Evaluation of the Immunogenicity and Biological Activity of the 

*Citrobacter freundii* Vi-CRM$_{197}$ Conjugate as a Vaccine for *Salmonella enterica* Serovar Typhi$^\dagger$†

Simona Rondini,1* Francesca Micoli,1 Luisa Lanzilao,1 Christine Hale,2 Allan J. Saul,1 and Laura B. Martin1

Novartis Vaccines Institute for Global Health, Via Fiorentina 1, 53100 Siena, Italy,1 and The Wellcome Trust Sanger Institute, The Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom2

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**Typhoid fever remains a major health problem in developing countries.** Young children are at high risk, and a vaccine effective for this age group is urgently needed. Purified capsular polysaccharide from *Salmonella enterica* serovar Typhi (Vi) is licensed as a vaccine, providing 50 to 70% protection in individuals older than 5 years. However, this vaccine is ineffective in infants. Vi conjugated to a carrier protein (i.e., an exoprotein A mutant from *Pseudomonas aeruginosa* [rEPA]) is highly immunogenic, provides long-term protection, and shows more than 90% protective efficacy in children 2 to 5 years old. Here, we describe an alternative glycoconjugate vaccine for *S. Typhi*, Vi-CRM$_{197}$, where Vi was obtained from *Citrobacter freundii* WR7011 and CRM$_{197}$, the mutant diphtheria toxin protein, was used as the carrier. We investigated the optimization of growth conditions for Vi production from *C. freundii* WR7011 and the immunogenicity of Vi-CRM$_{197}$ conjugates in mice. The optimal saccharide/protein ratio of the glycoconjugates was identified for the best antibody production. We also demonstrated the ability of this new vaccine to protect mice against challenge with Vi-positive *Salmonella enterica* serovar Typhimurium.

*Salmonella enterica* serovar Typhi (S. Typhi) is the causative agent of typhoid fever, a systemic disease which remains a major public health problem, predominantly in children in developing countries. Estimates of the global burden of typhoid fever range from 17 to 22 million cases per year with 216,000 to 600,000 associated annual deaths (2, 9). S. Typhi expresses a virulence (Vi) capsule encoded in the viaB locus of *Salmonella* pathogenicity island 7 (SPI7), which allows S. Typhi to modulate host responses during infection by evading innate immune surveillance (25, 26). Vi is also the main target for vaccines and a major protective antigen against typhoid fever. One of the two currently licensed typhoid fever vaccines is unconjugated Vi polysaccharide, available in more than 90 countries (41).

Vi consists of repeating (α1-4)-2-deoxy-2-N-acetyl galacturonic acid moieties and, similar to other polysaccharides consisting of repeating epitopes, is classified as thymus-independent type 2 (Ti-2) antigen (33). These types of polysaccharides provoke an immune response which is age related, with children under 2 years of age generally poor responders. Additionally, Ti-2 antigens lack affinity maturation of antibody response, generate limited immunologic memory and no booster effect, and produce predominantly immunoglobulin isotype IgM with limited class switching (1). The switch from IgM- to IgG-secreting B cells requires interaction with an activated antigen-specific CD4$^+$ T helper lymphocyte, which can be engaged by the conjugation of the polysaccharide to a carrier protein. Vi, as a Ti-2 antigen, does not generate immunological memory, and antibodies induced by Vi are not able to be boosted by repeated vaccinations (16, 40).

In contrast, Vi conjugated to a carrier protein provides a valid solution against those major drawbacks. Vi conjugated to tetanus toxoid as a carrier protein (Peda Typh) and to the nontoxic recombinant exotoxin A of *Pseudomonas aeruginosa* (Vi-rEPA) has been tested in humans. Although no efficacy data are published for Peda Typh, which is licensed only in India (28), Vi-rEPA showed 89% efficacy over 46 months in a double-blind, randomized, and placebo-controlled trial in 2- to 5-year-olds (17). Vi-rEPA has also been reported to be safe when delivered at 2, 4, and 6 months of age. Additionally, a recent Cochrane review (6) reported a protective efficacy of 55% (95% confidence interval [CI], 30 to 70%) at 3 years with one dose of Vi-polysaccharide vaccine and 87% (95% CI, 56 to 96%) at 2.3 years with two doses of Vi-rEPA.

The immunogenicity of the polysaccharide component of a conjugate is affected by different factors, like size and structure of the polysaccharide component, the carrier protein, the conjugation chemistry, the ratio of saccharide to protein, and the eventual presence of free saccharide in the vaccine conjugate (4, 23, 24, 27, 34, 36–38). These factors need to be carefully optimized to produce an effective vaccine.

