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Further Studies on the Immunogenicity of *Haemophilus influenzae* Type b and Pneumococcal Type 6A Polysaccharide-Protein Conjugates

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Conjugates were prepared by carbodiimide-mediated coupling of adipic acid hydrazide derivatives of *Haemophilus influenzae* type b (Hib), *Escherichia coli* K100, and pneumococcal 6A (Pn6A) polysaccharides with tetanus toxoid (TT), as an example of a "useful" carrier, and horseshoe crab hemocyanin (HCH), as an example of a "nonsense" carrier. These conjugates were injected into NIH mice, and their serum antibody responses to the polysaccharides and proteins were characterized. As originally reported, Hib conjugates increased the immunogenicity of the capsular polysaccharide and elicited greater than the estimated protective levels of anti-Hib antibodies in most recipients after one injection and in all after the third injection (Schneerson et al., J. Exp. Med. **152**:361–376, 1980). Both Hib conjugates induced similar anti-Hib responses. The K100-HCH conjugate was more immunogenic than the K100-TT conjugate and elicited anti-Hib responses similar to the Hib conjugates after the third injection. Simultaneous injection of the K100 and the Hib conjugates did not enhance the anti-Hib response. The Pn6A-TT conjugate induced low levels of anti-Hib antibodies; when injected simultaneously with the Hib conjugates, the anti-Hib response was enhanced, as all mice responded after the first injection and with higher levels of anti-Hib than observed with the Hib conjugates alone ($P < 0.05$). The Pn6A conjugates were not as immunogenic as the Hib conjugates. Pn6A-TT was more effective than was Pn6A-HCH; it elicited anti-Pn6A (>100 ng of antibody nitrogen per ml) in 6 of 10 mice after the third injection. The addition of the Hib-HCH conjugate to the Pn6A-TT conjugate increased the anti-Pn6A response with a higher geometric mean antibody titer, and 9 of 10 mice responded after the third injection. A preparation of diphtheria toxoid, TT, and pertussis vaccine increased the anti-Hib antibody levels after the first injection only in mice receiving Hib-TT, but not in mice receiving Hib-HCH, suggesting that additional carrier protein (TT) enhanced the anti-polysaccharide response. Simultaneous injection of Hib and Pn6A conjugates with the same or different carriers resulted in an enhanced serum antibody response to each polysaccharide. The anti-tetanus toxin response reached protective levels (>0.01 U/ml) in most mice after the first injection and in all mice after the second and third injections of TT conjugates. A progressive increase in the anti-HCH response with each additional injection was noted in animals receiving HCH conjugates. Animals receiving the diphtheria toxoid-TT-pertussis vaccine preparation responded with a greater increase in anti-carrier antibody than those receiving the conjugates alone. This method of synthesis provided conjugates capable of inducing protective levels of antibodies to both the polysaccharides and carrier proteins.

Prevention of invasive diseases, especially meningitis, due to *Haemophilus influenzae* type b (Hib) has been advocated because of their frequency and continuing morbidity and mortality despite antibiotic and supportive therapy and because of the increasing emergence of antibiotic-resistant organisms isolated from patients (55, 61). Serum antibodies to the capsular polysaccharide of Hib have been shown to confer protective immunity against invasive diseases caused by this organism (1, 3, 37, 39–41, 44, 45, 48, 50, 54). The purified Hib polysaccharide has

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limited usefulness as a vaccine because it does not induce a protective serum antibody response in infants, the age group with the highest incidence of invasive Hib diseases (37, 38, 41, 45, 58). In addition to this problem of age-related immunogenicity, the Hib polysaccharide does not induce a booster response; a single injection elicits the maximum response for each recipient (41, 58).

The ability of polysaccharide-protein conjugates to increase the immunogenicity of a bacterial capsular polysaccharide, pneumococcus type 3, was first reported by Goebel and Avery in 1929 (17). Recently, we have reported a synthetic scheme for conjugates composed of proteins and the capsular polysaccharide of Hib (47, 49, 51–53). The use of a spacer molecule, adipic acid dihydrazide (ADH), facilitated the coupling reaction between the Hib polysaccharide and proteins providing high yields of conjugates. In contrast to the slight or undetectable serum antibody response induced by the Hib polysaccharide in laboratory mice, the conjugates induced high levels of antibodies with bactericidal activity in almost all recipients. The immunogenicity of these conjugates was dose dependent within ranges that could be considered for human infant use, was increased sequentially after two and three injections, and was enhanced both by priming with the carrier protein and by incorporation with Freund adjuvant. It was proposed that the conjugates both increased the immunogenicity of the Hib polysaccharide and conferred upon it the properties of a "T-dependent antigen." In preliminary experiments, the Hib conjugates showed a similar increased immunogenicity when injected into primates. This synthetic approach for preparing Hib polysaccharide-protein conjugates was found to be applicable for preparing similar compounds with the capsular polysaccharides of other human pathogens, pneumococcus 6A and *Escherichia coli* K13 (49, 51). Other workers have reported similar results with meningococcal and Hib polysaccharides by different synthetic schemes (2, 7, 24; E. C. Beuvery, V. Kanhai, P. C. van Putten, A. van der Kaader, and J. Haverkamp, Abstr. Conference on Pathogenic *Neisseria*, Montreal, 4 to 6 August 1982).

