Its role during infection is to neutralize stomach acid by generating ammonia from urea (39), a function essential for the survival and pathogenesis of this microorganism in the host (12, 13, 23, 57). The enzyme

comprises two subunits, A and B, that assemble into a complex $[(\alpha\beta)_3]_4$ supramolecular structure (22). Antibodies against urease are common in people infected with *H. pylori* (3, 17, 31) and in animals that have been infected experimentally with *Helicobacter* (24, 29, 48). Immunization of mice with recombinant urease formulated in a variety of adjuvants induces strong antibody and cellular responses and affords protection against intragastric *Helicobacter* challenge (16, 21, 28, 38, 40, 42, 43). Oral administration of recombinant urease combined with heat-labile toxin (LT) from enterotoxigenic *Escherichia coli* protects nonhuman primates against *H. pylori* infection (11) and decreases *H. pylori* colonization levels in the stomachs of infected human volunteers (41).

Experiments in a mouse model have proved that using attenuated *Salmonella* strains (5), a variety of antigens, including *H. pylori* urease (1, 7, 8, 19), can be delivered to the immune system. Considerable progress has been made in humans with attenuated *Salmonella enterica* serovar Typhi strains, which can be used both as a more effective typhoid vaccine and for delivery of heterologous antigens. Among the most extensively evaluated vaccine candidates are serovar Typhi strains CVD908 (*Ty2 aroC aroD*) and CVD908-*htrA* (*Ty2 aroC aroD htrA*). Deletion mutations in their *aro* genes render these bacteria auxotrophic for aromatic amino acids as well as for *p*-aminobenzoic acid and 2,3-dihydroxybenzoate. The mutant

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bacteria become attenuated because they are unable to scavenge for these compounds in vivo (reviewed in reference 5). *htrA* encodes a periplasmic protease involved in degrading aberrant proteins. The *htrA* deletion attenuates *Salmonella* strains by impairing their response to stress and survival inside macrophages (27, 36). Upon a single oral immunization in humans, both strains have been found to be safely attenuated and strongly immunogenic, inducing cellular and antibody responses against autologous *Salmonella* antigens as well as against coexpressed heterologous antigens (9, 20, 25, 51–56). Attenuated serovar Typhi strains therefore constitute an attractive carrier system for the delivery of *H. pylori* urease in humans.

In a previous report, we described the use of *Salmonella enterica* serovar Typhimurium-expressing *H. pylori* urease for the oral immunization of mice and protection against *H. pylori* challenge (H. Kleanthous, P. Londoño-Arcila, T. Tibbits, J. Greenwood, R. Nichols, D. Freeman, T. Ermak, T. P. Monath, and M. Darsley, Abstr. Winter Biotechnol. Conf. Cold Spring Harbor: Molecular Approaches to Vaccine Design, p. 48, 1999). In this report, we describe the construction and characterization of serovar Typhi strains that can express urease under the control of an in vivo-inducible promoter never before used for heterologous antigen expression in serovar Typhi. A monocytic cell line was used to demonstrate stable maintenance of the expression plasmid during bacterial multiplication in human macrophages, and a refined model of intranasal infection in mice was used to assess plasmid retention during colonization of host tissue. Protection against *H. pylori* challenge was demonstrated in mice upon vaccination with a combined regimen, based on mucosal priming with serovar Typhi-delivered urease followed by parenteral boosting with urease formulated in alum. This study paves the way for clinical trials investigating the use of the serovar Typhi strains described here for vaccination against *H. pylori* in humans.

MATERIALS AND METHODS

Bacterial strains. Serovar Typhi strains were routinely cultivated in Luria broth (LB) or agar (Lennox modification; Sigma) enriched with L-phenylalanine, L-tryptophan, and L-tyrosine (40 µg/ml each) and *p*-aminobenzoic acid and 2,3-dihydroxybenzoic acid (10 µg/ml each) (LB enriched with aromatic compounds [LB-aro]). Ampicillin was used at 50 µg/ml when required.

Recombinant urease. Purified recombinant *H. pylori* urease was used in immunoassays and for mouse immunizations. The recombinant protein was expressed in *E. coli* as an assembled but inactive enzyme and purified by anion-exchange chromatography as described previously (33). Endotoxin contamination was removed using Sartobind Q filters (Sartorius), which reduced lipopolysaccharide (LPS) content to <1.5 ng/mg of urease. The purified protein was stored as a lyophilized powder.

Mice. Specific-pathogen-free BALB/c mice, purchased from Charles River, were used for all studies. Animal husbandry and experimental procedures were conducted according to the United Kingdom Animals (Scientific Procedures) Act 1986.

Construction of plasmid pHUR3 and transformation into serovar Typhi. Urease expression plasmid pHUR3 was constructed by subcloning the *ureA* and *ureB* genes into pTetnir15 (6) and substituting the *htrA* promoter (49) for the *nirB* promoter. The *ureA* and *ureB* genes were cloned by PCR amplification from plasmid pORV273 (50), using PfuTurbo DNA polymerase (Stratagene), the forward primer ORAFOR (5' TAG GGA ATT CTC ATG AAA CTC ACC CCA AAA G 3'), containing a *Bsp*HI site, and the reverse primer ORAREV (5' TCT ACT GCA GGA TCC AAA ATG CTA AAG AGT TGC G 3'), containing a *Bam*HI site. The *Bsp*HI-*Bam*HI-digested PCR fragment was inserted into *Nco*I-*Bam*HI-digested pTetnir15 to yield pNUR1. Subsequently, the *nirB* promoter was replaced with the *htrA* promoter by replacement of a *Pst*I-*Bgl*II

restriction fragment adjacent to the urease cassette in pNUR1 with the same fragment from a derivative of plasmid pTEThttrA1 which contained the *htrA* promoter (49) (kindly provided by Jingli Li [Medeva PLC]). Sequencing of the construct revealed an error at the 3' end of the *ureB* gene. The incorrect portion of the reading frame was excised by digestion with *Bam*HI to release a 750-bp fragment; it was replaced by a 750-bp *Bam*HI fragment from pORV273 containing the correct urease B sequence. pHUR3 was introduced into serovar Typhi bacterial cells by electroporation. The smooth phenotype of selected transformants was verified by anti-O9 antigen serum agglutination (Abbot) and LPS visualization in Tris-glycine gels (12% polyacrylamide) (58) stained with SilverX-press stain (Invitrogen).

