

Vi CAPSULAR POLYSACCHARIDE-PROTEIN CONJUGATES FOR PREVENTION OF TYPHOID FEVER

Preparation, Characterization, and Immunogenicity in Laboratory Animals

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Enteric fevers continue to cause considerable morbidity and mortality in nations that have not yet achieved control of drinking water and sewage disposal (1, 2). In these countries, the most frequent and serious cause of enteric fevers is *Salmonella typhi* (typhoid fever) (2). Immunoprophylaxis against typhoid fever on a world-wide basis has not been attempted because the two available vaccines have limitations; (a) cellular typhoid vaccines induce only a limited immunity and elicit side reactions that are frequent and severe enough to have discouraged their widespread acceptance (2, 3); and (b) an orally administered attenuated strain of *S. typhi*, Ty-21a, requires three to four doses to induce about 65% protection, it is expensive, and its mode of protection has not been identified, which has prevented precise standardization of the vaccine (1, 4).

Recently, two clinical evaluations in populations with high rates of typhoid fever (~1%/annum) have provided evidence that immunization with the capsular polysaccharide (CPS)¹ of *S. typhi* (Vi) confers immunity against typhoid fever (5, 6). The Vi vaccine, prepared under conditions that did not change its structure, elicited a fourfold or greater rise in serum antibodies in ~75% of children and adults in Nepal and in school children in the Eastern Transvaal, Republic of South Africa (2, 7, 8). The protective efficacy of the Vi in these two trials was ~70%. In contrast, the same Vi elicited a ≥ 4 -fold antibody rise in 97% of young adults in France and the United States (9). The seroconversion rate and efficacy of other CPS, e.g., meningococcal vaccines, were also lower in Africa than in Finland or the United States (10–12). This lesser immunogenicity and efficacy of meningococcal vaccines was attributed to the high burden of infections,

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¹*Abbreviations used in this paper:* Vi, Vi capsular polysaccharide; CPS, capsular polysaccharide; SPDP, *N*-succinimidyl 3-(2-pyridyldithio) propionate; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; TT, tetanus toxoid; DT, diphtheria toxoid; CT, cholera toxin; RIA, radioimmunoassay; NMR, nuclear magnetic resonance; FTIR, Fourier-transformed infrared spectroscopy.

including malaria, in the African population. Since the protective response elicited by CPS vaccines is serum antibodies, it could be predicted that a more immunogenic Vi would be more protective against typhoid fever in high-risk populations.

Originally, Goebel and Avery (13, 14) showed that the immunogenicity of pneumococcus type 3 polysaccharide could be increased by binding it chemically to a carrier protein. This principle has been applied successfully to increase the immunogenicity of CPS of other pathogens (15–23). We developed methods for synthesizing covalent bonds between the Vi and proteins to both increase the immunogenicity of and to confer the property of T-dependence to this antigen. Vi, a linear homopolymer of $-4\text{-D-}\alpha\text{-NacGalA-(1-}$ is *O*-acetylated up to 90% at C3 (24). The scheme used to prepare these conjugates used the carboxyl function of the NacGalA to form a thiol derivative. This thiol derivative was combined with proteins derivatized by the thiol-active compound, *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (25). The standardization and immunologic properties of several model Vi-protein conjugates formed by this method are reported here.

Materials and Methods

Vi CPS (Vi). The Vi used for the conjugates was prepared from *Citrobacter freundii*, strain WR 7011, kindly given to us by Dr. Lewis Baron, Walter Reed Army Institute of Research, Washington, DC. *C. freundii* was cultivated in medium containing yeast extract dialysate as described (3). Vi was precipitated from the culture supernatant with 1% hexadecyltrimethyl ammonium bromide (Sigma Chemical Co., St. Louis, MO) and sequentially treated with DNase, RNase, and then pronase (19, 27, 28). The enzyme-treated product was purified with cold phenol and the LPS was removed by centrifugation at 60,000 *g*, 10°C, for 5 h (10). The Vi was dialyzed exhaustively against pyrogen-free water and freeze-dried. Vi from *S. typhi* was prepared by a modification of this method by the Institut Merieux, Lyon, France (9). The final products contained <1% protein or nucleic acid (26) and <0.01% LPS as measured by SDS-PAGE (29). The molecular size of the Vi preparations was heterogeneous; the main peak had a molecular mass 5×10^5 kD as estimated by gel filtration through Sephacryl S-1000 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 0.2 M NaCl. A lower molecular size Vi, ~ 65 kD was prepared by ultrasonic irradiation (30).

Proteins. BSA (Sigma Chemical Co.) and cholera toxin (CT, Lot 582; Institut Merieux) were used without further purification. Tetanus toxoid (TT; Institut Merieux) and diphtheria toxoid (DT; Rijks Instituut voor Volksgezondheid, Bilthoven, Netherlands) were further purified by gel filtration through Sephacryl S-300 (Pharmacia Fine Chemicals) equilibrated in 0.2 M NaCl (17, 22). The fractions, corresponding to the molecular weight of the two toxoids, were concentrated by ultrafiltration and passed through 0.45- μm filters (Millipore Co., Bedford, MA). CT was additionally characterized for toxicity by the Chinese hamster ovary (CHO) cell assay and by the intradermal rabbit skin test (kindly performed by Dr. John Craig, State University of New York, Brooklyn, NY) (31).