Here, we describe a new conjugate Vi vaccine, Vi-CRM$_{197}$, where Vi was obtained from *Citrobacter freundii* strain WR7011. *C. freundii* produces the same Vi capsular polysaccharide as *S. Typhi* (3), and being a biosafety level 1 (BSL-1) organism rather than a BSL-3 organism like *S. Typhi*, its use provides several advantages in terms of safety and manufacturing costs.
C. freundii WR7011 is a nitrosoguanidine-mutated strain derived from the parent strain WR7004. Unlike WR7004, WR7011 stably expresses Vi (21, 32). After optimization of WR7011 growth conditions, Vi was purified and conjugated to the well-characterized diptheria toxin mutant CRM197, which was used as the carrier protein. This 58.4-kDa protein is an approved carrier for licensed childhood conjugate vaccines and has already been shown to be safe and effective in numerous clinical trials (10, 29, 31, 39).

We report antibody responses elicited by Vi-CRM197 conjugate at different doses, immunization schedules, Vi/CRM197 weight/weight (wt/wt) ratios, and in the presence of unconjugated Vi. Additionally, protection results are presented from a murine immunization challenge model using a virulent Salmonella enterica serovar Typhi murine immunization challenge model using a virulent Salmonella enterica serovar Typhi (S. Typhi) (8).

MATERIALS AND METHODS

Source of Vi antigens and CRM197. Citrobacter freundii WR7011 was obtained from the U.S. Public Health Service and used as a source of Vi. The Vi polysaccharide was either purified from fermentation supernatant at the Novartis Vaccines Institute for Global Health (NVIGH) or generously provided by S. Szn (Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health [NIH]). The carrier protein, CRM197, was obtained from Novartis Vaccines and Diagnostics (NV&D).

Growth conditions for C. freundii WR7011. C. freundii strain WR7011 was grown in baffled bottom shake flasks with vented caps (VWR) using both complex and chemically defined (CD) media. All cultures were grown at 37°C with constant agitation (200 rpm).

The following three complex media were evaluated: (i) Luria-Bertani (LB; 10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl); (ii) modified Luria-Bertani (Mod-LB; 5 g/liter yeast extract [Difco], 10 g/liter ultratrich yeast extract [PTK], 8 g/liter NaCl, 0.5% glycerol [not of animal origin]); and (iii) glutamine medium (Glu-medium; 10 g/liter glucose, 1 g/liter glutamine, 10 g/liter yeast extract, 0.07 M Na2HPO4, 0.03 M NaH2PO4, 0.01 M MgSO4·7H2O).

The following chemically defined medium was used with and without amino acid supplements: 13.3 g/liter KH2PO4, 4 g/liter (NH4)2HPO4, 1.7 g/liter citric acid monohydrate, 0.05 M MgSO4·7H2O, 0.1 M thiamine hydrochloride, and 5 mL/liter PTM4 trace salts. PTM4 trace solution is composed of 2 g/liter CuSO4·5H2O, 0.02 g/liter Na2MoO4·2H2O, 0.08 g/liter sodium iodide, 3 g/liter MnSO4·H2O, 0.2 g/liter NaNO3·2H2O, 0.02 g/liter boric acid, 0.5 g/liter CoCl2·6H2O, 0.5 g/liter CaSO4·2H2O, 7 g/liter ZnCl2, and 22 g/liter FeSO4·7H2O. The salts were dissolved in water containing 1 mL/liter H2SO4 and then an additional 0.2 g/ml D-biotin in 2N NH4OH was added. The carbon source used was either 0.5% glucose or glycerol.

CD medium with 0.5% glucose was also supplemented with different amino acid mixtures to evaluate the relevance of single or groups of amino acids for WR7011 growth. Amino acids were grouped according to Lederberg (16a) on the basis of the biosynthetic pathway leading to other amino acids (Table 1, groups 1 to 5) (15, 19). CD medium was initially supplemented with all amino acids but one. For example mix (−1) contained CD medium supplemented with amino acids from groups 2, 3, 4, and 5 but without amino acids from group 1; mix (−2) contained all amino acid groups but 2, etc. Growth of WR7011 in mix (−1), mix (−2), mix (−3), mix (−4), and mix (−5) media was recorded and compared to that of a positive control (CD medium supplemented with all amino acid groups) and a negative control (unsupplemented CD medium).

After this initial test to identify the amino acid group of interest (group 4; see Results), CD medium was supplemented with all amino acids except for those belonging to group 4 (for example, −1) contained CD medium plus all amino acids but histidine; −4) contained CD medium plus all amino acids but threonine, etc.). This test determined the most relevant amino acid for WR7011 growth. Finally, a confirmatory test was conducted by complementing CD medium with each of the amino acids belonging to group 4, one at a time. Thus, −4) contained CD medium supplemented with histidine, +4) contained CD medium plus threonine, etc.