Analysis of urease expression. Expression of urease was examined in lysates of bacteria harboring pHUR3 or plasmid-free parental controls. Broth cultures were grown at 37°C with shaking until mid-log phase and then incubated statically at 42°C for a further 4 to 16 h to induce expression of urease. Harvested bacteria were resuspended to an optical density at 650 nm of 10 and lysed by resuspension in sodium dodecyl sulfate-Tris buffer. Proteins were separated on Tris-glycine–12% polyacrylamide gels (Invitrogen) and either visualized by staining with Coomassie blue or electrotransferred onto nitrocellulose membranes for immunoblotting. Membranes blocked with 3% bovine serum albumin were probed with RPS-1, a hyperimmune rabbit serum raised against native *H. pylori* urease (33). Bound antibody was visualized with the ECL system (Pierce).

Evaluation of *Salmonella* survival in human monocytes. A modified version of the classic gentamicin protection assay (10) was used to study intracellular growth of recombinant serovar Typhi strains in the human monocytic cell line U937 (European Collection of Cell Cultures ref. no. 85011440). Cells were routinely cultured in RPMI 1640 medium enriched with 2 mM glutamine, 10% fetal calf serum, 100 U of penicillin, 100 µg of streptomycin/ml, and 20 mM HEPES (RPMI-10) at 37°C in a humidified 5% CO₂ atmosphere. For the assay, U937 cells were stimulated to differentiate into macrophages by exposure to 10 ng of phorbol myristate acetate (PMA)/ml for 72 h prior to infection. Cell monolayers were prepared for bacterial infection by dispensing a suspension of these PMA-stimulated cells in antibiotic-free RPMI-10, enriched with 50 ng of PMA/ml, into 24-well tissue culture clusters (5 × 10⁵ cell/ml/well). After overnight incubation, monolayers were washed twice with RPMI medium and infected with a suspension of 5 × 10⁶ CFU/0.1 ml/well of recombinant serovar Typhi grown statically at 37°C for approximately 18 h (multiplicity of infection = 10:1). After 1 h of incubation, noninternalized bacteria were killed by the addition of 200 µg of gentamicin/ml (time zero of the assay). After a further 1-h incubation, cells were washed twice and overlaid with medium containing a reduced concentration of gentamicin (10 µg/ml). At different time points, cell monolayers in triplicate wells were washed twice with antibiotic-free medium and lysed by incubation in 1.0% Triton X-100 (Sigma) at 37°C for 10 min. Dilutions of the lysates were made in phosphate-buffered saline (PBS), and the bacteria were plated on LB-aro agar with or without ampicillin (50 µg/ml) for enumeration. Results are reported as mean CFU/well of triplicate wells. All strains were tested concurrently on at least three separate occasions.

Enumeration of *Salmonella* bacteria in mouse organs. Groups of mice were immunized intranasally with recombinant serovar Typhi, as described below. On different days after immunization, five to ten randomly chosen individuals were euthanized from each group and the lungs, livers, and spleens were removed. The numbers of viable serovar Typhi bacteria present in each organ were determined by plating different dilutions of organ homogenates on LB-aro or on LB-aro with 50 µg of ampicillin/ml to indicate the proportion of cells that continued to harbor the plasmid.

Vaccination of mice with serovar Typhi. For intranasal infection with recombinant serovar Typhi, mice were anesthetized by inhalation of halothane and 20 µl of PBS containing 10⁸ live salmonella cells was deposited slowly over their nares using a Gilson Pipetman. Anesthesia was used to increase aspiration of inocula into the lung (56a). To produce the inocula, bacteria were grown statically for 18 h at 37°C in LB-aro (with ampicillin if required), harvested by centrifugation, and resuspended in PBS (pyrogen free; Sigma) at a concentration of 5 × 10⁹ bacteria/ml (as estimated from the optical density at 650 nm). Colony counts were performed for all inocula to verify the number of viable bacteria.

Vaccination of mice using the prime-boost regimen. A number of pilot experiments were carried out to optimize the immunization protocol and define blood and tissue sampling schedules. For the immunogenicity studies described here, mice were primed by intranasal immunization with serovar Typhi on day 0 (d0) and boosted on d32 with either a second salmonella intranasal immunization or a urease plus alum (urease-alum) parenteral booster immunization. For immunization with urease-alum, animals were injected subcutaneously with 10 µg of purified recombinant urease, formulated in 0.65% Alhydrogel. Blood samples were typically obtained on d27 or d28 after each immunization. Groups of three

to six animals, chosen randomly, were euthanized at the same time points to harvest splenocytes and carry out lung lavages. When indicated, a second urease-alum subcutaneous immunization was given 28 days after the first one.

Evaluation of antibody responses. Serum samples, collected from the tail veins of mice at different time points after immunization, were used to titrate humoral responses against *H. pylori* urease or *Salmonella* LPS. Serial dilutions of individual sera were tested in enzyme-linked immunosorbent assays (ELISAs), using plates coated with purified recombinant urease (10 µg/ml) or *S. typhosa* O901 LPS (50 µg/ml; Difco) in 0.1 M NaHCO₃ (pH 9.5). Biotinylated anti-mouse immunoglobulin G (IgG), IgA (Sigma), IgG1, or IgG2a (Pharmingen) was used as a secondary antibody. Bound antibody was visualized with an Extravidin-alkaline phosphatase conjugate (Sigma). Serum samples obtained from five naïve BALB/c mice were used as controls to establish end-point antibody titers during the course of this study. These were defined as the reciprocal of the sample serum dilution which would give an A_{492} value equal to the mean A_{492} value plus 2 times the standard deviation (SD) of control sera, tested at a 1/100 dilution for IgG and a 1/50 dilution for IgG1, IgG2a, and IgA.