Direct Binding of Vi to Proteins with EDAC (Vi-DT₁). Vi was bound to DT by the method of Beuvery et al. (16). Equal volumes of Vi and DT, containing 10 mg/ml each, were mixed, the pH was adjusted to 5.0 with 0.1 N HCl, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Bio-Rad Laboratories, Richmond, CA) was added to a final concentration of 0.1 M. The pH was maintained at 5.0 by addition of 0.1 N HCl for 3 h at room temperature. The reaction mixture was stirred at 3–8°C for an additional 24 h, dialyzed against 0.2 M NaCl for 48 h, and then centrifuged at 10,000 *g*, for 2 h, at 3–8°C. The supernatant fluid was subjected to gel filtration through 4B-Sepharose equilibrated in 0.2 M NaCl. The fractions were monitored by refractometry and by absorption

at 2,800 Å. The void-volume fractions, which contained the Vi-DT₁ conjugate, were pooled, made 0.01% in thimerosal (Eli Lilly and Co., Indianapolis, IN), and stored at 3–8°C.

Thiolation of Vi with Cystamine. Vi, 10 mg/ml in 0.2 M NaCl, was allowed to dissolve overnight at 3–8°C. Cystamine or cysteamine (Calbiochem-Behring Corp., La Jolla, CA) at twice the weight of the Vi was then added as a powder to the Vi solution. The pH of the reaction mixture was adjusted to 4.9 with 0.1 N HCl and the temperature was maintained at 37°C. EDAC, in an amount equal to the weight of the Vi, was added to the reaction mixture with stirring. The pH was maintained at about 4.9 by the addition of 0.1 N HCl until stable. The reaction mixture was then dialyzed exhaustively against distilled water at 3–8°C and freeze-dried. The extent of thiolation was estimated by the iodoplatinate assay (32) using cystamine as the reference. The thiol ester content was quantitated after reduction of the Vi derivative with DTT (Sigma Chemical Co.) and passage of the reaction mixture through Bio-Gel P10 (Bio-Rad Laboratories) to remove the low molecular weight materials. The material eluted in the void volume was then assayed for its SH content using cysteamine as a standard (33).

Derivatization of Proteins with SPDP. Protein solutions were made within a range of 5–20 mg/ml. Free SH groups were blocked by treatment with 0.01 M iodoacetic acid (Sigma Chemical Co.). The reaction mixture, containing the iodoacetic acid and the protein, was incubated at room temperature for 1 h and then equilibrated against 0.15 M Hepes (Boehringer Mannheim Biochemicals, Indianapolis, IN), 5 mM EDTA, pH 7.55 (HE buffer) by dialysis overnight at 3–8°C. SPDP (Pierce Chemical Co., Rockford, IL), 20 mM in 99.5% ethanol, was added to the protein solution with stirring (25). The final molar ratio of SPDP to protein ranged from 5–25 depending upon the extent of derivatization desired. The reaction was allowed to proceed for 1 h at room temperature. The excess reagent was removed by dialysis against HE buffer overnight at 3–8°C followed by gel filtration through Bio-Gel P10 in HE buffer. The void volume was pooled, concentrated to 5–10 mg/ml, and stored at 3–8°C. The molar ratios of 2-pyridyl disulphide in the derivatized protein were determined by reducing the disulfide bond in 50 mM DTT and calculating the amount of pyridine-2-thione released by using the extinction coefficient for pyridine of 8.08×10^3 /mol/cm at 3,430 Å (23, 25).

Synthesis of Vi-Protein Conjugates. The cystamine derivative of Vi was dissolved in HE buffer, at 5–10 mg/ml. DTT was added to a final concentration of 100 mM and stirred for 1 h at room temperature. After dialysis against HE buffer for 2 h, the reaction mixture was then passed through a Bio-Gel P10 column, equilibrated with HE buffer and the void volume peak was concentrated by ultrafiltration (Amicon Corp., Danvers, MA; molecular weight cutoff >5,000) under N₂ pressure. SPDP-derivatized protein was added to the reduced Vi derivative to achieve an equimolar ratio of *N*-pyridyl disulfide groups and SH groups. The reaction mixture was flushed with N₂ and allowed to incubate at room temperature for 1 h, and then at 3–8°C for 24 h. The release of pyridine during the reaction was measured by the change in OD 3,430 Å. The reaction mixture was concentrated by ultrafiltration (see above) and subjected to gel filtration through Sephacryl S-1000 in 0.2 M NaCl at room temperature. The void volume fractions were pooled, concentrated by ultrafiltration, dialyzed against 0.15 M NaCl, 0.01% thimerosal (Eli Lilly and Co.), 1 mM EDTA, pH 7.0, and stored at 3–8°C. The protein concentration of the conjugate was determined by the Coomassie Blue assay (Bio-Rad Laboratories) using the SPDP-derivatized protein as the standard (34). The concentration of protein and Vi in the conjugates were also determined by Fourier transformed infrared spectroscopy (FTIR) and by spectrophotometric titration by acridine orange (35).

