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Synthesis and Immunological Properties of Vi and Di-*O*-Acetyl Pectin Protein Conjugates with Adipic Acid Dihydrazide as the Linker

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The Vi capsular polysaccharide of *Salmonella typhi*, a licensed vaccine for typhoid fever in individuals ≥5 years old, induces low and short-lived antibodies in children, and reinjection does not elicit booster responses at any age. Its immunogenicity was improved by binding Vi to proteins by using *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) as a linker. Similar findings were observed with the structurally related, di-*O*-acetyl derivative of pectin [poly-α(1→4)-D-Gal*p*A] designated OAcP. Protein conjugates of Vi and OAcP were synthesized by carbodiimide-mediated synthesis with adipic acid dihydrazide (ADH) as the linker. Hydrazide groups were introduced into proteins (bovine serum albumin or recombinant *Pseudomonas aeruginosa* exoprotein A) by treatment with ADH and 1-ethyl-3(3-dimethylaminopropyl carbodiimide (EDC). The resultant adipic acid hydrazide derivatives (AH-proteins), containing 2.3 to 3.4% AH, had antigenic and physicochemical properties similar to those of the native proteins. The AH-proteins were bound to Vi and OAcP by treatment with EDC. The immunogenicity of Vi or OAcP, alone or as protein conjugates, was evaluated in young outbred mice and guinea pigs by subcutaneous injection of 2.5 and 5.0 μg, respectively, of polysaccharide, and antibodies were measured by enzyme-linked immunosorbent assay. All conjugates were significantly more immunogenic than Vi or OAcP alone and induced booster responses with 5- to 25-fold increases of antibodies. Vi conjugates were significantly more immunogenic than their OAcP analogs. A carboxymethyl derivative of yeast β-glucan enhanced the anti-Vi response elicited by an OAcP conjugate but had no effect on the immunogenicity of Vi or of OAcP alone. Vi and OAcP conjugates synthesized by this scheme will be evaluated clinically.

Based on its success in two randomized, double-blind and controlled clinical trials in areas with high rates of typhoid fever, Vi has been licensed for individuals ≥5 years of age in about 40 countries, and the World Health Organization has published requirements for this new vaccine (1, 17, 41). As with other polysaccharides (PSs), routine vaccination with Vi is limited because of its age-related and T-cell-independent immunologic properties (15, 18, 28); namely, Vi induces low and short-lived levels of serum antibodies in children <2 years old, and reinjection does not elicit a booster response in children or in adults (23, 30). To improve these immunologic properties, we prepared Vi-protein conjugates by using *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) as a linker (34, 39). These conjugates induced higher and longer-lasting levels of antibodies than Vi alone in adults and are now being evaluated for their efficacy in children (26, 27, 35).

Vi is a linear homopolymer of α(1→4)-D-Gal*p*A N-acetylated at C-2 and O-acetylated at C-3 (12, 16, 28, 37). The *N*- and *O*-acetyls dominate the surface and are essential for both antigenicity and immunogenicity of Vi (28, 33, 37). A structurally similar but immunologically unrelated plant PS, pectin [poly-α(1→4)-D-Gal*p*A], when O-acetylated at C-2 and C-3 (OAcP), is antigenically identical to Vi (5, 20). SPDP-linked OAcP-protein conjugates elicited serum Vi antibodies in animals (36).

The immunogenicity of the PS component of a conjugate is affected by its size, the carrier protein, the linker, and the ratio of the saccharide to the protein (2, 3, 8, 21, 22, 24, 32, 39). Fattom et al. reported that the immunogenicity of *Staphylococcus aureus* type 8 PS conjugates was related to the linker: conjugates prepared with adipic acid dihydrazide (ADH) elicited higher levels of type 8 antibodies than those prepared with SPDP (10). It is unclear whether the nature of the linker or the method of conjugation affected the immunogenicity of the PS component. We have been unable to prepare conjugates by binding adipic acid hydrazide (AH) derivatives of Vi to proteins (unpublished data).