In our original report, the protein was treated with ADH and a water-soluble carbodiimide to form an adipic acid hydrazide (AH) derivative. These AH derivatives were then bound to cyanogen bromide-activated Hib polysaccharide. This synthetic scheme posed some problems. (i) the immune response to the native protein induced by the conjugates was slight; and (ii) the AH derivatives of tetanus toxoid (TT) and horseshoe crab (*Limulus polyphemus*) hemocyanin (HCH) were poorly soluble at pH 4.7, which is considered optimal for the carbodiimide reaction (21).

Ideally, the conjugates would serve to induce protective antibodies to the polysaccharide as well as to the carrier protein. Accordingly, the conjugate should be constructed so as to preserve the immunogenicity of the native protein. In the present study, the conjugates were prepared by a modified scheme. The polysaccharides were first activated by cyanogen bromide and then reacted with ADH. The resultant AH-polysaccharide derivatives were bound to protein carriers by the carbodiimide reaction. The conjugates so prepared elicited both anti-polysaccharide and antiprotein antibodies.

The structures of the three polysaccharides used are shown in Fig. 1; all three contain a ribitol 5-phosphate in their repeat unit (12, 13, 27, 42). The Hib and K100 polysaccharides have the same composition and differ only in their ribose-ribitol bond; they have cross-reactive antigenic and immunogenic properties. *E. coli* K100 was studied to see whether the anti-Hib response could be accelerated by the addition of this closely related polysaccharide. The K100-induced anti-Hib antibodies in adult volunteers (23, 48, 50). The Pn6A polysaccharide was chosen for study because that pneumococcal type is a common cause of diseases in infants and children, its immunogenicity is age dependent like that of the Hib polysaccharide, it is a comparatively poor immunogen among the 14 types in the polyvalent pneumococcal vaccine, and it was not cross-immunogenic with the Hib polysaccharide in a clinical study (4, 5, 8, 9, 33, 43, 56, 57). Two protein carriers were studied: TT as an example of a "useful" carrier and HCH as an example of a "nonsense" carrier. Experiments were designed to increase the yield of bound polysaccharide in the conjugate. The effect of the additional injection of more than one conjugate and of the diphtheria toxoid-TT-pertussis vaccine (DTP) vaccine to the Hib conjugates was also studied.

MATERIALS AND METHODS

Carrier proteins. TT preparations were concentrated by vacuum dialysis (Union Carbide, Chicago, Ill.)

Pn6A
→2)-αD-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)α-L-Rha*p*-(1→3)-D-Ribitol-5-PO_4

Hib
→3)-D-Rib*f*-(1→1)-Ribitol-5→PO_4-

E. coli K100
→3)-D-Rib*f*-(1→2)-Ribitol-5→PO_4-

FIG. 1. Structure of capsular polysaccharides used for preparation of the polysaccharide-protein conjugates.

and equilibrated against 0.2 M NaCl at 3 to 8°C to a final concentration of about 40 mg of protein per ml. The concentrated TT preparations were centrifuged at 20,000 × *g* for 20 min and passed through a 5 by 100-cm S-300 Sephacryl (Pharmacia Fine Chemicals, Piscataway, N.J.) column equilibrated in 0.2 M NaCl–0.01% NaN_3 at 3 to 8°C. The peak corresponding to a molecular weight of 150,000 was pooled, concentrated by vacuum dialysis, and equilibrated against 0.2 M NaCl by exhaustive dialysis at 3 to 8°C. The final material was adjusted to 60 mg of protein per ml, centrifuged at 20,000 × *g* for 2 h at 4°C, passed through a 0.45-nm membrane (Nalge Co. Rochester, N.Y.), and stored at 3 to 8°C. The following TT preparations were used: Shanghai Institute of Biological Products, Shanghai, People's Republic of China, lot no. 713; Institute Armand Frappier, lot no. AT3/4 7, 2433 Lf/mg N protein, (courtesy of Jack Cameron); and Wyeth Laboratories lot no. 4371, 2939 Lf/mgN, (courtesy of Alan Bernstein).