Pneumococcus Type 6B-TT Conjugate (Pn6B-TT). Pneumococcus type 6B CPS, (Institut Merieux), was derivatized with adipic acid dihydrazide and bound to TT as described (17, 20, 22). The protein/polysaccharide ratio of this preparation was 2.8.

Immunization of Mice. Female, weanling, BALB/c mice, 16–20 g, were injected subcutaneously with 0.1 ml of either Vi, Vi conjugates or saline 1, 2, or 3 times at 2 wk apart. Mice from each experimental group were exsanguinated 10 d after each injection. Alum-adsorbed Vi-CT_{XII} was prepared with Alhydrogel (Superfos; Kemi A/S, Copenhagen,

Denmark). The Alhydrogel was centrifuged and Vi-CT_{XII} was added to the pellet to achieve a final concentration of 0.5 mg aluminium/ml and 5.0 μ g of Vi as a suspension. This mixture was tumbled overnight at room temperature and then stored at 3–8°C.

Immunization of Primates. Juvenile Rhesus monkeys, housed at the Division of Product Quality Control, Office of Biologics Research and Review, FDA, were injected subcutaneously twice, 1-mo apart with 0.5 ml of Vi or Vi-CT_{XII} containing 25 μ g of Vi. Controls were injected with Pn6B-TT containing 15 μ g of CPS (20). The monkeys were bled before and 3 wk after each injection.

Immunological Methods. Serum Vi antibodies were measured by RIA and expressed as μ g antibody/ml (9). The differences between the levels of antibodies in experimental groups were calculated by Fisher's exact *t*-test. The results were tabulated as the geometric mean and 80% confidence limits. Antibodies to CT were measured by ELISA (36). Rabbit anti-BSA serum was obtained from Cappel Laboratories, Cochranville, PA. Hyperimmune burro antiserum, (B-260) containing 660 μ g Vi antibody/ml, was prepared by multiple intravenous injections of formalin-fixed *S. typhi* Ty2 as described (37). The preparation and characterization of burro 241, hyperimmune CT antiserum, containing 16.5 mg antibody/ml, has been reported (38). Rocket immunoelectrophoresis and immunodiffusion were performed as described (23).

FTIR Spectroscopy. The composition of the conjugates was determined using the Vi and proteins as references. 1 mg of Vi, carrier protein, or Vi conjugate was added to 100 mg of KBr, and dissolved in 2.0 ml distilled water. The sample was freeze-dried and pressed into a pellet. FTIR spectra were recorded on a Nicolet 7199 spectrometer and analyzed as described (35).

[¹³C]Nuclear Magnetic Resonance (NMR). [¹³C]NMR spectra of the Vi (20 mg/ml D₂O) were recorded at 60°C in a Nicolet 270 spectrometer. A 5.0-mm sample cell was used and the spectrometer was operated at 67.9 MHz in the pulse Fourier-transformed mode with complete proton decoupling and quadrature phase detection. A 4-Hz line broadening was applied to the signal before Fourier transformation to enhance the signal-to-noise ratio.

SDS-PAGE. The molecular weights of the proteins and their Vi conjugates were assayed in 7.5% polyacrylamide gels (23). Samples containing 5–10 μ g of protein with or without 2-ME were electrophoresed concurrently with protein standards (Pharmacia Fine Chemicals). The gels were stained with Coomassie Blue (Sigma Chemical Co.).

Results

Vi Antigens. The Vi from *S. typhi* and some strains of *C. freundii* is a linear homopolymer of NacGalA acid, variably *O*-acetylated at the C3 position (24). FTIR and [¹³C]NMR spectroscopy showed that the structures of the Vi from the WR 7011 strain of *C. freundii* and the Ty2 strain of *S. typhi* were indistinguishable (39, 40) (Fig. 1). Immunodiffusion analyses showed a reaction of identity between Vi polysaccharides from *C. freundii* and *S. typhi* when reacted with B-260 anti-Vi antiserum (data not shown). The Vi used for immunization and for synthesis of the conjugates in these experiments contained about 80% *O*-acetyl per mole repeating unit as determined by [¹³C]NMR (Fig. 2). Since the Vi has no vicinal hydroxyl groups, the carboxyl served as an activation site in the strategy for the conjugation reaction.

Direct Binding of Vi with Proteins Using EDAC. The first Vi conjugates were prepared with EDAC, which catalyzed the direct conjugation between the carboxyls of Vi and the amino groups of DT (16). The yield from this reaction, illustrated by a representative product Vi-DT₁, was ~3% of the starting materials. Vi antibody levels elicited in mice by this conjugate and by the two controls, Vi and saline, are given in Table I.