We report the preparation and immunologic properties in mice and in guinea pigs of conjugates formed by binding Vi or OAcP to AH derivatives of proteins (AH-proteins) (bovine serum albumin [BSA] and *Pseudomonas aeruginosa* recombinant exoprotein A [rEPA]). In addition, we studied the adjuvant properties of a water-soluble carboxymethyl derivative of yeast β-(1→3)-D-glucan (CMG) with respect to the immunogenicity of OAcP alone or as a conjugate (6, 9, 25, 40).

MATERIALS AND METHODS

PSs. Vi from *Salmonella typhi* (Vi_s) was provided by Pasteur Mérieux Serum et Vaccins, Lyon, France. Vi purified from *Citrobacter freundii* WR 7011 (Vi_c;

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Walter Reed Army Institute of Research, Washington, D.C.) contained 2% nucleic acids, <1.0% protein, and <1.0% lipopolysaccharide (39). OAcP (69% O-acetylated) was prepared by treatment of pectin (GENU Pectin; Pectinfabrik, Copenhagen, Denmark) as described previously (36).

CMG (220 kDa; COOH/glucose molar ratio of 0.91) was purchased from the Institute of Chemistry, Slovak Academy of Sciences. CMG is a water-soluble chemically modified derivative of β-(1→3)-D-glucan branched at C-6 (1→3/1→6 linkages of 3:1) purified from baker's yeast *Saccharomyces cerevisiae* cell wall (25, 31, 40).

Proteins. BSA (lot 62HO1491) was purchased from Sigma Chemical Co., St. Louis, Mo. rEPA, a nontoxic derivative of exotoxin A (ETA) of *P. aeruginosa*, was isolated from the recombinant strain *Escherichia coli* BL21 as described previously (10).

Reagents and materials. Spectra/por tubing (molecular weight cutoffs, 6,000 to 8,000 and 12,000 to 14,000) was from Spectrum Medical Industries, Inc., Los Angeles, Calif. ADH (lot 77F5016), 1-ethyl-3(3-dimethylamino-propyl)carbodiimide (EDC; lot 32HO252) and 2-[*N*-morpholino]ethanesulfonic acid (MES) and its sodium salt (lot 14H5712) were from Sigma. BSA standard solution (2 mg/ml; lot 94052460) for determination of protein and the Coomassie blue protein assay reagent (lot 94052460) were from Pierce, Rockford, Ill. Sephacryl S-1000 was from Pharmacia, Inc., Piscataway, N.J., Bio-Gel P-10 (100/200 mesh) was from Bio-Rad Laboratories, Calif., rabbit BSA antiserum (lot 30716) was from Pel-Freez, Rogers, Ark., goat ETA antiserum (lot GAE-02A) was from List Biological Laboratories, Inc., Campbell, Calif., and protein standards (Mark^{12}; lot MRK40926) were from Novex, San Diego, Calif. Burro hyperimmune Vi antiserum B260 was prepared as described previously (35, 37).

Chemical assays. The Vi content of conjugates was estimated by measuring *O*-acetyl groups, using Vi and OAcP as references (11). Protein was measured by the Coomassie blue protein assay, using BSA as a reference (4), and hydrazide was measured by the trinitrobenzene sulfonic method with ADH as a reference (7, 14).

AH-protein. ADH (3.5 mg per mg of protein) was added to either BSA or rEPA (10 to 25 mg/ml in 0.15 M NaCl) and mixed. Then EDC was added at 0.1 mg per mg of protein unless otherwise stated (see Results). The reaction was carried out for 1 h at room temperature, pH 4.9 to 5.1 for BSA or pH 5.6 to 5.9 for rEPA. The pH was maintained with 0.1 N HCl or 0.1 M MES buffer (pH 5.6). The reaction mixtures were dialyzed overnight against 6 liters of 0.15 M NaCl at 4°C and concentrated by ultrafiltration. The concentrate was passed through a 2.5- by 46-cm column of Bio-Gel P-10; the void volume fractions were pooled, concentrated by ultrafiltration, and assayed for protein and hydrazide. The degree of derivatization of the protein with ADH is expressed as a percentage of the AH per weight of protein.