Lung lavages, performed postmortem, were used to measure mucosal antibody responses. Lavages were carried out with 1.5 ml of ice-cold PBS, flushed in and out of the lungs with a fine-tipped Pasteur pipette inserted via the trachea. Bovine serum albumin (1%) was added to lavage fluids before storage at -20°C. The total IgA content of individual lavage samples was assessed in a sandwich ELISA, using plates coated with anti-mouse IgA antibody. Specific antiurease IgA was titrated, using the same ELISA as for serum antibodies. Titers were expressed as ELISA units (EU) per microgram of total IgA. The number of EU was established by comparison to a reference mouse serum that contained a high titer of antiurease IgA antibodies and to which an arbitrary concentration of 10⁶ EU/ml was assigned.

Cell proliferation assays and cytokine quantification. Single-cell suspensions of splenocytes from three to six mice per group were prepared at different times after immunization. Splenocytes were resuspended in Dulbecco's minimal Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 10 mM HEPES (Sigma), and 4 × 10⁵ splenocytes were dispensed in 200-µl aliquots into 96-well tissue culture plates. Cells were stimulated in quadruplicate with recombinant *H. pylori* urease (10 or 30 µg/ml) or concanavalin A (ConA) (2.5 µg/ml; Sigma) for 96 h. Cellular proliferation was measured by incorporation of [³H]thymidine (1 µCi per well; Amersham, Little Chalfont, Buckinghamshire, United Kingdom) during the last 18 to 20 h of incubation. Background levels of proliferation were established with cells stimulated with antigen-free medium. For cytokine analysis, splenocytes were cultured in 48-well plates (10⁶/ml/well) and stimulated with antigens as for proliferation assays. Supernatants from duplicate samples were collected at 72 h and stored at -70°C until analyzed. For quantification of gamma interferon (IFN-γ), interleukin-10 (IL-10), IL-4, and IL-5 in culture supernatants, commercial ELISA systems were used (Pharmingen). The limits of detection were 8 pg/ml for IL-4, 15 pg/ml for IL-5, and 30 pg/ml for IL-10 and IFN-γ.

***H. pylori* challenge.** The challenge model and method used to determine *H. pylori* infection in gastric tissue specimens for this study have been described in detail before (14, 28). A mouse-adapted, streptomycin-resistant mutant of *H. pylori* strain X47-2AL (originally isolated from a domestic cat) was used to challenge mice intragastrically (ca. 10⁷ CFU per mouse). At 4 weeks after challenge, mice were euthanized and gastric tissue was harvested for assessment of urease activity and *H. pylori* culture. Urease activity was measured by incubating a longitudinal strip of the stomach in urea broth for 4 h. Urea hydrolysis was quantified spectrophotometrically, using phenol red as the pH indicator. Another longitudinal strip was homogenized in brucella broth (Difco), and the numbers of viable *H. pylori* present were determined by plating serial dilutions of homogenate in *Helicobacter*-selective agar.

Statistical analysis of data. Experimental results were plotted and analyzed for statistical significance with Prism3 software (GraphPad Software Inc.).

RESULTS

Expression of urease by serovar Typhi vaccine strains. Plasmid pHUR3, constructed as described in Materials and Methods, was used to coexpress the *H. pylori* *ureA* and *ureB* genes in serovar Typhi, under the control of the in vivo-inducible *htrA* promoter. In serovar Typhimurium, the *htrA* promoter is induced by heat shock, oxidative stress, and entry into macrophages (15, 49). Serovar Typhi vaccine strains CVD908 and CVD908-*htrA* were transformed with pHUR3 to give SY5915

TABLE 1. Serovar Typhi strains used in this study

Strain	Relevant characteristic	Source or reference
SY5915	CVD908 harboring pHUR3	This study
SY5917	CVD908- <i>htrA</i> harboring pHUR3	This study
CVD908	Serovar Typhi Ty2 Δ aroC Δ aroD	25
CVD908- <i>htrA</i>	Serovar Typhi Ty2 Δ aroC Δ aroD Δ htrA	55

and SY5917, respectively (Table 1). Transformants exhibited identical LPS profiles and showed the same growth pattern in LB-aro and minimal medium as the nontransformed parental strains (data not shown). Whole-cell lysates of recombinant strains SY5915 and SY5917 or of their parental control strains, grown at 42°C under low oxygen tension to induce *P_{htrA}*, were probed with a hyperimmune rabbit antiserum raised against native urease (RPS-1) (33). The blots revealed two major immunoreactive bands in SY5915 and SY5917 lysates that were absent from the CVD908 and CVD908-*htrA* lysates (Fig. 1). The electrophoretic mobility of these bands corresponded to that expected for the urease A and B subunits. Urease expressed from pHUR3 assembled into an inactive enzyme that was immunoreactive in a capture ELISA with IgA 71 (2), a monoclonal antibody directed against a conformational epitope in the B subunit (data not shown).

In vivo and in vitro stability of plasmid pHUR3. The human monocytic cell line U937 was used to study the effect of pHUR3 on the ability of serovar Typhi to replicate and persist in human macrophages. PMA-activated U937 cells were infected with SY5915, SY5917, or their parental strains, as described in Materials and Methods. At different time points, the intracellular bacteria were released from infected macrophages and enumerated by plating onto LB-aro agar with or without ampicillin, to which pHUR3 confers resistance. All the strains tested were able to persist intracellularly in macro-

FIG. 1. Urease expression by pHUR3-transformed serovar Typhi strains. Bacterial lysates, prepared as described in Materials and Methods, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel; 2 × 10⁷ bacteria per lane), blotted onto nitrocellulose, and probed with a rabbit polyclonal serum against native *H. pylori* urease (RPS-1). The A and B urease subunits were identified by comparison to a purified recombinant urease standard (100 ng/lane).