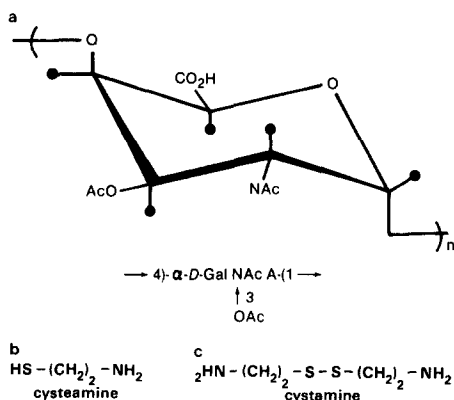


FIGURE 1. (a) Structure of the repeating unit of the Vi capsular polysaccharide of *Salmonella typhi*. (b) Structure of cysteamine. (c) Structure of cystamine.

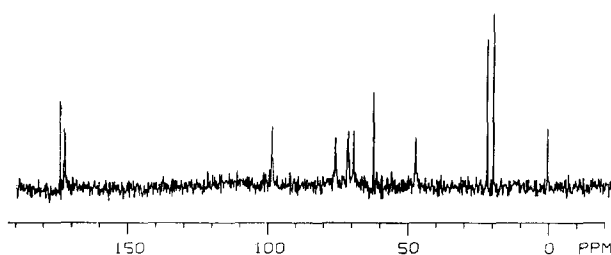


FIGURE 2. ^{13}C NMR chemical shift of Vi depolymerized by ultrasonic irradiation (65,000 mol wt, 20 mg/ml concentration), downfield from ^{15}C -enriched acetonitrile used as an internal standard. The spectra were recorded for a D_2O solution in a 5-mm tube with a Nicolet 270 spectrometer operating at 67.89 MHz in pulse Fourier-transformed mode.

TABLE I
Serum Antibody Responses of Female BALB/c Mice Injected with Vi or Vi-DT Conjugate (Vi-DT₁) Prepared with EDAC as the coupling reagent

Vaccine	Dose	Vi antibody			
		1st	2nd	3rd	
	μg	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
Vi	0.5	0.19* (0.05-0.72)	0.61 (0.30-1.23)	0.47	(0.19-1.15)
	5.0	0.56 (0.28-1.11)	0.43 [‡] (0.13-2.33)	1.11	(0.29-4.26)
	50.0	0.31 [§] (0.13-0.75)	0.24 [¶] (0.04-1.47)	0.20 [¶]	(0.04-0.97)
Vi-DT ₁	0.5	0.07** (0.04-1.12)	0.31 (0.06-1.52)	0.79	(0.28-2.24)
	5.0	0.63 ^{‡‡} (0.30-1.30)	2.79 ^{¶¶} (0.65-12.0)	2.85 ^{¶¶}	(1.09-7.46)
	50.0	1.40 ^{¶¶} (0.80-2.45)	2.65 ^{¶¶} (0.69-10.1)	3.00 ^{¶¶¶}	(1.39-6.46)

Mice were injected subcutaneously 2 wk apart with 0.1 ml of each vaccine or saline. There were eight mice for each experimental group. 10 d after each injection mice were exsanguinated and their sera were assayed for Vi antibodies by RIA (9). Results are expressed as the geometric mean and 80% confidence limits.

* vs. **: $p = 0.0014$, ** vs. ^{‡‡} ^{¶¶}: $p = 0.001$, [§] vs. ^{¶¶}, [‡] vs. ^{¶¶}: $p = 0.001$, [¶] vs. ^{¶¶}, [¶] vs. ^{¶¶¶}: $p = 0.0001$, ^{‡‡} vs. ^{¶¶}: $p = 0.003$.

There were no Vi antibodies in preimmune sera or from mice injected with saline (controls) in this and subsequent experiments. Dosages of 0.5, 5.0, or 50.0 μg of Vi elicited similar levels of antibodies after the first, second, and third injections; the slightly higher levels of antibodies in the group injected with 5.0 μg of Vi after the third injection were not statistically different. The levels of antibodies after the second and third injections of 5.0 or 50.0 μg of Vi-DT₁ were

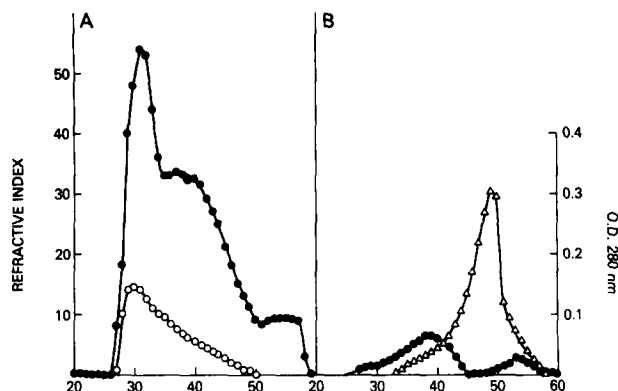


FIGURE 3. Gel filtration profile of Vi-CT_{xII} and its components, Vi and CT, through S1000 Sephacryl equilibrated in 0.2 M NaCl. (A) Conjugate. (●) refractive index, (○) OD 2,800 Å. (B) Vi-cystamine. (●) refractive index, and CT, (Δ) OD 2,800 Å.