Conjugates of Vi or OAcP with AH-protein. Equal amounts of PS and AH-protein, at a final concentration of 1.8 to 3.7 mg of each/ml, and 5 or 10 mM EDC were used for conjugation.

EDC was added to solutions of Vi or OAcP and mixed for ~2 min at room temperature. AH-protein was added dropwise, and the conjugation was carried out for 3 h at room temperature and at a pH of 5.6 to 5.9 maintained with 0.1 M HCl or 0.1 M MES buffer (pH 5.6). To stop the reaction, the pH was raised to 6.5 to 7.0 with 1.0 M sodium phosphate buffer (pH 7.0). The reaction mixture was dialyzed overnight against 6 liters of 0.15 M NaCl at 4°C and centrifuged at 7,300 × *g* for 3 min. The supernatant was passed through a 1.5- by 90-cm column of Sephacryl S-1000 in 0.15 M NaCl; the void volume fractions were pooled and assayed for protein and Vi.

SDS-PAGE. AH-proteins and conjugates were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 8% gels stained with Coomassie brilliant blue R-250. Prestained wide-range protein standards were used as M_r markers.

Immunization of mice and guinea pigs. Six-week-old female general-purpose mice, 8 to 10 per group, were injected subcutaneously with 100 μl of immunogen in saline containing 2.5 μg of Vi or OAcP in either free or conjugated form; controls received 100 μl of saline. Vi and OAcP conjugates were administered two and three times, respectively, at 2-week intervals. The PSs alone and saline were injected once. Mice from each experimental group were exsanguinated 7 days after each injection. CMG (200 μg/dose) was mixed with the immunogen and injected with the same regimen as that for immunogens alone (40).

Groups of four female 6-week-old Duncan-Hartley guinea pigs were injected subcutaneously on days 0, 21, and 42 with 5 μg of PS alone or as a conjugate. Guinea pigs were bled prior to each injection and on day 50. Anti-Vi immunoglobulin G was measured by enzyme-linked immunosorbent assay (ELISA) (performed by Pasteur-Mérieux).

Double immunodiffusion. Double immunodiffusion of Vi, OAcP, AH-proteins, and conjugates against B260, goat anti-ETA, and rabbit anti-BSA was performed in 1% agarose gels.

Sera after the second injection of the Vi conjugates and the third injection of the OAcP conjugates were screened for precipitating Vi and protein antibodies by double immunodiffusion against 100 μg of Vi or protein per ml.

ELISA. Vi antibodies in mouse sera were measured by ELISA, using Vi_c as the coating antigen (37). Hyperimmune Vi antiserum, containing 230 μg of anti-Vi (total antibody)/ml (35–37), was used as the reference. Antibody levels of individual sera were calculated by parallel-line analysis toward the reference and summarized as the geometric mean of micrograms of total Vi antibody/milliliter.

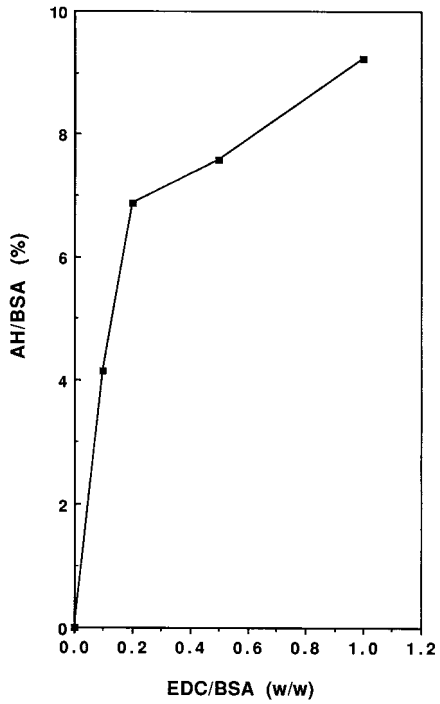

FIG. 1. Effect of the concentration of EDC on the derivatization of BSA with ADH.

Vi antibody titers in sera from guinea pigs were expressed in ELISA units/milliliter, referred to a guinea pig reference serum of 140 endotoxin units/ml (35).