The HCH was prepared from cell-free hemolymph (donated by T.-Y. Liu, Food and Drug Administration) (25). The hemolymph was centrifuged at 150,000 × *g* at 10°C for 5 h. The supernatant was discarded, and the pellet was dissolved in 0.2 M NaCl and recentrifuged at the same conditions. The blue pellet was dissolved at approximately 100 mg/ml and passed through an S-300 Sephacryl column (see above), and the peak corresponding to a molecular weight of 65,000 was pooled, concentrated by vacuum dialysis, equilibrated against 0.2 M NaCl, and centrifuged at 48,000 × *g* for 2 h at 10°C, and the supernatant was passed through a 0.45-nm membrane and stored at 3 to 8°C.

Polysaccharides. The Hib polysaccharide, prepared from strain 1482, contained less than 1% (wt/wt) protein and nucleic acid and less than 0.04% (wt/wt) lipopolysaccharide and had a *Kd* of 0.4 (molecular size) (44, 47, 63, 64). The Pn6A polysaccharides were donated by Allen Woodhour, Merck Sharp & Dohme Research Laboratories, West Point, Pa., and Frank Cano, Lederle Laboratories, Pearl River, N.Y. Both preparations passed the specifications for this type (10).

Synthesis of the conjugates. The polysaccharides were converted into sodium salt by passage through Dowex 50Wx8 (14). They were then activated with cyanogen bromide (Eastman Chemical Products, Inc., Rochester, N.Y.) at pH 10.5 for 6 min and reacted with 0.1 M ADH by tumbling overnight at 3 to 8°C. The reaction mixture was dialyzed against 0.2 M NaCl with two changes for 24 h, followed by gel filtration through G-100 Sephadex equilibrated in water, and freeze-dried. The resultant polysaccharides contained about 2.0% (wt/wt) adipic hydrazide (22).

The AH polysaccharide derivatives and the proteins were used at 20 ml/mg each, and coupling was done with 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride (Eastman) for 3 h at 4°C. The reaction mixture was passed through a CL-4B Sepharose column, and the void volume peak was pooled, passed through a 0.45-nm filter, and stored at 4°C. The concentrations of proteins and polysaccharides were assayed as described previously (26, 32, 47).

Serology. Serum anti-Hib antibodies and anti-Pn6A antibodies were measured by radioimmunoassays (45, 46). All samples were performed in duplicate, and the results are expressed as the geometric mean (GM) for all animals in an experimental group and the number of responders (anti-Hib > 0.15 μg of antibody per ml; anti-Pn6A, >100 ng of antibody nitrogen (AbN) per ml (45, 46). Hyperimmune sera, used as references, were taken from mice injected with 10 μg of TT or HCH in complete Freund adjuvant (62). The antitoxin titers were assayed as described by Barile et al. (6) with male mice. Two instead of three mice were used for each serum dilution. The mouse reference anti-TT was assayed for its antitoxin activity by twofold dilutions and found to contain 200 U of anti-toxin when compared with the U.S. reference tetanus antitoxin; other sera were titrated at 10-fold dilutions. An enzyme-linked immunosorbent assay (ELISA) was performed by the method of Engvall et al. (15). Samples of 4 μg of TT or HCH per ml were coated to polyvinyl chloride plates. Goat anti-mouse gamma globulin was conjugated to alkaline phosphatase, and *p*-nitrophenyl phosphate disodium (Sigma) was used as the substrate. The concentration of anti-HCH antibodies in the reference serum was measured by the quantitative precipitin reaction.

Chemicals. The Hib and K100 polysaccharides were measured by the Bial reaction with a Hib polysaccharide, dried over P_2O_5, as a reference (26). The Pn6A polysaccharide was measured by the orcinol reaction, and the protein was measured by the method of Lowry et al. (32). The hydrazide was measured by the method of Inman (22).