about sevenfold higher than those elicited by the Vi alone ($p = 0.001$). Each of the three dosages of Vi-DT_I elicited a booster response after the second injection. No differences in the levels of antibodies elicited by the 5.0- and 50.0- μg doses of Vi-DT_I were observed; both of these doses were more immunogenic than 0.5 μg of this conjugate after the first injection only ($p = 0.001$). Based upon these data, the reports of Gaines et al. (41) and Landy (42), and our experience with other conjugates (17, 19), all mice were immunized with 2.5 μg of the Vi, alone or as a component of a conjugate, in ensuing experiments.

Vi-Protein Conjugates with SPDP. An alternate conjugation procedure, which covalently bound thiolated derivatives of the Vi- and the SPDP-derivatized proteins, was studied in order to increase the yield and immunogenicity of the Vi conjugates.

Introduction of thiol groups onto the Vi was attempted by forming amide bonds between the amino groups of cysteamine (Fig. 1*b*) and the carboxyls of the Vi in the presence of EDAC. The yield of thiol added to the Vi by this scheme was <1% wt/wt, probably because the SH groups of cysteamine were oxidized. To avoid this possibility, cystamine was used, the thiols of which are linked by disulphide bonds and are thus protected from oxidation (Fig. 1*c*). Using EDAC to catalyze amide bond formation, ~6% wt/wt of cystamine was bound to the Vi. After reduction of the disulphide bonds by DTT, the yield of thiol esters was 0.5–2% of the repeating monosaccharide. The freeze-dried cystamine derivative of the Vi was stable at -20°C .

Physicochemical Characterization of the Vi Conjugates. The disulfide bonds of the Vi-cystamine derivative were reduced with DTT before the conjugation reaction. The molecular size of a representative conjugate, Vi-CT_{VIII} (<0.1 kD) was larger than that of the proteins (0.64 kD) or the Vi (0.36 kD), as illustrated by the gel filtration profile (Fig. 3). The composition of the thiolated Vi, the SPDP-protein derivatives, and the conjugates used in these studies are listed in Table II. The change in the molecular size of the intermediate and final products of the conjugation reaction was further analyzed by SDS-PAGE (Fig. 4). The BSA-SPDP derivative exhibited a pattern similar to that of native BSA, demonstrating that aggregation did not occur during the reaction with SPDP. The Vi-BSA_{IV}, as did all the Vi conjugates, failed to enter the gel, probably due to its

TABLE II
 Characterization of Vi-Protein Conjugates Prepared with *N*-Succinimidyl 3-(2-pyridylthio) Propionate (SPDP)

Conjugate	Yield*	SH/Vi	SPDP/protein		Protein/Vi
		% wt/wt	mol/mol	wt/wt	wt/wt
Vi-BSA _{IV}	63.0	1.2	17.0	0.078	0.7
Vi-CT _{VIII}	5.4	1.0	5.4	0.019	0.4
Vi-CT _{XII}	18.0	0.5	4.5	0.016	1.5
Vi-DT _{XV}	14.9	0.5	5.0	0.024	1.6
Vi-DT _{XX}	16.8	0.4	3.8	0.018	1.5
Vi-TT _{XXV}	6.2	0.7	12.0	0.025	1.4

* Percent of Vi recovered in conjugate.

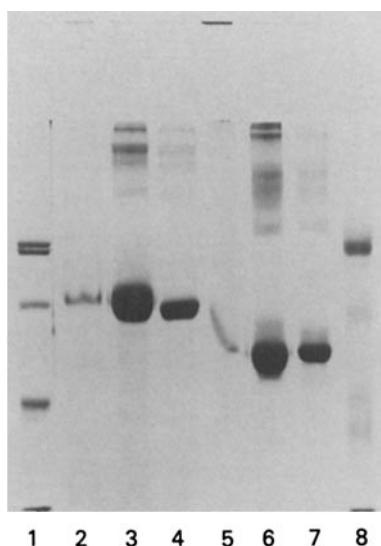


FIGURE 4. Polyacrylamide gel (7.5%) electrophoresis pattern of BSA and its conjugate with Vi. (Lanes 1-4) The migration pattern of samples reduced by 2-ME, (5 to 8) samples not treated by 2-ME. (2 and 5) Vi-BSA_{IV}, (3 and 6) BSA-SPDP, (4 and 7) BSA, (1 and 8) protein standards.