Statistical analysis. Comparisons of the geometric means were performed with the two-sided *t* test or Wilcoxon analysis.

RESULTS

AH-protein. Hydrazide was introduced into the proteins by treatment with ADH and EDC (29). In excess of ADH, the derivatization of protein with ADH is controlled by EDC.

BSA was derivatized with ADH (Materials and Methods) in the presence of EDC/protein ratios of 0.1:1, 0.2:1, 0.5:1, and 1.0:1. Ratios of AH to protein in the resultant protein derivatives exhibited a hyperbolic-like dependence on the concentration of EDC (Fig. 1). Concentrations of EDC above 0.2 mg/mg of protein did not result in a proportionally higher ratio of AH to protein. Protein aggregates in AH-BSA prepared with equal amounts of EDC and protein were detected by SDS-PAGE (not shown). To avoid cross-linking, AH derivatives of either BSA or rEPA (Table 1) were prepared at EDC/protein ratios of 0.1 to 0.15 and yielded 2.3 to 3.4% derivatization. BSA was derivatized at pH 4.9 to 5.1. The rEPA was derivatized at pH 5.6 to 5.9, since below this range it precipitated.

SDS-PAGE patterns of the AH-proteins were similar to those of the native proteins (not shown). All AH-proteins displayed a line of identity with the native proteins by double immunodiffusion (Fig. 2).

PSs. Gel filtration profiles of Vi_s, Vi_c, and OAcP on Sephacryl S-1000 are depicted in Fig. 3. Both Vi_s and Vi_c were eluted within the resolution range of the column. The OAcP exhibited a narrower M_r distribution, with a single peak K_d of 0.68. Double immunodiffusion of Vi_s, Vi_c, and OAcP displayed a line of identity between these PSs when reacted against B260 (not shown).

Conjugation of PS to AH-protein. The EDC-mediated reaction was also used for conjugation of Vi or OAcP to AH-

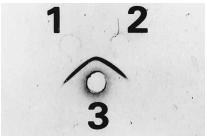

FIG. 2. Double immunodiffusion of rEPA and its AH derivative against anti-*P. aeruginosa* ETA serum. Wells: 1, rEPA, 100 μg/ml; 2, AH-rEPA$_5$, 100 μg/ml; 3, anti-ETA serum, 5 μl. The line of identity was observed also by double immunodiffusion of BSA and AH-BSA against anti-BSA serum.

protein (Table 1). The conjugation was influenced by the concentration of the reactants and the pH. Experiments carried out at ≥3.7 mg each of PS and AH-protein per ml with >10 mM EDC led to dense gels. Soluble conjugates were synthesized by mixing equal amounts of PS and AH-protein in the range of 1.8 to 3.7 mg of each/ml. The concentrations of EDC were 5 mM for conjugates 1 to 7 and 10 mM for Vi$_s$-rEPA$_8$. The pH was maintained at 5.6 to 5.9 for all conjugates. The local formation of gel particles while 0.1 N HCl was added to adjust the pH was avoided by using 0.1 M MES buffer (pH 5.6). Under these conditions, the reaction mixture appeared slightly viscous throughout the reaction.

Except for Vi$_c$-BSA$_1$, the Vi and OAcP conjugates emerged as a single peak on Sephacryl S-1000, represented in Fig. 4; Vi conjugates were more homogeneous in molecular size than Vi alone (Fig. 3 and 4). Vi$_c$-BSA$_1$ was eluted from the column in two peaks: Vi$_c$-BSA$_{1A}$ with a K_d of 0.15 and PS/protein ratio of 1.17 and Vi$_c$-BSA$_{1B}$ with a K_d of 0.42 and PS/protein ratio of 1.53. The PS/protein ratios of the conjugates ranged from 0.41 (OAcP-rEPA$_7$) to 1.62 (Vi$_s$-rEPA$_5$).

The Vi and OAcP conjugates did not enter 8% gels, and no bands corresponding to free AH-protein were observed by SDS-PAGE (Fig. 5).

