Physical and Chemical Characterization and Immunologic Properties of *Salmonella enterica* Serovar Typhi Capsular Polysaccharide-Diphtheria Toxoid Conjugates

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Typhoid fever, a serious systemic infection caused by *Salmonella enterica* serovar Typhi, remains a major public health problem in developing countries, especially among young children. Recent studies showed more than 50% of typhoid cases are in children under 5 years old. Licensed vaccines, such as *Salmonella enterica* serovar Typhi capsular Vi, did not confer protection against typhoid fever for this age group. Vi conjugate, prepared by binding Vi to *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA), induces protective levels of antibody at as young as 2 years old. Because of the lack of regulatory precedent for rEPA in licensing vaccines, we employed diphtheria toxoid (DT) as the carrier protein to accommodate accessibility in developing countries. Five lots of Vi-DT conjugates were prepared using adipic acid dihydrazide (ADH) as the linker. All 5 lots showed consistency in their physical and chemical characteristics and final yields. These Vi-DT conjugates elicited levels of IgG anti-Vi in young mice significantly higher than those in mice injected with Vi alone and induced a booster response upon reinjection. This booster effect was absent if the Vi replaced one of the two conjugate injections. Vi-DT was stable under repeated freeze-thaw (20 cycles). We plan to perform clinical evaluation of the safety and immunogenicity of Vi-DT when added to the infant combination vaccines.

Typhoid fever, a serious public health problem in developing countries, especially among young children. As was shown for other capsular polysaccharides, such as *Haemophilus influenzae* type b (8, 37); meningococcus groups A, C, and W135; and *Streptococcus pneumoniae* (12, 20), Vi covalently bound with protein conferred T-cell dependence and increased immunogenicity (48–50). To date, diphtheria toxoid (DT), tetanus toxoid (TT), cholera toxins (CT), the B subunit of the heat-labile toxin (LT-B) of *Escherichia coli*, recombinant outer membrane protein of *Klebsiella pneumoniae* (rP40), and iron-regulated outer-membrane proteins (IROMPs) of *S. Typhi* have served as carriers for Vi polysaccharide in laboratory studies (16, 17, 32, 48–50; personal communications). An improved method was developed (24), utilizing adipic acid dihydrazide (ADH) as the linker and *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA) as the carrier. Clinical trials of Vi-rEPA conjugates conferred 89% protection in Vietnamese children 2 to 5 years old for 46 months (23, 26, 28). The level of serum IgG anti-Vi induced by Vi-rEPA conjugates was correlated with prevention of typhoid fever in these studies (7, 21–23, 26, 28).

One limitation of using rEPA as the carrier protein is the lack of regulatory precedent in licensing vaccines. In this report, five lots of Vi conjugates using DT manufactured by pharmaceutical companies in China and India were prepared.
(24, 48, 49). Modifications of conjugation procedures were made for the purposes of easy adoption and scale up by manufacturers. The stability of Vi-DT was studied for the feasibility of stockpiling in disaster relief.

Another important aspect of conjugate vaccine implementation is the optimum immunization formulation and schedule using alternating injections of polysaccharide and conjugate. Priming or boosting effects of polysaccharide on its conjugate vaccine have been observed in infants injected with pneumococcal and meningococcal vaccines (3, 4, 31, 40). There was no consistent conclusion about various types of polysaccharides studied (6, 9, 31, 40, 41). Here, we compared the immune response of Vi polysaccharide injected before or after the administration of Vi-DT with the responses of those receiving 2 injections of Vi-DT. We also investigated the dosage effect for the purpose of better formulation.

MATERIALS AND METHODS
Vi purification and characterization. Vi was extracted and purified from S. Typhi strain Ty2 (ATCC 19430) as described previously (54). Briefly, the culture supernatant was mixed with 0.2% hexadecyl-trimethyl-ammonium bromide (Cetavlon; Sigma Life Science). The precipitate was washed with 0.1 M NaCl and extracted with 1.0 M NaCl. The supernatant was brought to 25% ethanol and centrifuged to remove nucleic acids and then was brought to 75% ethanol. The precipitate was washed with 98% ethanol, freeze-dried, and extracted four times with buffered phenol at 10°C. The water phase of the extraction was precipitated with 75% ethanol, dialyzed extensively against pyrogen-free deionized water, and freeze-dried.