C. freundii WR7011 fermentation. For Vi production, C. freundii WR7011 was grown in a 7-liter bioreactor (EZ-Control, Applikon), containing 4 liters of either Mod-LB or Glut-medium. The bacteria were grown overnight from glycerol stocks in a shake flask containing the same medium to be used for fermentation. Cultures were diluted to obtain an inoculum with an optical density at 600 nm (OD600) of between 0.06 and 0.1 (UV-visible spectrophotometer Biomate 3UV/VIS). The culture pH was controlled (Applibens pH electrode, Applikon) at 7.2 by the automatic addition of 14% NH4OH. The temperature was maintained at 37°C with a heating blanket and closed-loop water circulation. The air sparging flow rate was set at 1 liter/min of air per liter of culture volume, and the dissolved oxygen (DO) was controlled at ≥30% saturation (calibrated polarographic electrode, oxygen sensor low drift; Applikon). DO was achieved by automatic variation of the agitation rate in the range of 500 to 1,000 rpm (stirrer motor P100; Applikon). When needed, glucose concentrations were measured by the glucose oxidase method using a glucose analyzer (GM8 Micro-Stat analyzer; Analox Instruments).

Approximately 10 h post inoculation, when the cultures reached stationary phase, the fermentation broth was harvested by centrifugation (8,000 × g for 1 h) followed by sterilization via filtration with a 0.2-μm pore-size filter (Stericup; Millipore). Vi expression by C. freundii WR7011 was checked using anti-Vi polyclonal rabbit antiserum (anti-Salmonella Vi; BioStat Sifin) by slide agglutination. The Vi concentration in culture medium was measured by high-performance anion-exchange chromatography, coupled with pulsed amperometric detection (HPAEC-PAD) (19). Purification of Vi and synthesis and purification of Vi-CRM197 conjugates.

Vi was purified by a process optimized at NVIGH that combined precipitation, resolubilization, and filtration steps without the use of phenol (F. Micoli et al., unpublished data). Briefly, the Vi polysaccharide was precipitated from the culture supernatant by the addition of cetyltrimethyl ammonium bromide, re-suspension of the pellet in ethanol, and filtration. The Vi solution in ethanol was then precipitated with sodium chloride (NaCl), and the pellet was resuspended in an aqueous solution of NaCl and filtered. Compared with Vi obtained from NIH, Vi purified with this new protocol presented higher O acetylation levels. The purity was confirmed by analytical HPLC analysis (19).

Conjugates were synthesized according to a method based on that already reported by Kossackza et al. (14) and as detailed by Micoli et al. (19). Briefly, CRM197 was derivatized with adipic acid dihydrazine. Then, Vi was activated with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride and linked to the derivatized protein. All conjugates were prepared using the same conditions but with different ratios of Vi/CRM197 (wt/wt). Conjugation reaction mix-

| Group | Amino acid | Amt of individual amino acids (mg/ml) | Mix: final OD600 (inoculum OD600 = 0.2) |
|-------|------------|-------------------------------------|--------------------------------------|
| G1    | Lysine     | 30                                  | Mix(−1): 0.4                         |
|       | Arginine   | 10                                  | Mix(−1): 0.4                         |
|       | Methionine | 25                                  | Mix(−1): 0.4                         |
|       | Cystine HCl| 65                                  | Mix(−1): 0.4                         |
| G2    | Leucine    | 30                                  | Mix(−2): 0.5                         |
|       | Isoleucine | 30                                  | Mix(−2): 0.5                         |
|       | Valine     | 150                                 | Mix(−2): 0.5                         |
|       | Asparagine | 10                                  | Mix(−2): 0.5                         |
| G3    | Phenylalanine | 60                              | Mix(−3): 0.6                         |
|       | Tyrosine   | 5                                   | Mix(−3): 0.6                         |
|       | Tryptophan | 25                                  | Mix(−3): 0.6                         |
|       | Glutamine  | 10                                  | Mix(−3): 0.6                         |
| G4    | Histidine  | 25                                  | Mix(−4): 0.2                         |
|       | Threonine  | 200                                 | Mix(−4): 0.2                         |
|       | Glutamate  | 10                                  | Mix(−4): 0.2                         |
|       | Proline    | 10                                  | Mix(−4): 0.2                         |
|       | Aspartate  | 100                                 | Mix(−4): 0.2                         |
| G5    | Alanine    | 10                                  | Mix(−5): 0.4                         |
|       | Glycine    | 10                                  | Mix(−5): 0.4                         |
|       | Serine     | 400                                 | Mix(−5): 0.4                         |

*CD medium was supplemented with the following: mix (−1), G2, G3, G4, G5 amino acid mixtures; mix (−2), G1, G3, G4, G5 amino acid mixtures; mix (−3), G1, G2, G4, G5 amino acid mixtures; mix (−4), G1, G2, G3, G4 amino acid mixtures; mix (−5), G1, G1, G3, G4 amino acid mixtures.*