FIG. 2. Persistence and stability of serovar Typhi strains harboring pHUR3. (A) Intracellular survival of recombinant serovar Typhi strains in the human monocytic cell line U937. The plot shows the number of salmonellae recovered from cultures of PMA-activated U937 cells (5×10^5 per well) infected with strains carrying pHUR3 or with their plasmid-free parental controls (multiplicity of infection = 10:1). SY5915 and SY5917 CFU were enumerated in plain LB-aro agar (solid lines) or LB-aro agar containing ampicillin (dotted lines). Values represent the mean CFU/well of triplicate wells. Results shown are representative of three separate experiments in which the four bacterial strains were tested concurrently. (B) Persistence and stability of recombinant serovar Typhi strains in mice infected intranasally (10^8 CFU per mouse). Plot shows the number of recombinant salmonellae recovered from the lungs of mice infected with CVD908, SY5915, CVD908-*htrA*, or SY5917. Bacteria were enumerated in LB-aro agar (L; solid symbols) or LB-aro with ampicillin (Ap; open symbols). Data shown have been compiled from two separate experiments in which plasmid-free control and pHUR3-harboring strains were tested concurrently. Bars represent geometric means. *, $P = 0.008$ in Student's *t* test.

phages for at least 72 h (Fig. 2A). As expected, strains harboring an *htrA* mutation in addition to the *aro* mutations (CVD908-*htrA* and SY5917) were less persistent than those carrying the *aro* mutations alone (CVD908 and SY5915). The presence of pHUR3 reduced intracellular bacterial growth, as indicated by the lower numbers of SY5915 and SY5917 recovered at 24, 48, and 72 h after infection compared to those of their respective plasmid-free parental controls. However, there was no significant difference in the numbers of viable SY5915 or SY5917 bacteria recovered in the presence or absence of

antibiotic selection ($P > 0.05$ in Wilcoxon signed rank test), indicating that pHUR3 was stably maintained by both serovar Typhi strains throughout macrophage infection. Similar results were observed in macrophage assays carried out for 125 h (data not shown). The intracellular location of serovar Typhi in these experiments was confirmed by fluorescence-activated cell sorter analysis and fluorescence microscopy. A fluorescein isothiocyanate-labeled commercial antibody against *Salmonella* common structural antigen gave a positive signal only in permeabilized macrophages (data not shown).

Serovar Typhi is a human pathogen to which other mammals, and mice in particular, are resistant. However, transient colonization of mouse lungs can occur if the bacteria are administered via the intranasal route (18, 44, 46). We introduced modifications to the published infection model (as described in Materials and Methods) which resulted in serovar Typhi persisting in the lungs of mice for at least 2 weeks after intranasal infection, as opposed to the 3 days reported before. Using this refined infection model, SY5915 and SY5917 transiently colonized the lungs of mice without significant loss of pHUR3 (Fig. 2B). The numbers of bacteria recovered on ampicillin agar on d7 after infection were not significantly different from those recovered on ampicillin-free agar ($P > 0.05$ in paired Student's *t* test). As seen for the human monocyte persistence assays described above, bacteria of the strain harboring the *htrA* mutation, SY5917, were recovered at lower levels after infection than bacteria of the strain harboring only *aro* mutations, SY5915 ($P = 0.008$ in Student's *t* test). However, viable, ampicillin-resistant, SY5917 bacteria were still recovered in significant numbers from lungs of mice 14 days after infection (Fig. 2B). The pHUR3-harboring bacteria recovered from mouse lungs had retained the ability to express urease, as determined by immunoblot studies using lysates from randomly chosen bacterial colonies.

Characterization of the immune response to serovar Typhi-delivered urease. The immunogenicity of the strains described here was evaluated by vaccination of BALB/c mice via the intranasal route. Groups of mice were immunized with ca. 10^8 CFU of pHUR3-harboring strain SY5915 or SY5917 or the respective parental control, CVD908 or CVD908-*htrA*. On different days after immunization, serum samples were obtained to measure antibody responses against urease and serovar Typhi LPS, and some animals were euthanized for assessment of cellular responses. Figure 3 shows that both SY5915 and SY5917 generated serum IgG responses against urease after a single intranasal immunization. The IgG antiurease titers generated by SY5915 were significantly higher than those generated by SY5917 ($P = 0.015$; one-tailed Student's *t* test), suggesting that immunogenicity correlated with in vivo persistence (Fig. 2A). Both strains induced an IgG2a-dominated antibody response, as indicated by the ratios between IgG2a and IgG1 titers in individual mice (Fig. 3B and data presented below). As expected, no antiurease antibodies were detected in mice immunized with the parental strain CVD908 or CVD908-*htrA* (Fig. 3). All strains tested induced similar levels of serum IgG against *Salmonella* LPS (data not shown).

The presence of antiurease antibodies of different IgG isotypes in mice vaccinated with SY5915 or SY5917 (Fig. 3B) suggested that these strains were also able to engender T-helper responses against the carried antigen. To evaluate the

FIG. 3. Humoral antibody response elicited by serovar Typhi-delivered urease. IgG responses to urease (A) and urease-specific IgG2a/IgG1 antibody titer ratios (B) in mice immunized with CVD908, SY5915, CVD908-*htrA*, or SY5917 via the intranasal route (10^8 CFU per mouse) are shown. Titers shown were determined in individual serum samples obtained on d28 after immunization. Bars represent geometric means. *, $P = 0.015$ in one-tailed Student's t test.

magnitude of these responses, cell proliferation assays were carried out with splenocytes obtained from vaccinated mice. Splenocytes from mice immunized on one occasion via the intranasal route with either SY5915 or SY5917 exhibited proliferative responses to urease (Table 2). Quantification of cytokines in the supernatants of proliferating splenocytes revealed the presence of IFN γ (Table 2) but not IL-4, IL-5, or IL-10 (data not shown). However, these proliferative and cytokine secretion responses were modest and heterogeneous, as they could only be detected in some of the immunized mice

(Table 2). A second *Salmonella* immunization increased the cellular responses only marginally (data not shown).