The antigenicity of Vi was confirmed by immunodiffusion reaction with burro Vi hyperimmune serum (provided by NIH) (30). The molecular size of Vi was estimated by the size exclusion chromatography (SEC) method in a 1.5 by 95-cm Sepharose CL-4B column (GE Health). The O-acetyl content of Vi was determined by Hestrin assay, using acetylcholine chloride as a standard (50). The nucleic acid content was determined with UV spectrometry at a wavelength of 260 nm. The protein concentration was measured by the Coomassie protein assay (Thermo Scientific), using bovine serum albumin (BSA) as a standard (5). The DT content was determined by Coomassie protein assay (Method of Bradford) with an Ohpak SB-806 M HQ column (Shodex, Japan) was used to estimate the molecular sizes of the conjugates. Dextrins 110, 500, and 2000 (Pharmacia) were used as references.

Immunization. Outbred female mice (CD1; Charles River Laboratories) were used for all experiments unless otherwise specified. Hyperimmune Vi sera were raised in 8-week-old mice by multiple subcutaneous (s.c.) injections of formalin-inactivated S. Typhi (1, 30). DT hyperimmune sera were raised in 8-week-old mice by multiple s.c. injections of DT with incomplete Freund adjuvant (Pierce Biotechnology), as described previously (24).

For immunogenicity evaluation, 6-week-old mice (10/group) were injected twice s.c. at 4-week intervals with 100 µl of the immunogen in saline containing 2.5 µg of Vi as a conjugate or as a polysaccharide. Blood samples were collected through eye bleeding 2 weeks after each injection.

Immunogenicity of Vi-DT C1 was compared with that of Vi-eEPA (lot 13106), a clinical lot prepared at NIH. Following the NIH research protocol, 5- to 6-week-old female mice (Swiss Webster; 10/group) were injected s.c. twice 2 weeks apart with conjugates containing 2.5 µg of Vi per injection. The mice were exsanguinated 7 days after each injection. The negative controls were mice injected with 100 µl saline.

As a priming or boosting vaccine, the effect of Vi on the immunogenicity of Vi conjugate was evaluated in five groups of mice (6 weeks old; 10/group) by injecting Vi polysaccharide s.c. at the first or the second dose and was compared with those receiving 2 doses of conjugate C5. Vi and C5 were injected into mice in various sequences (two injections 4 weeks apart): Vi/C5, C5/Vi, and C5/C5. Serum samples were collected via eye bleeding 2 weeks after each injection. The control groups were those injected with C5 once or Vi alone. Serum samples were taken at 2 and 6 weeks after the injection.

Dosage study. Three groups of mice (6 weeks old; 10/group) were injected s.c. with various amounts of C4 containing 2.5 µg, 1.25 µg, and 0.5 µg of Vi, respectively. Mice were boosted with the same dosage 4 weeks later. Blood samples were taken 2 weeks after each injection via eye bleeding.

Serology. Double immunodiffusion was performed in 1% agarose in saline. Serum IgG Vi and DT antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) using Nunc Maxisorp plates as described previously (13, 48). ELISA data were processed with Program ELISA for Windows (Centers for Disease Control and Prevention) (33). IgG anti-Vi and IgG anti-DT levels were expressed in ELISA units (EU) using hyperimmune sera as references. International Vaccine Institute (IVI) anti-Vi hyperimmune serum was calibrated with NIH reference serum (100 EU) and assigned a value of 82 EU (47–50). IVI anti-DT hyperimmune serum was assigned 100 EU.

The results were tabulated as the geometric mean (GM) and 95% confidence interval (CI).

Statistical evaluation. Antibody levels were expressed as the GM of EU. Sera with antibodies below detectable levels were assigned 0.02 EU (one-half of the lowest detectable level) for the purpose of GM calculation. Statistical significance was calculated using a two-tailed Student’s t test with a confidence level of 95% (P = 0.05).

RESULTS
Vi polysaccharide. The purified Vi met WHO requirements (in parentheses) for polysaccharide typhoid vaccine: the O-acetyl content was 2.56 mmol/g of Vi (≥2.0 mmol/g of Vi), and 57.6% of Vi (≥50%) was eluted from a Sepharose CL-4B column after the Kav reached 0.25. The purity of the polysaccharide was as follows: nucleic acid content < 2.0% (<2.0%), protein content < 0.25% (<1.0%), and an endotoxin level < 0.3 EU per µg of Vi (54).