TABLE 1. Composition of AH-proteins and related Vi or OAcP conjugates

AH-protein	AH/protein (%)	Conjugate	PS/protein (wt/wt)	K_d[a]
AH-BSA$_1$	3.4	Vi$_c$-BSA$_{1A}$	1.17	0.15
		Vi$_c$-BSA$_{1B}$	1.53	0.42
AH-BSA$_2$	2.9	OAcP-BSA$_2$	0.58	0.1
	2.9	OAcP-BSA$_3$	0.5	0.1
AH-rEPA$_3$	3.3	Vi$_c$-rEPA$_4$	0.59	0.1
	3.3	Vi$_s$-rEPA$_5$	1.62	0.37
AH-rEPA$_4$	2.5	Vi$_c$-rEPA$_6$	0.67	0.1
AH-rEPA$_5$	2.3	OAcP-rEPA$_7$	0.41	0.1
	2.3	Vi$_s$-rEPA$_8$	1.17	0.1

[a] Partition coefficient of conjugates on a 1.5- by 84-cm column of Sephacryl S-1000 in 0.15 M NaCl.

Double immunodiffusion of Vi and the OAcP conjugates revealed a precipitin line with anti-Vi and with anti-ETA sera that did not cross but did not fuse (Fig. 6).

Serum Vi antibodies in mice. Vi was administered only once, since reinjection does not elicit booster response (19, 34, 37, 39). After one dose, the Vi conjugates were more immunogenic than Vi alone ($P < 0.0001$) (Table 2). The second dose of Vi conjugates induced booster responses with a 5- to 25-fold rise in Vi antibody levels over the first dose ($P = 0.007$ to $P < 0.0001$). Conjugates of lower molecular size, Vi$_c$-BSA$_{1B}$ (K_d of 0.42) and Vi$_s$-rEPA$_5$ (K_d of 0.37), elicited similar antibody responses as the larger conjugates (K_d of ≤0.15).

Of 54 sera raised with two doses of the Vi conjugates, forty-five (84%) contained precipitating Vi antibodies that exhibited a partial identity with B260 and complete identity with the murine hyperimmune Vi serum (not shown). Of 38 sera raised with two doses of Vi-rEPA conjugates, 13 (34%) precipitated

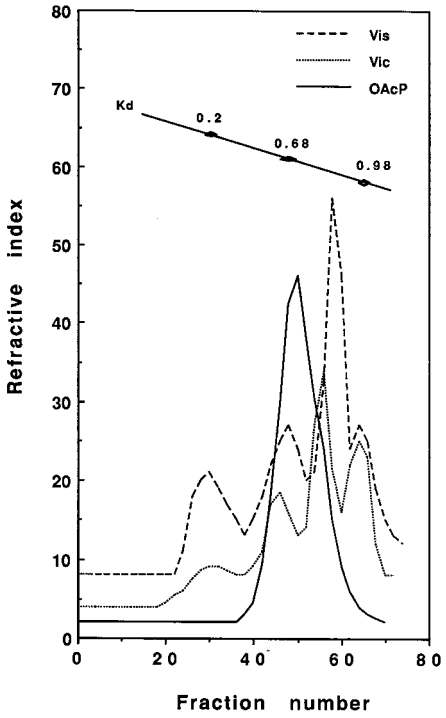

FIG. 3. Gel filtration profiles of the Vi$_s$, Vi$_c$, and OAcP (K_d of 0.68) on a 1.5- by 90-cm column of Sephacryl S-1000 in 0.15 M NaCl.

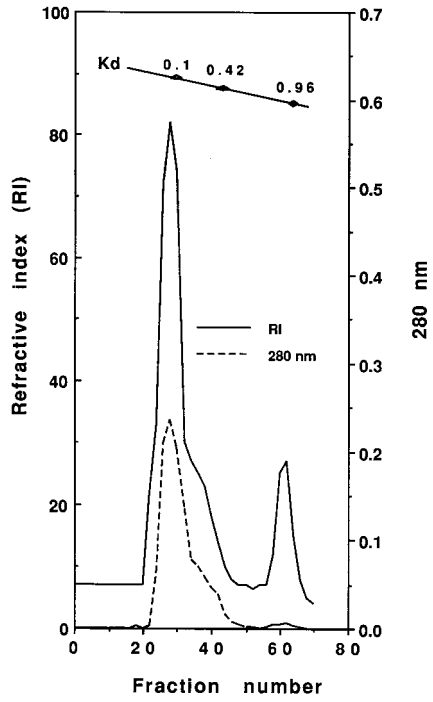

FIG. 4. Gel filtration profile of Vi$_c$-rEPA$_4$ conjugate on Sephacryl S-1000.