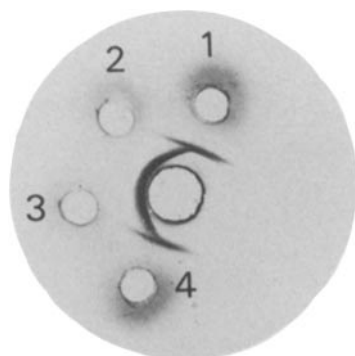


FIGURE 5. Immunodiffusion pattern of Vi-BSA_{IV} conjugate reacting with various antisera in 1% agarose gel. The center well contained 15 μ l of Vi-BSA_{IV} (0.5 mg/ml). Wells 1 and 4, Burro 260 anti-*S. typhi* Ty2 antiserum; wells 2 and 3, rabbit anti-BSA antiserum.

large size. Vi-BSA_{IV}, reduced with 2-ME, exhibited a band similar to the native BSA, indicating that the Vi was bound to this protein by a disulphide bond.

Antigenic Analysis. The Vi conjugates were analyzed by immunodiffusion with antisera to each of its components (Fig. 5). The anti-Vi and anti-BSA sera formed

TABLE III
Serum Antibody Responses of Mice Injected with the Vi or Vi Conjugates with BSA, CT, DT or TT Crosslinked with SPDP

Immunogen	n	Antibody*		
		1st injection	2nd injection	3rd injection
		$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
Vi	7	0.56 [‡] (0.22-1.43)	ND	ND
Vi-BSA _{IV}	7	2.86 [§] (1.25-6.53)	ND	ND
Vi-CT _{VIII}	4	1.35 [¶] (0.27-6.71)	4.53 (2.74-7.46)	ND
Vi-CT _{XII}	10	2.12 [¶] (1.29-3.47)	9.04 ^{**} (4.84-16.9)	6.28 ^{**} (1.98-19.9)
Vi-CT _{XII} ^{§§}	10	1.01 ^{¶¶} (0.57-1.79)	1.16 (0.54-2.53)	0.87 (0.37-2.07)
Vi-CT UD ^{¶¶}	10	2.94 ^{***} (1.55-5.55)	4.23 (1.75-10.2)	ND
Vi-DT _{XV}	10	2.57 ^{¶¶¶} (1.27-5.17)	4.61 (2.49-8.52)	ND
Vi-DT _{XX}	10	1.76 ^{§§§} (0.92-3.38)	1.26 (0.63-2.52)	ND
Vi-TT _{XXV}	9	2.56 ^{¶¶¶} (1.40-4.69)	3.15 (1.71-5.80)	4.18 (2.74-6.36)

Female, 16-20 g, BALB/c mice were injected subcutaneously with 0.2 ml containing 2.5 μg Vi or Vi conjugate 2 wk apart. Mice of each group were exsanguinated 10 d after their last injection and Vi antibodies were measured by RIA (59).

* Numbers indicate geometric mean (80% confidence limits).

[‡] vs. [§], [¶], ^{¶¶}, ^{¶¶¶}, ^{§§}, ^{¶¶¶}: $p = 0.001$; [‡] vs. [¶]: $p = 0.002$; [‡] vs. ^{¶¶}: $p = 0.04$; ^{**} vs. [¶]: $p = 0.0003$; ^{**} vs. ^{¶¶}: $p = 0.003$.

^{§§} Adsorbed onto aluminium hydroxide, 0.5 mg Al^{3+} /ml.

^{¶¶} Molecular size of Vi reduced to $\sim 65,000$ by ultrasonic irradiation (30).

a partial identity reaction with the conjugate indicating that the Vi and Vi-BSA were covalently bound. The Vi antiserum reacted with the Vi and Vi-BSA_{IV} with a partial identity reaction; a spur of precipitation overrode the Vi line on to the reaction of the antiserum with the Vi-BSA_{IV} (not shown). Conjugates prepared with DT, TT, and CT failed to react with their homologous antiprotein antisera by immunodiffusion but did precipitate with these antisera by rocket immunoelectrophoresis (not shown).

Residual Toxicity of Vi-CT_{XII} Conjugate. The toxicity of CT toxin was reduced by its conjugation to Vi. A 10^4 -fold reduction was observed in the CHO cell assay. The skin test in rabbits however, showed only a 10^3 -fold reduction in toxicity. Although injection with 0.5 ml of Vi-CT_{XII} (one human dose) had no effect on mice, injection of 5.0 ml i.p. of Vi-CT_{XII} resulted in the death of two of three guinea pigs. This conjugate, therefore, did not pass the safety requirements of the Code of Federal Regulations (CFR 600:16).

Immunogenicity in Laboratory Mice. None of the preimmunization sera or sera from mice injected with saline had detectable levels of Vi antibodies (Table III). All of the conjugates elicited higher levels of antibodies than the Vi alone ($p = 0.001$). The highest level of antibodies after the first injection were elicited by the Vi-CT prepared from the Vi depolymerized by ultrasonic irradiation, but these differences were not statistically significant. Booster responses after the second injection, as defined by a fourfold or greater increase in the geometric mean antibody levels, were observed in the animals injected with all the conjugates except the Vi-CT adsorbed. Vi-TT_{XXV} also elicited an increase in antibodies after the third injection. The levels in this group however, were similar to those observed with the other conjugates after two injections. The poorest response