To assess whether the immune responses elicited by *Salmonella*-delivered urease conferred protection against *H. pylori* colonization, groups of 10 mice were challenged intragastrically with a mouse-adapted *H. pylori* strain after receiving one (d0) or two (d0 and d21) intranasal vaccinations with SY5915, SY5917, CVD908, or CVD908-*htrA* (as described in Materials and Methods). Mice vaccinated with SY5915 or SY5917 showed no detectable reduction in their gastric urease activity or *H. pylori* burden compared to unvaccinated mice or to those vaccinated with the parental control strains (data not shown).

Enhancement of cellular responses to urease following boosting with purified antigen plus alum. Because it had been established in previous studies that urease-engendered protection against *H. pylori* requires cell-mediated immune responses (14, 28), we embarked on an investigation of ways to enhance the cellular response to urease in mice primed by serovar Typhi-delivered antigen. It has been demonstrated that antibody and, in particular, systemic cellular responses to serovar Typhimurium-delivered antigens can be boosted by parenteral administration of as little as 1 μ g of purified recombinant antigen (35). It has also been reported that protocols combining mucosal and parenteral immunization with urease plus LT can confer protection against *H. pylori* challenge in mice (14, 28) and primates (32). To investigate whether the cellular response to serovar Typhi-delivered urease could be boosted by parenteral inoculation with purified antigen, proof-of-principle studies were carried out with strain SY5915. For this purpose, mice primed by a single SY5915 intranasal immunization were given subcutaneous immunizations with urease-alum. PBS- or CVD908-primed mice were boosted in the same manner to serve as controls.

SY5915-primed mice showed a fivefold increase in their systemic cellular response to urease following subcutaneous immunization with urease-alum (Fig. 4A). The urease-alum immunization also generated proliferative responses in splenocytes from PBS- or CVD908-primed mice (Fig. 4A). However, the responses of these mice were significantly lower than those of SY5915-primed mice ($P < 0.01$ in one-tailed Student's t test comparisons of results obtained with either 30 or 10 μ g of urease/ml), an observation consistent with the presence of an anamnestic cellular response to urease in SY5915-primed mice. Following parenteral urease-alum boosting, splenocytes from all groups of mice secreted IFN γ , IL-4, IL-5, and IL-10 in

TABLE 2. Cellular response to urease induced by a single intranasal immunization with serovar Typhi-delivered urease

Strain	Proliferation (Δ cpm) ^a			IFN γ secretion ^c (pg/ml)
	Group mean	Range	No. of responders/total no. tested ^b	
SY5915	1,321	226–2,436	3/4	<30.0
CVD908	724	698–751	0/4	<30.0
SY5917	3,991	1,699–5,566	4/5	113.5 $\pm$ 85.5
CVD908- <i>htrA</i>	1,018	57–2,121	0/5	<30.0

^a Proliferation assays were performed with splenocytes from individual mice, obtained on d28 after immunization, and tested in quadruplicate with 10 μ g of urease/ml. Δ cpm, [3 H]thymidine incorporation with antigen – [3 H]thymidine incorporation with medium alone.

^b Responders were mice whose splenocytes exhibited a Δ cpm value $\geq$ (mean Δ cpm + 2 SD) that of the respective parental strain control group. The same response rates were obtained when 10 or 30 μ g of urease/ml was used for stimulation.

^c IFN γ was quantified in supernatants of pooled splenocyte populations from three animals/group stimulated with 30 μ g of urease/ml for 72 h. All splenocyte populations secreted IFN γ in response to ConA stimulation but not when cultured with medium alone.

FIG. 4. Cellular responses to urease in mice primed with SY5915 and boosted with urease-alum. Urease-specific proliferative response (A) and cytokine secretion (B) of splenocytes from mice primed by intranasal immunization with SY5915, CVD908, or PBS (d0) and boosted by subcutaneous administration of urease-alum (+ Ure/al; d32) are shown. Responses after primary immunization (prime) are also shown for comparison. Splenocytes were collected on d27 after primary immunization or on d28 after urease-alum boost. Proliferative responses (A) are shown after stimulation with 30 or 10 μ g of purified recombinant urease/ml. Columns represent the mean ($\pm$ SD) stimulation indices of splenocytes from three to six animals per group, tested individually in quadruplicate. Stimulation index = [3 H]thymidine incorporation with antigen/[3 H]thymidine incorporation with medium alone. *, $P < 0.01$ in one-tailed Student's t test comparison to the group of mice immunized with SY5915 followed by urease-alum. Cytokine secretion (B) was quantified in supernatants of pooled splenocyte populations from three mice per group, stimulated with 10 μ g of urease/ml. Columns represent the means ($\pm$ standard errors of the means) of samples tested in duplicate. None of the cytokines tested was detected in splenocyte populations stimulated with medium alone. All cytokines were detected in splenocytes from SY5915-, CVD908-, or PBS-primed mice upon stimulation with ConA.

response to in vitro urease stimulation (Fig. 4B). This indicated that the urease-alum parenteral boost not only enhanced but also broadened the spectrum of cellular responses elicited by serovar Typhi-delivered urease.