TABLE 1. Grouping of amino acids used to supplement CD medium


TABLE 2. Characteristics of Vi-CRM<sub>197</sub> conjugates used in immunogenicity studies

| Conjugate     | Vi manufacturer | % Vi O acetylation by nuclear magnetic resonance | Vi content by HPAEC-PAD (µg/ml) | Protein content by Micro BCA (µg/ml) | Vi/protein ratio experimentally determined (nominal ratio before conjugation) | Immunogenicity studies |
|---------------|-----------------|-----------------------------------------------|---------------------------------|--------------------------------------|--------------------------------------------------------------------------------|-----------------------|
| Vi (NVGH)-CRM<sub>197</sub> | NVGH            | >90                                           | 11.7                            | 13.8                                 | 0.9 (1:1)                                                                          | Influence of different Vi-CRM<sub>197</sub> ratios; impact of unconjugated Vi |
|                | NVGH            | >90                                           | 28.2                            | 13.8                                 | 2.1 (2:1)                                                                          | Influence of different Vi-CRM<sub>197</sub> ratios; impact of unconjugated Vi |
|                | NVGH            | >90                                           | 61.4                            | 6.1                                  | 10.1 (10:1)                                                                        | Influence of different Vi-CRM<sub>197</sub> ratios; impact of unconjugated Vi |
| Vi (NIH)-CRM<sub>197</sub> | NIH             | 68                                            | 99.6                            | 137.6                                | 0.7 (0.5:1)                                                                        | Dose escalation; bacterial challenge |
|                | NIH             | 68                                            | 14.3                            | 16.7                                 | 0.9 (1:1)                                                                          | Influence of different Vi-CRM<sub>197</sub> ratios; impact of unconjugated Vi |
|                | NIH             | 68                                            | 22.4                            | 11.9                                 | 1.9 (2:1)                                                                          | Influence of different Vi-CRM<sub>197</sub> ratios; impact of unconjugated Vi |
|                | NIH             | 68                                            | 32.9                            | 5                                    | 6.6 (10:1)                                                                        | Influence of different Vi-CRM<sub>197</sub> ratios; impact of unconjugated Vi |

ELISA method. Anti-Vi and anti-CRM<sub>197</sub> antibody levels in mouse sera were determined by enzyme-linked immunosorbent assay (ELISA). The wells of 96-well ELISA plates (Maxisorp; Nunc) were coated with 100 µl of either 1 µg/ml Vi or 2 µg/ml CRM<sub>197</sub> in carbonate buffer (0.05 M, pH 9.6) and left overnight at 4°C. Vi used for coating was purified from C. freundii WR7011 obtained from NIH; CRM<sub>197</sub> was supplied by NV&V. The following morning, the plates were blocked for 1 h at room temperature (RT) with 200 µl of 5% fat-free milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). After being washed with PBST (ELISA washer ELA405, BioTek), 100 µl of mouse serum (at least 1:200 diluted in PBST containing 0.1% BSA) was incubated for 2 h at RT. After three more washes, 100 µl of alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma A3438, diluted 1:10,000 in PBST, 0.1% BSA) was incubated for 1 h at RT. Alkaline phosphatase substrate (p-nitrophenyl phosphate; Sigma) dissolved in diethanolamine buffer (1 M, pH 9.8) was added after wash and incubated for 1 h at RT. Color development was read at 405 and 490 nm using an ELISA reader (ELX800; BioTek). Absorbance values for antibody ELISA unit determination were obtained by subtracting OD<sub>490</sub> values from OD<sub>405</sub> values. ELISA units are expressed relative to a mouse anti-Vi or anti-CRM<sub>197</sub> antibody standard serum curve, with the best four-parameter fit determined by a modified Hill plot. One ELISA unit is defined as the reciprocal of the dilution of the standard serum that gives an absorbance value equal to 1 in this assay. Each mouse serum was run in triplicate.

Immunization studies. To evaluate the antigenicity of Vi and Vi-CRM<sub>197</sub>, CD1 female mice of approximately 5 weeks of age were used. Vaccinations were given by subcutaneous injection (200 µl) at 2-week intervals. In all cases, “dose” refers to the amount of Vi injected, not the mass of the conjugate.

Dose response studies. Two dose escalation studies were performed with six mice per group immunized with increasing doses of Vi-CRM<sub>197</sub> conjugate (from 0.125 to 16 µg of Vi injection). Mice were given either one or two injections, and blood was collected at days 0 (preimmune serum), 14, 28, and 42. The Vi for these Vi-CRM<sub>197</sub> conjugates was obtained from NIH.

Polysaccharide-to-protein ratio study. Six mice per group were immunized twice at 14-day intervals with increasing amounts of Vi-CRM<sub>197</sub> (from 0.125 µg to 8 µg Vi injection) prepared at 1:1, 2:1, and 10:1 (weight/weight) polysaccharide-to-protein ratios. Vi conjugates used in this study were Vi-CRM<sub>197</sub> with Vi either purified at NVGH or obtained from NIH. Another group of mice was given unconjugated Vi (NVGH) polysaccharide. Blood was obtained at days 0 (preimmune), 14, 28, 42, and 56.