TABLE IV
 Serum Antibody Responses of Juvenile Rhesus Monkeys Immunized with Vi, Vi Conjugated to CT (Vi-CT_{XII}), or Pneumococcus Polysaccharide type 6B Conjugated to TT (Pn6B-TT)

Vaccine	n	Antibody*		
		Pre-immune	1st injection	2nd injection
		$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
Vi	6	0.07 (0.05-0.10)	0.23 [‡] (0.06-0.82)	0.07 [§] (0.05-0.09)
Vi-CT _{XII}	8	0.07 [‡] (0.03-0.19)	0.93 [‡] (0.24-3.55)	3.65** (0.72-18.5)
Pn6B-TT	8	0.08 (0.06-0.11)	0.10 (0.05-0.21)	0.10 (0.04-0.23)

* Geometric mean (80% confidence limits). Measured by RIA (9). [‡] vs. [‡]: $p = 0.004$, [‡] vs. **: 0.02, [‡] vs. **: $p = 0.0003$, [‡] vs. [‡]: $p = 0.009$, ** vs. [§]: $p = 0.001$. Monkeys were immunized subcutaneously with 0.5 ml containing either 25 μg of Vi or 15 μg of Pn6B-TT at 3 wk intervals. The monkeys were bled before each injection and 3 wk after the last injection.

was elicited by Vi-CT_{XII} adsorbed; this preparation elicited the lowest levels of antibodies after the first injection and there were no booster responses after the second or third injections. There were differences in the immunogenicity of conjugates prepared with the same components. The first injection of Vi-CT_{VIII} and Vi-CT_{XII} induced an ~30-fold increase in CT antibodies, which also rose fourfold after the second and third injections (data not shown). Vi-CT_{XII} elicited higher levels of CT antibodies, as well as Vi antibodies, than Vi-CT_{VIII}.

Juvenile Rhesus. A single injection of Vi elicited Vi antibodies in five of the six monkeys ($p = 0.004$) (Table IV). The levels of Vi antibodies declined in all six monkeys after the second injection of the Vi. Seven of eight monkeys injected with Vi-CT_{XII} responded with a ~20-fold increase in antibodies after the first injection. The one nonresponding monkey had a preimmune level of 0.35 μg antibody per milliliter. The second injection of the conjugate in this group elicited about a threefold increase over that induced by the first injection of Vi-CT_{XII} ($p = 0.02$) and ~60 times the preimmunization level ($p = 0.0003$). The monkey that did not respond to the first injection of Vi-CT_{XII} had a twofold increase in antibodies after the second injection. No change in Vi antibodies was observed after injection of the Pn6B-TT (control).

Discussion

The pathogenic and protective roles of the Vi in typhoid fever have been controversial (1, 2, 4, 7, 41-43). There are data that the Vi exerts the same "shielding" effect upon *S. typhi* as do the CPS of the encapsulated bacterial pathogens (2, 44-47). Now there is clinical evidence that antibodies elicited by Vi confer immunity against typhoid fever (5, 6). The pathogenic and protective roles of the Vi are therefore, similar to the CPS of other encapsulated bacterial pathogens (47, 48).

The main, if not the sole, protective immune response elicited by CPS is serum antibodies. Both the seroconversion rates and postimmunization levels of antibodies induced by CPS have been correlated with their protective actions. Serum antibody responses induced by Vi in habitants of areas where typhoid fever is endemic, where there is also a high rate of malnutrition and other acute and chronic infections diseases, are less than optimal (2, 5, 10-12). Accordingly, we

covalently bound the Vi to T-dependent proteins both to increase its immunogenicity and to confer upon it the properties of T-dependency (19, 21, 49). This synthesis offers several advantages, namely: (a) there is no crosslinking of either component; (b) the reactions are conducted in aqueous solutions at neutral pH; (c) the synthesis is applicable to other polysaccharides with carboxyl functions. We have synthesized protein conjugates of *Escherichia coli* K93, pneumococcus type 12 and *Staphylococcus aureus* type 8 by this scheme and are studying the immunological properties of these conjugates (Fattom, A., W. Vann, S. C. Szu, R. Schneerson, et al.); and (d) the carrier protein is only slightly modified and can elicit antibodies to the native protein (25). The resultant conjugates elicited higher levels of antibodies than the Vi alone and induced a booster response in weanling mice and juvenile primates. Similar studies of *Haemophilus influenzae* type b-protein conjugates were predictive of their enhanced antibody responses in humans (15–17, 19–22). Whether Vi conjugates will elicit a higher seroconversion rate and levels of antibodies in habitants of areas with high rates of typhoid fever must be ascertained by clinical evaluation.

IgG Vi antibodies protected mice challenged with *S. typhi* (41, 50, 51). Indirect evidence was provided that the Vi elicited IgG antibodies in humans (50). *H. influenzae* type b-protein conjugates elicit IgG CPS antibodies (19–22). It is likely therefore, that Vi conjugates will also elicit IgG antibodies. We plan to evaluate the isotypes, IgG subclasses and the isoelectric pattern of Vi antibodies elicited by Vi conjugates.