Immunization. Six-week-old, female N:NIH(S) (NIH) mice were injected subcutaneously with saline solutions of the conjugates containing 2.5 μg of polysaccharide chosen on the basis of previous experiments and because this amount of conjugate seemed compatible with proposed dosage in human infants (37, 41, 44, 56, 58). When two or more conjugates were injected simultaneously they were mixed in a single syringe. When Hib and K100 conjugates were injected simultaneously, each was injected at 1.25 μg of polysaccharide. When Hib or K100 (or both) was injected with Pn6A, 2.5 μg of each was used. Groups of 10 mice were injected three times at 2-week intervals with each conjugate or combination of conjugates and were bled 2 weeks after the first immunization and 1 week after the second and third immunizations. DTP (Lederle Laboratories, Pearl River, N.Y.; lot. no. 661-506) was injected in 0.1-ml amounts at a different site in three experimental groups. Control animals, receiving only polysaccharide, were omitted in most experiments based upon an extensive experience in our laboratory and others which has uniformly showed little or no detectable antibody in most mice injected with a wide dosage range of these polysaccharides (2, 47).

Statistics. The antibody levels were converted to logarithms to the base 10, and all statistical analyses were performed on the log data. The term "mean" refers to the mean of log antibody levels. The antilog of a mean is the GM of a group of antibody levels. Group 5 (Pn6A-HCH) in Table 2 was excluded from statistical analyses, as there were no positive responses.

Among groups receiving the same number of injections, means were compared by using the F test of analysis of variance. If means were significantly different, then they were arranged in increasing order and divided into subgroups in which means are not signifi-

cantly different from each other ($P < 0.05$). The Newman-Keuls method of the multiple comparison test was used to create these subgroups which may be overlapping, i.e., a mean may belong to more than one subgroup (59). In Tables 2, 3, 5, and 6, subgroups are identified by vertical and horizontal bars. For example, in Table 2 for the first injection, group 6 has the smallest mean and group 11 has the largest. The first subgroup contains groups 6 and 4, the second subgroup contains groups 4 and 3, the third subgroup contains groups 3, 8, 2, and 9, and the fourth subgroup contains groups 8, 2, 9, 14, 13, 7, 1, 10, 12, and 11. This shows that groups 6 and 4 are not different and groups 4 and 3 are not different but groups 6 and 3 are statistically different, etc.

RESULTS

Efficiency of coupling reaction. The effect of the concentration of the protein and the Hib polysaccharide during the coupling reaction upon the yield of conjugates was studied. In preliminary studies, it was found that the yield of conjugates, based upon the amount of polysaccharide bound, was variable; the concentration of the reactants in the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide - hydrochloride – mediated coupling reaction seemed to be important in determining this variation. Figure 2 shows a representative experiment with TT to study this problem. The concentrations of both the AH-Hib polysaccharide and TT were kept equal and increased from 5.0 to 30.0 mg/ml. The concentration of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride was kept constant. After incubation for 3 h at 4°C and pH 4.9 ± 0.1, the reaction mixture was dialyzed against phosphate-buffered saline overnight at 3 to 8°C and centrifuged at 20,000 × *g* at 4°C for 20 min, and the supernatant was passed through a 4B Sepharose column. There was a progressive increase in the efficiency of the coupling reaction as the concentration of the reactants increased; there was 8% recovery when the reactants were 5 mg/ml and a 62% recovery when the reactants were 30 mg/ml. In addition, the protein/polysaccharide ratio decreased from about 3.0/1 at 5.0 mg/ml to 1.4/1 to 1.6/1 at 30 mg/ml. On the basis of these data, and because of the difficulty using higher concentrations of proteins with limited amounts of tetanus toxoid, the protein and polysaccharide were used at 20 mg/ml for the coupling reactions.

Not shown are experiments in which the ratio of protein to polysaccharide was varied from 1/3 to 3/1. No increased efficiency of binding was observed throughout this range of reactants.

Characterization of the conjugates. The yield and composition of the conjugates used in these experiments are shown in Table 1. The protein/polysaccharide ratio varied from 2.8 for Pn6A-HCH to 1.4 for Hib-TT. The yields ranged from a low of 2.2% (K100-HCH) to 52% (Pn6A-HCH).