Impact of unconjugated Vi. Vi-CRM<sub>197</sub> doses containing 1 µg of Vi were spiked with increasing amounts of unconjugated “free” Vi (from 0 to 50% of total Vi content; i.e., 0 to 1 µg/dose). Ten mice per group were given two injections, and blood was obtained at days 0 (preimmune), 14, 28, 42, and 56.
RESULTS

Growth conditions for C. freundii WR7011. C. freundii WR7011 grew well in all complex media, but no growth was observed in the chemically defined medium. Supplementation of CD medium with amino acid mixes resulted in weak growth in shaker flasks (OD_{600} of ~0.5 after 5 h growth starting from an OD_{600} of 0.2) (Fig. 1). To evaluate the relevance of individual or groups of amino acids, different amino acids were divided into five groups (Table 1) and CD medium was initially supplemented with all but one of the amino acid groups, from mix(−1) to mix(−5). From this experiment, growth was not supported in mix(−4); thus, WR7011 needed one or more amino acids present in group 4 (Table 1) to grow in defined medium.

A second experiment was designed to identify the most relevant amino acid(s) of group 4. When the medium was deficient in proline (−Pro group), growth of WR7011 was completely impaired (Fig. 1); the exclusion of the other amino acids present in group 4 (histidine, threonine, glutamate, and aspartate) had little impact. The auxotrophic requirement for proline was confirmed by growth of WR7011 in CD medium supplemented with each of the amino acids in group 4, where the highest OD_{600} value was obtained in the proline-containing medium (Fig. 1). Thus, proline was identified as the single most important amino acid required to support the growth of WR7011.

Fermentation of C. freundii WR7011. The best growth of WR7011 in shaker flasks was obtained using Mod-LB and Glut-medium (OD_{600} of 4.4); therefore, these media were used for fermentations (Table 3). Different carbon sources (e.g., glycerol, glucose, shift of carbon source), nutrient feeds (e.g., yeast extract, glutamine), and fermentation strategies (e.g., batch versus fed batch) were evaluated. The maximum OD_{600} value reached in the bioreactor was 7 to 9 at 10 h postinoculation.

Glut-medium with glutamine and glucose feed supported the highest growth (OD_{600} of 9; duplication time of 1 h for the first 5 h, entering stationary phase after about 8 h) (Table 3). Higher OD_{600} readings resulted in greater amounts of total Vi released into the culture supernatant. Vi (approximately 4 µg/ml) was detectable by HPAEC-PAD at an OD_{600} of 4, and the level progressively increased, reaching about 100 µg/ml at approximately an OD_{600} of 9. In contrast, when yeast extract was used as feed, a very low Vi yield (about 4 µg/ml) was obtained.

Vi conjugate synthesis and characterization. At the end of each fermentation, Vi was purified from culture medium, obtaining an overall yield >50%, compared to the Vi amount obtained in the fermentation supernatant. The polysaccharide was thoroughly characterized for appearance, purity, identity, and integrity before performing conjugation. Vi purified at NVGH contained higher acetylation levels than Vi obtained from NIH (>90% compared to 68%).

Conjugation reaction mixtures containing 1:1, 2:1, and 10:1 (wt/wt) nominal amounts of Vi (both NVGH and NIH) per CRM_{197} were used to generate conjugates for immunogenicity studies on the influence of different Vi/CRM_{197} ratios. A conjugation reaction mixture containing 0.5:1 (wt/wt) amount of Vi (NIH) was used to generate the conjugate for dose escalation and challenge studies. Conjugates were purified by gel filtration chromatography on Sephacryl S1000 gel, separating the conjugate by both free Vi and unconjugated CRM_{197}. Experimentally determined Vi/CRM_{197} ratios of the purified used conjugates are reported in Table 2, as measured by HPAEC-PAD and the Micro BCA kit.

Dose response studies. Mice were immunized once (Fig. 2A and B) or twice (Fig. 2C and D) with increasing doses of Vi (NIH)-CRM_{197} conjugate. Anti-Vi and anti-CRM_{197} antibodies (total IgG) were measured by ELISA. Anti-Vi antibodies from mice receiving a single or a double immunization of Vi-CRM_{197} were detected at the lowest dose tested (0.125 µg).