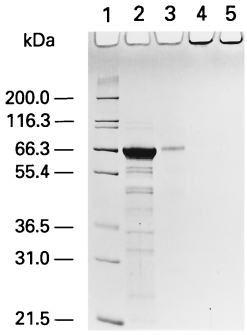

FIG. 5. SDS-PAGE (8% gel) patterns of Vi and OAcP conjugates. Lanes: 1, protein molecular weight markers, 10 μl; 2, rEPA, 3 μg; 3, AH-rEPA$_5$ containing 0.7 μg of protein; 4, Vi-rEPA$_8$ containing 3.4 μg of protein; 5, OAcP-rEPA$_7$ containing 3.4 μg of protein. The gel was stained with Coomassie brilliant blue R-250.

with ETA, but no precipitation against BSA was observed with sera raised with the Vi-BSA conjugates (not shown).

To test the need for covalent binding of Vi to AH-protein to enhance the anti-Vi response and to induce a booster, equal amounts of Vi and AH-BSA were mixed and stored for 3 weeks at 4°C. The mixture was passed through a 1.5- by 90-cm column of Sephacryl S-1000, and peak fractions with a K_d of 0.1, containing both Vi and BSA in the ratio of 0.41 (the K_d of BSA on this column is 0.96), were pooled and injected into mice as Vi conjugates. A single dose elicited a threefold-higher level of Vi antibodies than the Vi alone ($P = 0.002$). A second injection, however, failed to induce a booster response.

As reported previously (36), the OAcP alone did not elicit Vi antibodies, probably because of its low M_r. The first injection of the OAcP conjugates induced higher Vi antibody levels than Vi alone ($P < 0.03$), and the second elicited seven- to eightfold rises ($P < 0.002$). The third injection of the OAcP conjugates did not elicit statistically significant rises of anti-Vi (not shown). The levels of Vi antibodies after the second injection of OAcP conjugates were two to seven times lower than those of Vi conjugates ($P < 0.04$ to $P < 0.0001$). Nevertheless, after the third injection, 21 (70%) of 30 mice had precipitating Vi antibodies that displayed a line of identity with sera raised with Vi conjugates (Fig. 7).

Adjuvant effect of CMG on OAcP-rEPA. CMG did not enhance the antibody response to either OAcP or Vi alone (not shown). After one injection, OAcP-rEPA$_7$ plus CMG elicited threefold-higher Vi antibody levels than the conjugate alone ($P = 0.02$) (Table 2). A significant rise was elicited by the second injection ($P < 0.0005$). The rise following the third injection (45.0 versus 30.0 μg/ml) was not statistically significant.

TABLE 2. Serum Vi antibodies of mice injected with Vi or OAcP alone, as conjugates or a mixture of Vi and AH-BSA[a]