Immunomodulatory effect of the urease-alum boost on antibody response to urease. Boosting with urease-alum significantly enhanced the antiurease antibody response in mice

FIG. 5. Antibody responses to urease in mice primed with SY5915 and boosted with urease-alum. IgG responses to urease (panel A) and the urease-specific IgG2a/IgG1 antibody titer ratios (panel B) in mice immunized on one or two occasions with SY5915 via the intranasal route or primed intranasally with SY5915 or CVD908 and boosted subcutaneously with urease-alum on one (+Ure/al) or two (+2Ure/al) occasions are shown. Total IgG, IgG1, and IgG2a antiurease antibody titers and the IgG2a/IgG1 antibody ratios were determined for individual serum samples obtained from four to six animals per group. Columns correspond to group means ($\pm$ SD). *, $P < 0.05$ in Student's t test. Serum samples were obtained for *Salmonella*-primed groups on d28 after the primary inoculation, d27 after the first boost, and d10 after the second boost. See Materials and Methods for inoculation protocol.

primed by intranasal immunization with SY5915 ($P < 0.05$ in a Student's t test comparison of responses after priming) (Fig. 5A). Antiurease IgG attained a higher titer in mice boosted with urease-alum than in those boosted with SY5915 (as determined on d28 after boost) (Fig. 5A). Urease-alum immunization also elicited IgG antibody responses in CVD908-primed mice (Fig. 5A). However, the responses were significantly higher in SY5915- than in CVD908-primed animals (mean titers of 254,700 and 89,810, respectively, on d28 after boost; $P = 0.024$ in Student's t test comparison). Moreover, pilot studies indicated that the postboost IgG titers rose faster in mice primed with urease-expressing serovar Typhi than in those primed with control serovar Typhi strains or PBS (as determined on d7 and d19 after boost) (data not shown).

More striking than the enhancement of antibody titers was

TABLE 3. Urease-specific IgA responses induced by different immunization regimens

Immunization regimen ^a		Mucosal IgA ^b (EU/ μ g of IgA)	Circulating IgA (end-point titer)
Prime	Boost		
SY5915 i.n.	SY5915 i.n.	790.5 $\pm$ 339.4	300.0
SY5915 i.n.	Urease-alum s.c.	116.6 $\pm$ 75.0	59.5
CVD908 i.n.	CVD908 i.n.	12.0 $\pm$ 11.0	<50.0
CVD908 i.n.	Urease-alum s.c.	15.0 $\pm$ 9.9	<50.0
PBS i.n.	Urease-alum s.c.	16.7 $\pm$ 8.1	<50.0
SY5915 i.n.	None	ND	<50.0

^a Mice were boosted on one occasion with serovar Typhi strains (d32 after primary immunization) or on two occasions with urease-alum (d32 and d60). i.n., intranasal; s.c., subcutaneous.

^b Responses were determined as described in Materials and Methods, using individual lung lavage fluid specimens from 4 to 6 mice per group carried out on d59 (for groups primed and boosted with SY5915 or CVD908) or d70 (for urease-alum-boosted mice). Values shown represent the group means $\pm$ SDs. ND, not done.

^c Values shown correspond to the geometric mean titers determined in serum samples collected on the day lung lavages were performed. Similar results were obtained with samples obtained on d28 after the first urease-alum boost. For the group that received only the primary SY5915 intranasal immunization, similar results were obtained with serum samples collected on d14, d28, d33, or d46 after immunization.

the modulating effect exerted by the parenteral urease-alum boost on the balance of antiurease IgG1 and IgG2a circulating antibodies (Fig. 5B). The ratio of urease-specific IgG2a and IgG1 antibody titers in individual mice indicated that mucosal SY5915 priming induced on average twofold greater IgG2a than IgG1 titers (Fig. 5B and 3B). A second SY5915 mucosal immunization skewed the response even more substantially towards IgG2a, raising IgG2a and IgG1 titers by an average of 316- and 13-fold, respectively, compared to titers after priming. (Note that these are the mean increases in individual antibody titers rather than increases in the mean antibody titers per group.) In contrast, administration of a urease-alum parenteral boost increased the IgG2a and IgG1 titers 1,089- and 250-fold, respectively, thus maintaining an only moderate IgG2a dominance. A second urease-alum boost reduced the magnitude of the IgG2a bias, enhancing the IgG2a titers only twofold and the IgG1 titers only threefold. In CVD908-primed mice, which were naïve with respect to urease, the first urease-alum boost induced a balanced IgG2a/IgG1 response ratio, which was maintained after the second boost (Fig. 5B). In PBS-primed mice, the urease-alum immunization induced a response moderately biased towards IgG1 (IgG2a/IgG1 ratio = 0.5 ± 0.08).

Effect of different vaccination schedules on the mucosal response to urease. Antiurease IgA was detected in lung lavage fluids from mice vaccinated on two occasions with SY5915 or primed with SY5915 and boosted with urease-alum (Table 3). Strong circulating antiurease IgA responses were detected only in mice immunized on two occasions with SY5915. Parenteral immunization alone was not sufficient to generate either circulating or mucosal IgA responses against urease, as indicated by the absence of these antibodies in PBS- or CVD908-primed mice following a urease-alum boost (Table 3). Therefore, the development of mucosal IgA responses to urease in mice immunized with the combined mucosal prime-parenteral boost regimen was the result of SY5915 priming. The fact that in these mice, IgA responses to urease were detected in lung lavage fluids but not sera indicated that the antibodies had a

FIG. 6. *H. pylori* challenge after vaccination with the combined mucosal prime-parenteral boost immunization schedule. The results for *H. pylori* recovered from gastric tissue samples of mice primed by intranasal vaccination with SY5915, SY5917, or the plasmid-free parental controls (CVD908 and CVD908-*htrA*) and boosted subcutaneously with urease-alum on days 21 and 35 are shown. Results are also shown for mice that received the priming immunization with SY5915 or SY5917 alone and for control mice immunized with urease-alum alone or with PBS. For details of immunization and challenge protocols, see text. Bars represent the geometric means. *, $P < 0.05$ in Wilcoxon rank sum test comparison to group immunized with PBS.

mucosal origin, as they could not have been transudated from sera.