Characterization of DT Vi. The level of derivatization of DT Vi was between 2.9% and 3.5% AH/DT (wt/wt), corresponding to an AH/DT molar ratio of 10 to 12 (Table 1). Based on the 14% SDS-PAGE pattern, the molecular weight of DT Vi was similar to that of the native DT (data not shown).
Double immunodiffusion with DT antiserum showed a line of identity between DT and DT\textsubscript{AH} (Fig. 1).

**Chemical compositions of Vi-DT conjugates (Table 1).** The Sephacryl S1000 gel filtration profiles of all five lots of the conjugate reaction mixtures were similar and were exemplified by C2 in Fig. 2. One major peak was eluted from the void volume (\(K\text{av} = 0\)) and trailed through the rest of the column. Only the fractions eluted before the \(K\text{av} = 0.4\) were pooled as the final conjugate C2. Since C2 was eluted before Vi and DT\textsubscript{AH}, this indicated C2 had a higher molecular weight than the individual components. The size distribution was further confirmed by the HPLC analysis (Fig. 3, left). All five conjugates showed similar profiles, with a single major peak at 6.06 min (range, 5.04 to 7.18 min), ahead of both Vi and DT.

The Vi/DT ratios of the conjugates ranged from 0.80 to 1.09 (Table 1). The conjugates failed to enter the 14\% SDS-polyacrylamide gel, and no free protein was detected (data not shown).

The Vi/DT ratios of the conjugates ranged from 0.80 to 1.09 by weight, equivalent to 198 to 270 Vi repeating units per DT molecule. Based on the recovery of Vi, the yield was between 42\% and 52\% (Table 1).

The conjugates formed a line of identity with Vi when reacting with anti-Vi serum in immunodiffusion (data not shown). In contrast, none of the conjugates formed visible precipitation when reacting with anti-DT in immunodiffusion.

**Stability test.** Conjugate C3 underwent repeated freeze-thaw cycles 20 times and showed no visible change in solubility and immunogenicity (data not shown). The HPLC profile of the treated C3 was identical to that of C3 kept at 4\°C. In contrast, C3 stored at 25\°C or 37\°C for 5 weeks showed obvious size reduction, evident from the appearance of trailing in the HPLC profile. No dissociation of C3 was observed when it was stored at \(-20\°C\) for the same length of time (Fig. 3, right).

**Immunogenicity (Table 2).** Vi elicited 0.39 EU (GM) of serum IgG Vi antibodies. All Vi-DT conjugates elicited levels of IgG anti-Vi antibodies in mice significantly higher than those elicited by Vi alone: anti-Vi levels were about 16 and 42 times higher than those in the Vi groups after the first and second injections, respectively (\(P < 0.001\)). The IgG Vi antibody levels increased from 6.5 to 11.1 EU after the first injection and to 16.9 to 18.7 EU after the second injection (1st versus 2nd injections, \(P < 0.05\) for all conjugates except C4, \(P = 0.19\)). The GM levels of IgG Vi antibodies after the 1st and 2nd injections were similar among the 5 conjugates (\(P > 0.083\) and \(P > 0.69\), respectively).

One injection of the conjugates in mice did not elicit detectable IgG anti-DT antibodies. After the second injection, anti-DT antibodies were detected in all groups, with C3 having the highest level. Since dosages of DT varied in different groups, no statistical analysis was performed.

**Dosage effect (Table 3).** As observed in humans injected with Vi-rEPA, the serum IgG Vi antibody response was related to the dosage of Vi-DT: the higher the dosage, the higher the level of antibodies after each of the two injections (7). The group of mice that received 2.5 \(\mu\)g elicited levels of antibodies significantly higher than in those that received 0.5 \(\mu\)g after each of the two injections (\(P < 0.001\)). There was a difference between the groups that received 2.5 \(\mu\)g and those that received 1.25 \(\mu\)g after the first injection (\(P < 0.05\), but there was no difference following the second injection (\(P > 0.3\)). IgG anti-Vi levels were significantly higher in the 1.25-\(\mu\)g group than in the 0.5-\(\mu\)g group after either of the two injections (\(P < 0.002\) and \(P < 0.003\), respectively).