Immunogen	*n*	Geometric mean μg of antibody/ml (25th–75th centiles)	
		1st injection	2nd injection
Saline	8	0.21 (0.07–0.3)	ND[b]
OAcP	8	0.31 (0.25–0.43)	ND
Vi$_s$	8	0.9 (0.52–1.9	ND
Vi$_c$-BSA$_{1A}$	8	5.66 (2.9–10.1)	144.0 (119–191)
Vi$_c$-BSA$_{1B}$	8	11.1 (7.76–17.2)	80.0 (48.9–148)
OAcP-BSA$_2$	10	2.33 (0.72–6.15)	15.1 (7.9–33.9)
OAcP-BSA$_3$	10	1.63 (0.89–5.88)	10.8 (5.36–29.6)
Vi$_c$-rEPA$_4$	9	6.09 (3.3–11.9)	80.7 (59.6–374)
Vi$_s$-rEPA$_5$	10	10.1 (5.76–15.2)	90.4 (40.0–196)
Vi$_c$-rEPA$_6$	9	9.6 (7.4–19.1)	44.2 (34.0–142)
OAcP-rEPA$_7$	10	1.8 (1.04–4.28)	12.8 (6.45–26.6)
OAcP-rEPA$_7$ + CMG	10	5.31 (2.38–15.5)	30.0 (21.2–49.8)
Vi$_s$-rEPA$_8$	10	12.6 (9.5–16.5)	79.5 (55.2–106)
Vi$_c$ + BSA	8	3.29 (1.58–6.87)	5.92 (3.0–14.1)

[a] Vi and OAcP conjugates elicited higher levels of anti-Vi compared to Vi after the first injection ($P < 0.0001$ and $P < 0.03$); both conjugates elicited booster responses after the second ($P < 0.007$ and $P < 0.0001$). Vi conjugates were more immunogenic than their OAcP analogs ($P < 0.04$ to $P < 0.0001$). Addition of CMG had no effect on the immunogenicity of Vi and OAcP alone (not shown) but increased the anti-Vi level elicited by OAcP-rEPA$_7$ ($P = 0.02$).
[b] ND, not done.

The geometric mean Vi antibody level after the second injection of OAcP-rEPA$_7$ plus CMG was higher than that of the conjugate alone ($P = 0.02$) as well as the other OAcP conjugates. Vi antibody levels after the second injection of the Vi conjugates, however, were uniformly higher than those elicited by CMG plus OAcP-rEPA$_7$. CMG had also a stimulatory effect on the immunogenicity of the protein component of OAcP-rEPA$_7$. Precipitating ETA antibodies were found in 7 (70%) of 10 sera after three injections of OAcP-rEPA$_7$ plus CMG, as opposed to only 3 (30%) of 10 sera of mice injected with the conjugate alone. No reactivity of these sera with CMG was observed by double immunodiffusion.

Serum Vi antibodies in guinea pigs. As reported previously (35), Vi did not elicit antibodies in this species. Only one conjugate, Vi$_s$-rEPA$_5$, elicited Vi antibodies after the first injection (Table 3). All of the conjugates elicited antibodies after the second injection. Although the number of animals is small and the differences between the groups are not large, the tendency for Vi conjugates to elicit higher levels of antibodies than OAcP conjugates was observed in guinea pigs as it was in mice.

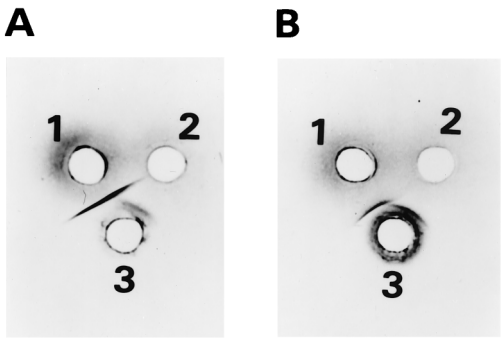

FIG. 6. Double immunodiffusion of Vi (A) and OAcP (B) conjugates against anti-Vi (B260) and anti-ETA sera. (A) Wells: 1, anti-Vi, 2 μl; 2, anti-ETA, 5 μl; 3, Vi-rEPA$_8$ conjugate (200 μg of Vi/ml and 170 μg of rEPA/ml), 20 μl. (B) Wells: 1, anti-Vi, 2 μl; 2, anti-ETA, 5 μl; 3, OAcP-rEPA$_7$ conjugate (63 μg of OAcP/ml and 170 μg of rEPA/ml), 20 μl.

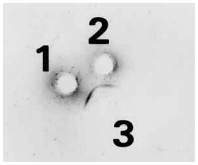

FIG. 7. Representative double immunodiffusion of murine sera raised with Vi and OAcP conjugates against Vi. Wells: 1, 20 μl of serum from a mouse injected twice with Vi-rEPA$_8$; 2, 20 μl of serum from a mouse injected three times with OAcP-rEPA$_7$; 3, Vi$_s$ (100 μg/ml), 20 μl.