Effect of the urease-alum boost on protection against *H. pylori* challenge. Challenge experiments were performed to evaluate whether the enhanced immune responses to urease observed following mucosal prime-parenteral boost vaccination (Fig. 4 and 5) conferred protection against *H. pylori*. Groups of ten BALB/c mice were vaccinated via the intranasal route with SY5915 or SY5917 or their respective parental strain and given boosts on two occasions (21 and 35 days later) by subcutaneous injection of urease-alum. As controls, groups of mice were given the urease-alum parenteral immunization or the SY5915 or SY5917 mucosal immunization alone and another group was sham immunized with PBS. Thirty-five days after the second urease-alum boost, all animals were challenged intragastrically with 10^7 CFU *H. pylori*, and levels of protection were determined 1 month later by directly quantifying bacterial burden and urease activity (as the result of *H. pylori* colonization) in gastric tissue (Fig. 6). Parenteral urease-alum immunization alone, mucosal priming with SY5915 or SY5917 alone, or mucosal priming with control serovar Typhi strains followed by parenteral boosting afforded no significant reduction in *H. pylori* burden (Fig. 6) or gastric urease activity (data not shown) compared to immunization with PBS ($P > 0.05$ in Wilcoxon rank sum test). These results were consistent with previous findings showing that parenteral urease-alum immunization was poorly effective in protecting mice against *H. pylori* (14). In contrast, significantly reduced *H. pylori* burden (Fig. 6) and gastric urease activity (data not shown) were

observed in the stomachs of mice primed with serovar Typhi expressing urease and boosted with urease-alum ($P < 0.05$ in Wilcoxon rank sum test). The combined prime-boost vaccination protocol afforded SY5915- and SY5917-primed mice decreases in the gastric *H. pylori* burden of $\log_{10} = 1.68$ and $\log_{10} = 1.65$, respectively, compared to sham-immunized animals. The levels of protection afforded by priming with SY5915 or SY5917 were not significantly different ($P > 0.05$ in Wilcoxon rank sum test comparisons of gastric urease activity or bacterial burden), a finding that highlights the potential of either strain as an antigen carrier in combined prime-boost immunization regimens.

DISCUSSION

The feasibility of the use of *Salmonella*-delivered urease to vaccinate humans against *H. pylori* infection was suggested by studies of mice showing that serovar Typhimurium strains expressing urease engender solid protection against *H. pylori* challenge (7, 19; Kleanthous et al., Abstr. Winter Biotechnol. Conf. 1999). An early proof-of-principle study in humans showed that serovar Typhi Ty800 ($\Delta phoP$, $\Delta phoQ$), expressing urease from an episomal vector, was unable to generate antiurease antibody responses when given orally to volunteers (8). A $\Delta phoP$, $\Delta phoQ$ serovar Typhimurium strain harboring the same expression plasmid was slightly more effective, as it induced weak antibody responses to urease in three out of six vaccinees (1). In a more recent study, the licensed typhoid vaccine strain serovar Typhi Ty21a (*galE*, *via*), expressing urease from a different episomal vector, also failed to elicit humoral responses against urease in nine volunteers, although it induced weak cellular responses in three of them (4). The reasons for the disappointing results of these trials probably relate to the nature of the urease expression systems employed, as in all three cases, the antigen was expressed from a constitutive promoter. Constitutive expression of high levels of foreign protein in *Salmonella* results in rapid loss of the expression vector, particularly during host colonization (1, 6–8). The choice of carrier strain could have been a second factor contributing to the poor immunogenicity of urease in these studies. Levine concluded from data compiled from clinical trials carried out at the Center for Vaccine Development, University of Maryland, that Ty800 is not as immunogenic in humans as other typhoid vaccine candidates, such as CVD908, CVD908-*htrA*, or $\chi 4073$ (34). Strain Ty21a, although licensed as a typhoid fever vaccine, has not been thoroughly evaluated as a carrier of heterologous antigens, mainly because of its weak immunogenicity (34). Consequently, additional work is needed to define a suitable serovar Typhi carrier strain and a stable expression system for delivery of *H. pylori* urease to humans.

For this investigation, we chose to express urease in serovar Typhi strains CVD908 and CVD908-*htrA*. These strains have been tested in human volunteers, both as typhoid vaccine candidates and as antigen delivery vectors (20, 52–56). To ensure stable in vivo expression of the antigen, we cloned the urease genes on a multicopy plasmid, under the control of the in vivo-inducible *htrA* promoter. Significant antigen expression from this promoter should only occur in response to specific environmental signals encountered within the host (15, 45, 49). Hence, in contrast to that of constitutive promoters, the bur-

den of heterologous antigen expression is delayed until the carrier bacteria have reached a site optimal for induction of immune responses. The results shown in Fig. 1 confirm that *P_{htrA}* is suitable for heterologous antigen expression, particularly that of *H. pylori* urease, in serovar Typhi vaccine candidates.

Two key determining factors for effective delivery of heterologous antigens by *Salmonella* are the persistence of the carrier bacteria in host tissues and their ability to retain the antigen-expression vector during host colonization (6). We have adapted an in vitro model of macrophage infection for measurement of intracellular bacterial persistence and plasmid stability (10). The results obtained provided direct experimental evidence that the *htrA* gene plays a role in survival of serovar Typhi in human macrophages, as higher numbers of CVD908 than of CVD908-*htrA* bacteria were consistently recovered from the infected macrophages over the course of the assay (Fig. 2A). This observation underpins clinical findings which indicate that CVD908-*htrA* is more attenuated in humans than its double *aro* mutant parent strain, CVD908 (55, 56). The results obtained also demonstrated that pHUR3 was not segregated out of the carrier serovar Typhi during intracellular bacterial multiplication (Fig. 2A). Although it is uncertain whether the findings in U937 cells will extrapolate to the human reticuloendothelial system, we have found a strong correlation between the persistence and stability of attenuated serovar Typhimurium strains multiplying in vitro in J-774 murine macrophages and their persistence and stability in livers and spleens of orally infected mice (E. Rees and P. Londoño-Arcila, unpublished observations).