**The effects of Vi polysaccharide on the immunogenicity of Vi-DT (Table 4).** After one injection, as observed before, groups that received C5 had significantly higher anti-Vi levels than those that received Vi (11.32, 12.95, and 13.20 versus 0.35 and 0.20; \(P < 0.001\) (47–50)). After 2 injections, the C5/C5 group had significantly higher levels of anti-Vi than those injected with either Vi/C5 or C5/Vi (16.88 versus 6.31 and 5.81; \(P < 0.001\)). However, IgG anti-Vi levels were comparable in the C5 and Vi/C5 groups 2 weeks after C5 injection (11.32 versus 5.81; \(P = 0.12\)), indicating that previous immunization...

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**TABLE 1. Chemical compositions of S. Typhi Vi-DT conjugates**

| Conjugate | AH/DT (% wt/wt) | Molar ratio | Vi concn (\(\mu\)g/ml) | Yield\(^a\) (%) | Vi/DT ratio (wt/wt) | Vi repeating units/DT\(^b\) |
|-----------|-----------------|-------------|------------------------|---------------|--------------------|------------------------|
| C1        | 3.5             | 12          | 58.05                  | 42            | 0.80               | 198                    |
| C2        | 3.1             | 11          | 53.83                  | 48            | 1.06               | 263                    |
| C3        | 3.2             | 11          | 47.00                  | –             | 0.87               | 216                    |
| C4        | 2.9             | 10          | 50.77                  | 44            | 0.91               | 226                    |
| C5        | 3.4             | 12          | 56.04                  | 52            | 1.09               | 270                    |

\(^a\) Yield was based on the recovery of Vi.

\(^b\) Molar ratio calculated based on the following: DT, \(M_r = 62,000\); Vi repeating unit, \(M_r = 250\); ADH, \(M_r = 174\).

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**FIG. 2.** Gel filtration profiles of Vi, DT, and Vi-DT conjugate (exemplified by C2) through a 2.5-\(\times\) 95-cm column of Sephacryl S-1000 in 0.2 M NaCl (pH 7.4). Fractions (15 ml/fraction) in which \(K\text{av} \approx 0.40\) were collected as conjugates. Detector wavelength, 280 nm (milliaborbance unit).
with Vi did not impair or enhance the immune response to Vi-DT.

There was a slight increase in the IgG anti-Vi level in the C5/Vi group compared with those that received C5 only, but the difference was not statistically significant (6.31 versus 2.66; $P < 0.05$).

**Immunogenicity comparison between Vi-rEPA and Vi-DT** (Table 5). The effects of different carriers on immunogenicity were examined by comparing conjugates C1 and Vi-rEPA in mice. The polysaccharide/protein molar ratios were similar in C1 and Vi-rEPA. One week after each of the two injections, the IgG anti-Vi level induced by Vi-rEPA was higher than that elicited by C1, but the difference was not statistically significant (43.99 versus 35.01; $P < 0.05$). Both conjugates induced levels of IgG anti-Vi statistically higher than those elicited by Vi ($P < 0.001$) (Table 5). The slightly higher response in C1 (35.01 EU) than those observed in immunogenicity evaluation (18.74 EU) (Table 2) could be due to the different strains of mice used in the studies.

**DISCUSSION**

In this study, Vi was conjugated to DT, a licensed vaccine for routine infant immunization. Physical and chemical characterization of all 5 lots of Vi-DT showed similar polysaccharide/protein ratios, yields, and antigenicities. Similar levels of serum IgG Vi antibodies elicited by all Vi-DT conjugates that were significantly higher than those elicited by Vi alone were also observed. These levels were also comparable to those elicited by Vi-rEPA, a clinical lot prepared at the National Institutes of Health. These findings demonstrated that the synthesis of Vi conjugates could be reproduced with another carrier protein in a different laboratory.

Because dosage-related immunogenicity has been shown for

**FIG. 3.** HPLC profiles of Vi-DTs using refractive index detectors (milliabsorbance units). The column was a Shodex OHpak SB-806 HQ (8 by 300 mm), the eluent was 0.2 M saline (pH 7.2), and the elution speed was 1 ml/minute. (Left) All five conjugates showed similar profiles, with a single major peak at 6.06 min (spanning from 5.04 to 7.18 min, ahead of both Vi and DT). (Right) Conjugate C3 underwent repeated freeze-thaw cycles 20 times and showed a profile identical to that of C3 kept at 4°C. In contrast, C3 stored at 25°C or 37°C for 5 weeks showed obvious size reduction. No dissociation of C3 was observed when it was stored at −20°C for the same period.