TABLE 3. Serum Vi antibodies of guinea pigs injected with Vi alone or with Vi or OAcP conjugates[a]

Immunogen	Geometric mean Vi antibody (ELISA units/ml)		
	1st injection	2nd injection	3rd injection
Vi_s	<10	<10	<10
Saline	<10	<10	<10
Vi_s-$rEPA_5$	10	43	64
Vi_c-BSA_{1A}	<10	13	61
Vi_s-$rEPA_8$	<10	60	54
OAcP-BSA_2	<10	12	48
OAcP-BSA_3	<10	12	49
OAcP-$rEPA_7$	<10	7	13

[a] Groups of four guinea pigs were injected subcutaneously with saline solutions of 5.0 μg of PS alone or as a conjugate every 2 weeks. The animals were bled 7 days after each injection, and their sera were assayed for Vi antibodies by ELISA. Preimmune sera from all the guinea pigs had <10 ELISA units/ml.

DISCUSSION

We describe the preparation of Vi and OAcP conjugates by using ADH as a linker bound first to the protein rather than to the saccharide component. This approach to the preparation of PS-protein conjugates was reported by Schneerson et al. (29). Seven conjugates, of either Vi or OAcP bound to BSA or rEPA, were synthesized. The concentrations of the reactants and the pH affected the conjugation. To avoid gel formation, the concentrations of both PS and AH-protein had to be <3.7 mg/ml, and the pH had to be >5.5. The pH of the conjugation reaction was maintained at 5.5 to 6.0, since the optimal pH of EDC-mediated reactions is 4.9 ± 0.2 (13). The concentration of EDC in the conjugation reaction mixture was 5 or 10 mM. Variations in these parameters affected the ratio of PS to protein and the size of the conjugates. Despite differences in molecular sizes (K_ds of 0.1 to 0.42), PS/protein ratios (0.59 to 1.62), origin of Vi (Vi_s or Vi_c), and carrier protein (BSA or rEPA), all Vi conjugates elicited similar levels of Vi antibodies in either mice or in guinea pigs.

The antigenic properties of Vi are mimicked by di-*O*-acetyl pectin (36). Although not immunogenic alone, OAcP conjugated to protein induces serum Vi antibodies with booster responses (36). In those experiments, the immunogenicity of SPDP-linked Vi and OAcP conjugates in mice were similar. We found that unlike the SPDP-linked conjugates, ADH-linked Vi conjugates were significantly more immunogenic in mice than their OAcP analogs. Although OAcP conjugates were less immunogenic than Vi conjugates, most sera from the second and third injections had precipitating Vi antibody. Double immunodiffusion with Vi revealed antigenic identity between mouse sera elicited by OAcP and by Vi conjugates.

There are advantages to using pectin as the PS component of conjugate vaccines for typhoid. Pectin is abundant, has no lipopolysaccharide, and requires a simple chemical modification to prepare its di-*O*-acetyl derivative. Because it may not be reliable to extrapolate data from animals to humans, clinical evaluation of both types of conjugates is under way.

Double-immunodiffusion patterns of all conjugates showed lines of precipitation with the antiprotein and the anti-Vi that did not fuse but did not cross. If the polysaccharide and the protein were not covalently linked, one would expect precipitin lines that cross each other, and this was not the case. The ability of Vi and OAcP conjugates to induce anti-Vi booster responses in mice, in contrast to the mixture of Vi and AH-BSA, provides evidence that the PS and the protein were covalently bound. This observation is further supported by experiments with guinea pigs demonstrating that while Vi alone was not immunogenic, both Vi and OAcP conjugates elicited Vi antibody and booster response.

Immunogenicity of OAcP conjugate was enhanced two- to threefold by CMG. CMG also enhanced the immunogenicity of the protein carrier. Since no stimulatory effect of CMG on the antibody response to either OAcP or Vi alone was observed, it is likely that the CMG functions through activation of T cells. Further studies with this CMG are planned.

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