Convalescence from typhoid fever does not always confer immunity to *S. typhi* (52, 53). Recent data may provide an explanation for this finding; convalescence from typhoid fever does not always result in an elevation of Vi antibodies (31, 54–56). Tsang et al., reported that the Vi alone was a better immunogen in mice than *S. typhi* strain 560Ty (56). Increasing evidence points to superior immunogenicity of CPS-protein conjugates compared with that of the homologous bacteria in certain circumstances, namely: (a) conjugates of *H. influenzae* type b elicit higher levels of CPS antibodies in infants and young children than do systemic infections (15, 21); and (b) one to three injections of these conjugates elicited higher levels of antibodies in mice than that elicited by the homologous bacteria (Schneerson, R., and J. B. Robbins, unpublished observations).

Unlike the CPS of many encapsulated bacterial pathogens, Vi induced serum antibodies and conferred protection against lethal challenge with *S. typhi* in mice (8). Heidelberger et al. (49) showed that reinjection of several types of pneumococcal CPS did not induce a booster response. Landy reported similar data with the Vi in mice and humans (8, 42). We confirmed that reinjection of Vi did not induce a booster effect in mice. In Rhesus monkeys, reinjection reduced the levels of Vi antibodies. This could explain the findings of Gaines et al. (57) who injected chimpanzees three times with Vi. The immunized chimpanzees had no detectable Vi antibodies and were not protected against challenge with *S. typhi*.

The serum antibody responses elicited by conjugates prepared with SPDP were higher than those prepared by direct binding with EDAC. An explanation for this finding is that crosslinking reagents, such as SPDP, form conjugates with a "spacer" between the two macromolecules. This property may enable a more

effective interaction between the carrier protein and helper T lymphocytes (15, 18, 27, 58).

The higher levels of Vi antibodies elicited by the Vi-CT conjugates could be explained by the adjuvant effect exerted by the residual activity (toxicity) of the CT (20, 60, 61). The formation of a conjugate with the Vi reduced the toxicity of the CT $\sim 10^3$ – 10^4 -fold. The resultant conjugate was lethal for the guinea pig, but not mice, in the general safety test for bacterial vaccines described in the Code of Federal Regulations. This was probably due to the greater sensitivity of guinea pigs to the lethal effects of cholera toxin. We plan to conjugate the Vi to the β subunit of CT to avoid the problem of toxicity and yet induce serum antibodies that may exert protective effects against other enterotoxigenic bacterial pathogens (62).

Some of the primates in these experiments and healthy individuals in the United States had preexisting serum Vi antibodies (19). Since *S. typhi* or *S. paratyphi C* are rare in the United States, it is improbable that either of these two pathogens were the stimulus for these Vi antibodies. One possible source for the stimulus for these crossreacting antibodies could be the CPS of several strains of *S. aureus* that have an aminogalacturonic acid moiety in their repeat unit (62). The higher levels of Vi antibodies in young adults in countries where typhoid fever is endemic are likely to have been stimulated by infection with *S. typhi*.

S. typhi is an inhabitant of and a pathogen for humans only; there are yet no animal models or in vitro correlates of immunity that could be used to predict the protective activities of Vi-conjugates. Only the mucin-enhanced lethal infection of mice has been shown to correlate with the clinical effectiveness of cellular typhoid vaccines (7). The mouse protection model has been shown to be a measure of Vi antibodies, whether actively induced or passively administered (42). We have chosen to measure the Vi antibody response induced by the conjugates in laboratory animals by RIA rather than this bioassay (9). Accordingly, we plan to standardize the Vi conjugates by physicochemical and bioassays as has been done for other conjugates, and to evaluate their clinical properties of safety, immunogenicity, and then their protective actions against typhoid fever without the use of animal models (22).

Summary

The Vi has proven to be a protective antigen in two double masked, controlled clinical trials in areas with high rates of typhoid fever ($\sim 1\%$ per annum). In both studies the protective efficacy of the Vi was $\sim 70\%$. $\sim 75\%$ of subjects in these areas responded with a fourfold or greater rise of serum Vi antibodies. In contrast, the Vi elicited a fourfold or greater rise in 95–100% of young adults in France and the United States. Methods were devised, therefore, to synthesize Vi-protein conjugates in order to both enhance the antibody response and confer T-dependent properties to the Vi (and theoretically increase its protective action in populations at high risk for typhoid fever). We settled on a method that used the heterobifunctional crosslinking reagent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), to bind thiol derivatives of the Vi to proteins. This synthetic scheme was reproducible, provided high yields of Vi-protein conjugates, and was

applicable to several medically relevant proteins such as diphtheria and tetanus toxoids. The resultant conjugates were more immunogenic in mice and juvenile Rhesus monkeys than the Vi alone. In contrast to the T-independent properties of the Vi, conjugates of this polysaccharide with several medically relevant proteins induced booster responses in mice and in juvenile Rhesus monkeys. Clinical studies with Vi-protein conjugates are planned. This scheme is also applicable to synthesize protein conjugates with other polysaccharides that have carboxyl functions.

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