**TABLE 2.** Immunogenicity evaluation in mice for S. Typhi Vi-DT conjugates

| Conjugate | Anti-Vi IgG$^a$ | Anti-DT IgG$^b$ |
|-----------|----------------|----------------|
|           | 1st (GM of IgG (95% CI) 2 wk after indicated injections) | 2nd | 1st | 2nd |
| C1        | 8.95 (4.67–17.20) | 0.02 (0.02–0.02) | 18.74 (13.28–26.42) | 0.20 (0.07–0.62) |
| C2        | 6.45 (3.95–10.53) | 0.02 (0.02–0.03) | 16.85 (10.09–28.14) | 1.89 (0.73–4.95) |
| C3        | 9.34 (5.53–15.79) | 0.02 (0.02–0.02) | 17.39 (13.62–22.20) | 0.04 (0.02–0.12) |
| C4        | 11.09 (7.06–17.41) | 0.03 (0.02–0.07) | 17.38 (9.57–31.55) | 0.73 (0.28–1.87) |
| C5        | 10.24 (6.73–15.56) | 0.02 (0.02–0.03) | 18.05 (12.14–26.82) | 0.63 (0.14–2.81) |
| Vi        | 0.39 (0.24–0.64) | ND | 0.40 (0.15–1.07) | ND |
| DT        | ND$^d$ | 0.02 (0.02–0.02) | ND | 0.44 (0.21–0.95) |
| Saline    | 0.02 (0.02–0.02) | ND | ND | ND |

$^a$ Female mice (6 weeks old; 10/group) were injected s.c. twice, 4 weeks apart, with 2.5 µg of Vi as a conjugate or Vi alone per injection. Serum samples were taken 2 weeks after each injection. IgG anti-Vi is expressed as EU with respect to reference Vi and DT sera.

$^b$ All conjugates versus the Vi group (for all injections), $P < 0.001$; within each conjugate group, 1st versus 2nd injection, $C1$, C2, C3, and C5, $P < 0.05$; C4, $P = 0.19$. There was no statistical difference among conjugates after the 1st and 2nd injections ($P > 0.083$ and $P > 0.69$, respectively).

$^c$ Conjugates induced anti-DT responses in mice similar to those with DT. Dosages of DT varied in different groups, so no statistical analysis was performed.

$^d$ ND, not done.
other polysaccharide conjugates, such as Haemophilus influenzae type b and S. pneumoniae, here we tested various dosages of Vi-DT in mice (7, 8, 23, 48–50). Within the range of dosages studied here, the higher the dosage, the higher the level of anti-Vi elicited. This dosage dependence was also consistent with results observed in the clinical study of Vi-rEPA in 2- to 5-year-old children (7, 26).

The Vi-DT conjugates did not form a line of precipitation with the anti-DT serum in immunodiffusion. This could be due to the distinct physical conformations of the two molecules: the compact globular conformation of DT as opposed to the long unstructured form of Vi (Vi molecular mass, >1,000 kDa). It is probable that Vi exerted “shielding” effects on the antigenic sites of DT. However, the immunogenicity of DT was retained, as evidenced by the induction of anti-DT in mice.

In our study, two different sources of DT were used to prepare Vi-DT, and there was no significant difference among the conjugates prepared. Utilizing DT as a carrier protein has the following advantages: (i) both DT and Vi are licensed vaccines, and the safety concerns are minimal; (ii) both vaccines are already produced locally in many developing countries, so no new facilities would be required for raw material preparation, and the cost of production could be lower; (iii) anti-DT elicited by Vi-DT bears clinical advantages; and (iv) not introducing a new carrier protein, such as rEPA, will potentially simplify Vi conjugate licensing. Finally, the production of DT is economical due to its unusually high yield from fermentation.

Recently, a locally produced Vi conjugate using TT as a carrier protein was licensed in India (45). Several other manufacturers in developing countries have proceeded to perform clinic trials and are in the process of licensing Vi conjugates (personal communications). We evaluated the effects of priming and/or boosting of Vi polysaccharide on conjugates by alternate immunization. Our results showed that injecting conjugates twice induced the highest level of anti-Vi; replacing either injection with Vi significantly lowered the level of antibody elicited. However, priming with Vi did not weaken or enhance the immune response to Vi-DT. Other reasons for such trials were to explore alternative immunization schedules and formulations. There were several attempts to study the immunological memory induced by polysaccharide on the conjugate, or vice versa (3, 4, 6, 9, 31, 40, 41). For certain types of pneumococcus, there were enhancements of immune response in groups primed with the conjugate vaccines and benefits to the subsequent injection of polysaccharide (31, 40). This phenomenon was not obvious in the cases of meningococcal or Haemophilus influenzae type b conjugates, where the best immune response still came from using the conjugate vaccine for the full course of immunization (3, 4, 6, 9, 41).