TABLE 3. Growth of WR7011 under different (5-liter) bioreactor conditions

| Medium    | OD_{600} (inoculum) | OD_{600} (final) | Carbon source | Feeda |
|-----------|----------------------|------------------|---------------|-------|
| Mod-LB    | 0.07                 | 4                | Gly           | Gly and Glu |
|           | 0.01                 | 6                | Gly           | Glu |
|           | 0.06                 | 4.5              | Glu           | Glu and YE |
|           | 0.02                 | 8.2              | Glu           | YE |
| Glut-medium | 0.09                 | 8                | Glu           | Glutamine |
|           | 0.07                 | 7                | Glu           | Glutamine |
|           | 0.13                 | 9                | Glu           | Glutamine |

a DO, pH, and temperature were controlled similarly in all fermentation runs.

b Gly, glycerol; Glu, glucose; YE, yeast extract.
and showed a significant relationship between dose and antibody response following either vaccination regimen (Spearman rank correlation for day 28 samples following one immunization, \( r = 0.5 \) and \( P = 0.004 \); two immunizations, \( r = 0.6 \) and \( P = 0.0006 \)). The highest anti-Vi antibody levels were detected in day 28 samples and did not increase further. In contrast, anti-CRM197 antibodies increased at each successive time point in both studies (Fig. 2B and D). Anti-CRM197 antibody responses were also dose dependent in both studies (Spearman rank correlation for day 28 samples following one immunization, \( r = 0.8 \) and \( P = 2.1 \times 10^{-7} \); two immunizations, \( r = 0.7 \) and \( P = 0.00001 \)).

**Influence of different Vi/CRM197 ratios on antibody responses.**

To evaluate the influence of the polysaccharide/carrier ratio, mice were immunized twice with increasing doses of Vi conjugate (from 0.125 \( g \) to 8 \( g/\text{injection} \)) prepared at 1:1, 2:1, and 10:1 Vi/CRM197 ratios (wt/wt). In this study, Vi (NVGH)-CRM197 was also compared with Vi (NIH)-CRM197 at 0.7 g/dose.

Conjugates containing similar amounts of Vi (NVGH) and CRM197 by weight (actual postconjugation ratios of 0.9 and 2.1) induced higher IgG antibody responses than conjugate with postconjugation ratios of 10.1, as shown in Fig. 3A. A significant difference was observed only at a 0.125-\( g \) dose of Vi (NVGH)-CRM197, with the 0.9 and 2.1 ratio conjugates being more immunogenic than the 10.1 ratio conjugate (Kruskal-Wallis ANOVA: chi-squared value of 10.9, post hoc analysis \( P \) value of 0.004).

Anti-CRM197 antibody levels also declined with increasing Vi/CRM197 ratios (Fig. 3B), with significant differences detected for all Vi (NVGH)-CRM197 doses at ratios of 0.9 and 2.1 [and ratios of 0.9 and 1.9 of Vi (NIH)-CRM197], compared with a 10.1 conjugate ratio [and 6.6 ratio of Vi (NIH)-CRM197] (Kruskal-Wallis ANOVA: chi-squared range, 9.8 to 12.5; post hoc analysis \( P \) value range of 0.002 to 0.007).

Similarly, when delivered at a dose of 1 \( g \), Vi (NIH)-CRM197 conjugates (Fig. 4) prepared at 0.9, 1.9, and 6.6 postconjugation ratios induced high IgG antibody responses that were not statistically different. Comparison of immunogenicity of Vi (NVGH)-CRM197 and Vi (NIH)-CRM197, prepared at...
different Vi/CRM₁₉₇ ratios and delivered at a 1-μg dose, showed no significant difference for either anti-Vi (Fig. 4) or anti-CRM₁₉₇ (see Fig. S1 in the supplemental material). As previously reported by others (5, 7), unconjugated Vi elicited very weak IgG antibody responses even at a dose of 8 μg/injection (Fig. 4).

**Influence of unconjugated Vi on antibody response.** To determine the influence of unconjugated Vi on the generation of antibody response following Vi (NVGH)-CRM₁₉₇ conjugate vaccination, mice were immunized twice with a fixed immunization dose of 1 μg conjugate spiked with increasing amounts of unconjugated “free” Vi. No significant difference in anti-Vi (Fig. 5) or anti-CRM₁₉₇ (data not shown) antibody responses was detected by Spearman rank correlation (P > 0.05).

**Protection elicited by Vi-CRM₁₉₇ immunization on bacterial challenge.** Mice were immunized intranasally on days 0 and 8 followed by subcutaneous immunization on day 22 with 10 μg of Vi (NIH)-CRM₁₉₇, with or without the addition of 1 μg LT, as previously described by Hale et al. (8). Bacterial challenge was performed by IP administration with 10⁴ CFU of virulent...
Vi-positive *S. Typhimurium* (strain C5.507) on days 61, 104, and 111 and with 10⁴ CFU of virulent Vi-negative *S. Typhi-

murium* (strain SGB1) on day 104 to evaluate the specificity of immune protection. One day after challenge, mice were sacrificed and viable bacterial counts determined in spleens and livers.