To determine whether the pHUR3 plasmid was retained during replication of serovar Typhi in the host milieu, we took advantage of the fact that serovar Typhi has been found to colonize the lungs of mice upon administration via the intranasal route (18, 46, 46). We introduced some refinements to the published infection model which resulted in enhanced delivery of bacterial cells to the lung lumen and significantly longer persistence (14 days, as opposed to 72 h). In this model (Fig. 2B), CVD908 and CVD908-*htrA* could persist for several days in mouse lungs, as could their pHUR3-harboring counterparts SY5915 and SY5917. pHUR3 was not significantly segregated from either serovar Typhi carrier strain during lung colonization, indicating that it did not interfere with bacterial survival in vivo. In agreement with previous reports (44, 46), we were consistently unable to recover significant numbers of viable serovar Typhi bacteria from the spleens or livers of mice infected via the intranasal route. Therefore, we could not assess whether pHUR3 would be stably maintained during systemic dissemination of the carrier strain. Encouragingly, we have found that pHUR3 is retained by serovar Typhimurium *aro* or *aro htrA* mutants during systemic colonization of mice infected via the oral route (Kleanthous et al., Abstr. Winter Biotechnol. Conf. 1999).

Both SY5915 and SY5917 were clearly able to deliver urease to the mammalian immune system, as demonstrated by the presence of solid antibody responses to the antigen in mice immunized with either of these strains via the intranasal route (Fig. 3 and 5). The strains could also elicit cellular responses to the carried antigen, as evidenced by the detection of antiurease antibodies of different T-cell-dependent isotypes in all vaccinated mice (Fig. 3B and 5B) (Table 3) and of urease-specific

splenocyte proliferation responses in a proportion of them (Table 2). Although these responses were not sufficient to confer protection against *H. pylori* challenge, they provided adequate immunological priming for the development of protective responses upon subsequent parenteral boosting with purified recombinant antigen (Fig. 6).

Most of the early studies which addressed the role of urease-induced responses in protection against *H. pylori* concentrated on the role of antibodies (16, 21, 28, 38, 40, 42, 43). However, it is now well documented that cellular responses are not only required but also sufficient for protection (14, 28, 37). Our data showed that greater antiurease cellular responses were observed in response to a mucosal prime-parenteral boost urease immunization regimen than in response to immunization via only one of these routes (Table 2 and Fig. 4). Significantly, neither intranasal delivery of urease by serovar Typhi alone nor parenteral immunization with urease-alum alone engendered anti-*H. pylori* protection, whereas a combination of the two immunization regimens did (Fig. 6).

It has been reported that immunization with urease in adjuvants capable of inducing a mixture of Th1 and Th2 responses confers strong protection against *H. pylori* challenge (14, 21, 28; Kleanthous et al., Abstr. Winter Biotechnol. Conf. 1999). Although neither the IgG2a/IgG1 ratio (21) nor the individual IgG1 or IgG2a antiurease titers (58) are predictors of protection, a dominant IgG2a response is more protective than a dominant IgG1 response (21, 28, 37, 43; Kleanthous et al., Abstr. Winter Biotechnol. Conf. 1999). In this study, the protective heterologous prime-boost immunization regimen generated a Th1/Th2 response profile not seen with either of the individual immunization regimens (Fig. 4 and 5). Using the relative levels of antiurease IgG2a and IgG1 antibodies as markers of Th1 and Th2 responses, we observed that a parenteral urease-alum boost enhanced the antigen-specific response of SY5915-primed mice dramatically but maintained a moderate Th1 bias. In contrast, homologous prime-boost immunization with SY5915 led to a very strong Th1 bias, while parenteral immunization with urease plus alum alone induced a Th2 bias. This suggested that a moderately dominant Th1 response to urease was essential in curtailing *H. pylori* colonization.

Immunization regimens based solely on parenteral inoculation with urease (14, 21, 33, 59) can be as protective as those based solely on mucosal inoculation (7, 14, 19, 28; Kleanthous et al., Abstr. Winter Biotechnol. Conf. 1999). A correlation between mucosal antiurease responses and protection has been observed in mice immunized mucosally with urease plus LT (14, 28, 33). In the present study, only mice that had been primed with serovar Typhi-delivered urease exhibited mucosal IgA responses to urease (Table 2). Although these responses were not sufficient to confer protection to mice immunized solely with SY5915 or SY5917, they probably contributed to limiting *H. pylori* colonization in mice immunized with the combined mucosal prime-parenteral boost regimen. This observation is supported by the lack of protection observed in mice boosted with urease-alum after CVD908 or PBS priming (Table 3 and Fig. 6).

Live attenuated serovar Typhi strains, such as the ones described in this report, are intended for administration to humans via the oral route (i.e., in their natural host, via their natural route of infection). Upon administration, they are ex-

pected to disseminate throughout the reticuloendothelial system, where their potential for inducing solid, long-lasting immune responses should be significantly better than in mice infected via the intranasal route. We found that a single oral immunization with a serovar Typhimurium *aro* mutant strain harboring pHUR3 was sufficient to induce strong systemic cellular responses and protection against *H. pylori* colonization in mice (Kleanthous et al., Abstr. Winter Biotechnol. Conf. 1999). This suggests that in humans, SY5915 or SY5917 immunization via the oral route might be sufficient for protection against *H. pylori*. However, if it is found that secondary immunization is required to enhance protective responses, parenteral boosting with purified antigen coformulated in alum, an adjuvant considered safe for use in humans, constitutes an applicable option.

The results shown here reveal a novel, promising approach for the employment of attenuated serovar Typhi strains expressing urease in the development of a human vaccine against *H. pylori*. They confirm the value of CVD908 and CVD908-*htrA* as antigen delivery vehicles and pave the way for testing SY5915 and SY5917 in human volunteers. Finally, they provide useful insights into the modulation and optimization of immune responses to *Salmonella*-delivered antigens and highlight the potential of this mucosal prime-parenteral boost immunization regimen for vaccination against a variety of diseases.

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