Vi-DT and Vi-rEPA elicited similar levels of anti-Vi IgG in mice. Vi-rEPA was demonstrated to be safe and efficacious in 2- to 5-year-old children in an area of high endemicity for typhoid fever (23, 26). Since the mouse has been shown to be a reliable animal model for evaluation of the immune responses of polysaccharide-protein conjugates, we predict that Vi-DT is as immunogenic and protective as Vi-rEPA. We plan to perform clinical evaluation of the safety and immunogenicity of Vi-DT when added to the infant combination vaccines.

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### TABLE 3. Comparison of immune responses in mice injected with various dosages of Vi-DT C4

| Dose       | GM of IgG anti-Vi (95% CI), 2 wk after indicated injectiona |
|------------|-------------------------------------------------------------|
|            | 1st                                      | 2nd                                      |
| Vi-DT C4   |                                           |                                          |
| 2.5 µg     | 13.24 (9.36–18.73)                         | 24.04 (11.07–52.19)                     |
| 1.25 µg    | 8.81 (6.84–11.35)                          | 17.65 (8.89–35.04)                      |
| 0.5 µg     | 4.36 (2.12–5.11)                           | 4.33 (1.83–5.88)                        |
| Saline     | 0.02 (0.02–0.02)                            | ND                                       |

* Female mice (6 weeks old; 10/group) were injected s.c. twice, 2 weeks apart, with 2.5 µg of Vi as conjugates or Vi alone per injection. The mice were exsanguinated 7 days after each injection. The sera were assayed for IgG anti-Vi by ELISA.

### TABLE 4. Effects of Vi upon immunogenicity of Vi-DT conjugate

| Conjugate | GM of anti-Vi IgG (95% CI), 2 wk after indicated injectionb |
|-----------|-------------------------------------------------------------|
|           | 1st                                      | 2nd                                      |
| C5        |                                           |                                          |
|          | 11.32 (6.31–20.34)                           | 2.66 (1.12–6.34)                        |
| C5/Vi     | 12.95 (8.31–20.16)                           | 6.31 (3.20–12.46)                       |
| C5/C5     | 13.20 (9.35–18.64)                           | 16.88 (14.55–19.60)                     |
| Vi/C5     | 0.35 (0.19–0.66)                             | 5.81 (2.83–11.94)                       |
| Vi        | 0.20 (0.06–0.60)                             | ND                                       |
| Saline    | 0.02 (0.02–0.02)                             | ND                                       |

* Female mice (6 weeks old; 10/group) were injected s.c. twice, 4 weeks apart, with 2.5 µg of Vi alone or as a conjugate per injection. Serum samples were collected 2 weeks after each injection. For group C5 and group Vi, blood samples were collected 2 and 6 weeks after the injection.

### TABLE 5. Immunogenicity comparison of Vi-DT C1 and clinical lot of Vi-rEPA (lot 13106)c

| Conjugate       | GM of anti-Vi IgG (95% CI), 1 wk after indicated injection |
|-----------------|-----------------------------------------------------------|
|                 | 1st                                      | 2nd                                      |
| Vi-DT C1        |                                           |                                          |
|                 | ND                                       | 35.01 (15.34–79.91)                      |
| Vi-rEPA         | 0.64 (0.26–1.56)                             | 43.99 (30.09–64.32)                     |
| Vi              | 0.37 (0.14–1.03)                             | ND                                       |
| Saline          | 0.02 (0.02–0.02)                             | ND                                       |

* Female mice (5 to 6 weeks old; 10/group) were injected s.c. twice, 2 weeks apart, with 2.5 µg of Vi as conjugates or Vi alone per injection. The mice were exsanguinated 7 days after each injection. The sera were assayed for IgG anti-Vi by ELISA, using the NIH hyperimmune serum as a reference (100 EU).

b 35.01 versus 43.99, P = 0.58.

c 35.01 and 43.99 versus 0.37, P < 0.001.

d ND, not done.
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