The data collected, irrespective of challenge day, indicated that only mice immunized with Vi-CRM197 were able to control the growth of the *S. Typhimurium* Vi-positive strain, in comparison to control mice (Fig. 6, data shown are for day 105, both C5.507 and SGB1 challenge). The addition of LT to Vi-CRM197 reduced bacterial colonization, with significant differences in bacterial counts seen in the spleens of the Vi-CRM197 plus LT group compared with bacterial counts in the spleens of the PBS group on day 62 (*P* = 0.027) (see Fig. S2 in the supplemental material) and in liver samples on day 105 (*P* = 0.037) (Fig. 6C). On day 112, significantly fewer Vi-positive *S. Typhimurium* bacterial CFU were counted in both spleens and livers of mice after either Vi-CRM197 or Vi-CRM197 plus LT immunization compared to that in the PBS group (*P* values of <0.03) (see Fig. S2 in the supplemental material). *S. Typhimurium* Vi-negative bacteria infected all groups of mice, regardless of the recipient's vaccination or immune status. The protective effect seen was Vi specific, as no reduction in bacterial counts was recorded with Vi-negative *S. Typhimurium* (Fig. 6B and D).

Total anti-Vi IgG levels, measured in sera of mice bled at day 112 (1 day after challenge with Vi-positive *S. Typhimurium*), were detected in immunized mice and not in control groups as expected. Analysis of anti-Vi IgG subclasses indicated that IgG1 subclass contributed to the majority of total IgG responses. No significant IgG2 levels above the background were detected (*P* > 0.05) (see Fig. S3 in the supplemental material). To confirm that strain C5.507 recovered from immunized mice still expressed Vi antigen, colonies were screened by anti-Vi antibody immunoblotting. All bacteria recovered from tissues of mice challenged with Vi-positive *S. Typhimurium* expressed Vi (data not shown).

**FIG. 6.** Protection elicited by Vi-CRM₁₉₇ immunization following bacterial challenge. One hundred BALB/c female mice were divided into four groups and immunized on days 0, 8 (intranasally), and 22 (subcutaneously). Mice were immunized as follows: group 1, 10 µg Vi-CRM₁₉₇ plus 1 µg LT; group 2, 10 µg Vi-CRM₁₉₇; group 3, 1 µg LT; and group 4, PBS. Mice were challenged on days 61, 104, or 111 with Vi-positive *S. Typhimurium* (strain C5.507) and day 104 with Vi-negative *S. Typhimurium* (strain SGB1). Spleen and liver bacterial CFU of mice challenged with C5.507 were counted on days 62 (see Fig. S2 in the supplemental material), 105 (A and C), or 112 (see Fig. S2 in the supplemental material). Spleen/liver bacterial CFU of mice challenged with SGB1 were counted on day 105 (B and D). Vi-CRM₁₉₇ was prepared using Vi from NIH with a polysaccharide-to-protein ratio of 0.7.
DISCUSSION

The Vi-CRM197 vaccine described here is one of several new Vi conjugate vaccines with the potential of preventing typhoid fever, especially in young children. S. Typhi infection is a serious public health problem, and the increasing transmission of multidrug-resistant strains has led to untreatable typhoid fever cases (13, 20, 22, 41, 43). Although the oral attenuated Ty21a and the Vi polysaccharide vaccines are currently licensed, they have not been implemented as a routine public health measure in most countries where typhoid fever is endemic. This is despite low vaccine costs (a dose of Vi polysaccharide vaccine is $0.57 [42]), compared with the elevated costs of medical treatment for typhoid fever (World Health Organization [WHO] estimates that the total cost per case of typhoid fever requiring hospitalization ranged from $129 to $820 in India and approximately $334 in the rest of Asia [43]). A major drawback of the licensed vaccines, which may have also impacted their introduction, is their requirement for repeated booster vaccination, modest efficacy (~70%), and ineffectiveness in young children and infants, who are highly susceptible to the disease.

Conjugation of the protective antigen Vi to a carrier protein is an effective method to convert a thymus-independent antigen into a thymus-dependent antigen, with the consequence of eliciting an immune response in infants as well as developing high levels of long-lasting antibody. CRM197 is a well-characterized diphtheria toxin mutant with an excellent safety and effectiveness profile, as shown by its use in licensed childhood conjugate vaccines. Thus, CRM197 was selected by NVGH as the preferred carrier protein and used for conjugation to Vi polysaccharide.

The source of Vi used in these studies was obtained from C. freundii WR7011 and is identical to Vi from S. Typhi (3). Fermentation and handling of a biosafety level 1 (BSL-1) organism, like C. freundii, instead of S. Typhi, a BSL-3 organism, poses several advantages in terms of safety and manufacturing costs. The strain chosen, WR7011, has been mutagenized to constitutively express high Vi capsule levels, but the chemical mutagenesis may have altered other metabolic pathways, making it more difficult to grow to high cell density either at a small scale or in bioreactors.

Although Citrobacter isolates, including the WR7011 parent strain (WR7004), generally grow in defined media (8) (our unpublished observation), WR7011 cannot, probably as a result of alterations in metabolic pathways. Growth in defined synthetic media is preferred in biopharmaceutical production processes to reduce costs, ensure reproducible growth, and minimize regulatory concerns; therefore, it is an important asset when choosing an isolate as a potential producing source. Additionally, the quality of material originating from animals is hard to control and could easily have an impact on process performance.

We first observed that growth of WR7011 in CD medium was sustained at low levels by the addition of various amino acids to the medium. After grouping individual amino acids and verifying growth of WR7001, it was possible to identify proline as essential for the growth of the bacterium in such medium. This is probably only one of the several metabolic pathways that may have been damaged through chemical mutagenesis. Although we were unable to reach ODs higher than 9 even in fermentation conditions, Vi production per OD600 unit was satisfactory at 12.5 μg/ml/OD600 and was substantially higher than that reported by Jiang et al. (11) from an optimized fermentation process of S. Typhi Ty2 that resulted in 6.1 μg/ml/OD600. The Vi yield obtained from a 1,000-liter fermentor of C. freundii WR7011 followed by a purification process with >50% recovery would correspond to approximately 2 million 25-μg doses. However, further optimization of the Citrobacter fermentation to allow higher final cell densities would make this process commercially more attractive.

Vi obtained from WR7011 was finally conjugated to CRM197. Immunogenicity studies and bacterial challenge experiments in mice were performed to evaluate the usefulness of Vi-CRM197 as a new vaccine candidate against S. Typhi. Mice immunized once or twice, even with low doses (0.125 μg/dose) of Vi-CRM197, responded by generating antibodies to both Vi and CRM197. A correlation between Vi dose and specific antibody response was found with both immunization regimens.

Few studies have investigated the influence of the saccharide-to-protein ratio on immunogenicity of the conjugates, although this has a significant impact on manufacturing costs, safety, and immunogenicity. Our data showed that a ratio of Vi to CRM197 of 10.1 at low doses (0.125 μg) was suboptimal in antibody production compared to that of conjugates with a Vi-to-CRM197 ratio of 0.9 or 2.1. These data support the development of an equal weight ratio of Vi to CRM197.

The presence of free Vi in the conjugate preparation is unlikely to impact the immune response. This is in contrast to other polysaccharide conjugate vaccines; in the case of Streptococcus pneumoniae capsular polysaccharide-tetanus toxoid conjugate, the presence of more than 10% free polysaccharide significantly decreased the immunogenicity of the conjugate and led to a persistent state of unresponsiveness (27). To test the possible impact of free Vi, we used mixtures of Vi and Vi-CRM197, with a 10.1 ratio of Vi to carrier, because under these conditions the suboptimal antibody response to Vi may lead to a more stringent test. However, we did not observe any interference of free Vi on the level of anti-Vi antibodies even with a proportion of free Vi/Vi-CRM197 ratio of 1:1. This finding corroborates previous data from our group where conjugate preparations deliberately produced to contain free Vi were immunologically indistinguishable from purified Vi-CRM197 containing only conjugated antigen (19). Additionally, immunization with 8 μg of free Vi failed to induce a response above the background. Thus, there is no evidence that the presence of free Vi is likely to be a problem in Vi-CRM197 conjugate vaccines.

To assess whether the immune response generated after Vi-CRM197 immunization would be sufficient to protect mice upon bacterial challenge, we utilized a mouse infection model developed to test immunity to Vi. Mice immunized with Vi-CRM197 were protected against Vi-positive S. Typhimurium colonization. The immune protection was Vi specific, as no reduction in bacterial counts was recorded in the organs of animals challenged with Vi-negative S. Typhimurium. There are two features of this model that are particularly important. First, this S. Typhimurium has been transfected with the Salmonella pathogenicity island 7 (SPI7) from S. Typhi, thus providing further evidence that the anti-Citrobacter Vi response is
relevant for S. Typhi. Second, the BALB/c mice used in these challenge experiments constitute a hypersusceptible model of infection due to impaired macrophage function, and thus this constitutes a stringent challenge (8).

In summary, conjugates based on a novel source of Vi and a commercially available carrier, CRM₁₉₇, are highly immunogenic in the absence of adjuvant in mice. The antibodies produced protected mice against challenge with a Vi-positive S. Typhimurium. The antibody response was similar over a range of Vi-to-CRM₁₉₇ ratios and was not affected by the presence of free Vi. These findings indicate that Cıtrobacter as a source of Vi and a Vi-CRM₁₉₇ conjugate may be an industrially attractive route to an affordable conjugate vaccine for typhoid fever vaccines, especially in young children in low-income countries.

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