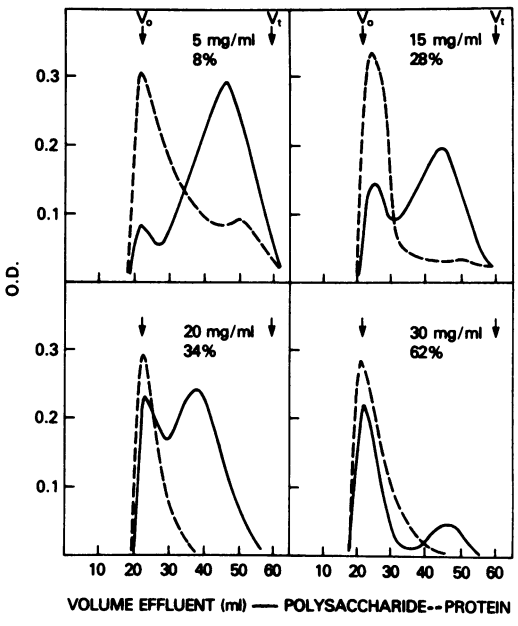

FIG. 2. Effect of concentration of reactants upon the composition and yield of Hib-TT conjugates. The solid line depicts the concentration of the polysaccharide, and the broken line shows the protein concentration of the effluent. The concentration of the AH derivative of the Hib polysaccharide and TT was varied; the concentration of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride was kept constant. After the 3-h coupling reaction at 4°C and at pH 6.0, the mixture was dialyzed against saline at 2 to 8°C for about 18 h, centrifuged at 12,000 × *g* at 4°C for 20 min, and passed through a 4B Sepharose column. Increasing the concentration of the Hib polysaccharide and TT increased both the yield of material passing through the void volume of the column (V_0) and the ratio of polysaccharide to protein in the final conjugate.

Anti-Hib antibody response. Table 2 shows the serum anti-Hib responses induced by the six conjugates. The GM of anti-Hib antibodies induced by the two Hib conjugates after the first immunization exceeded the estimated protective level (0.15 μg of antibody per ml). The GM and percentage of responders increased after the second and third immunizations with these two conjugates. After the third immunization, all animals responded with greater than 0.15 μg of antibody per ml. There were no differences between the anti-Hib responses to these two conjugates after any of the three immunizations.

The K100 conjugates elicited a lesser anti-Hib response than that of the Hib conjugates after the first immunization. Thereafter, the K100-HCH conjugate elicited both a higher GM and a

TABLE 1. Characterization of polysaccharide-protein conjugates[a]

Conjugate	Protein (μg/ml)	Polysaccharide (μg/ml)	Prt/Ps[b] ratio	Yield (%)
Hib-TT	407	252	1.6	33
Hib-TT[c]	490	342	1.4	45
Hib-HCH	565	268	2.1	25
K100-TT	544	236	2.3	25
K100-HCH	78	39	2.0	2.2
Pn6A-TT	90	53	1.7	50
Pn6A-HCH	504	180	2.8	52

[a] The polysaccharide was measured by the Bial reaction (26), and the protein was measured by the method of Lowry et al. (32). The yield was determined by calculating the amount of polysaccharide in the void volume of a calibrated 4B Sepharose column compared with the amount of starting material.

[b] Prt/Ps, Protein/polysaccharide.

[c] The second preparation of Hib-TT conjugate was not used for immunization in this experiment.

higher proportion of responders than the K100-TT conjugate ($P < 0.05$). After the second and third immunization, the K100-HCH conjugate induced anti-Hib antibodies similar to those elicited by the two Hib conjugates.

The Pn6A-TT conjugate elicited a slight anti-Hib response after the second and third immunizations; this response was less than that induced by the two Hib conjugates (in Pn6A-TT versus Hib-HCH, $P < 0.0025$ after the second immunization and $P < 0.001$ after the third immunization; in Pn6A-TT versus Hib-TT, $P < 0.001$ for the second and third immunizations) and the K100-HCH conjugate ($P < 0.01$). The Pn6A-HCH conjugate did not elicit anti-Hib antibodies after any of the three injections.

The effect of injecting both Hib conjugates was similar to that observed with the monovalent preparations. The total Hib polysaccharide dose was 2.5 μg in the mice receiving either the monovalent or the bivalent preparations. There were no differences between the anti-Hib antibodies in the groups that received both Hib conjugates after any of the three immunizations by using the criteria of either the GM or the percentage of responders. Similarly, there was no enhancement of anti-Hib when Hib conjugates were injected simultaneously with either of the K100 conjugates, although the GM of anti-Hib antibodies induced by the K100-HCH and the Hib conjugates were higher than those elicited by the K100-TT along with the Hib conjugates ($P < 0.05$).

The GM and percentage of responders was greater after the first injection in mice receiving Hib-HCH and either of the Pn6A conjugates ($P < 0.05$). There was a 100% response, and the GMs were 1.79 for Pn6-HCH and 1.86 for Pn6A-TT when combined with the Hib-HCH as compared with 0.42 for Hib-HCH alone. After the

TABLE 2. Serum anti-Hib response of mice injected with HiB, K100, Pn6A polysaccharides conjugated to HCH or TT alone or with DTP adsorbed[a]

Group	Conjugates	GM μg of anti-Hib antibody per ml (no. of responders/total) after injection		
		1	2	3
1	Hib-HCH	0.42 (8/10)	0.74 (8/10)	7.49 (11/11)
2	Hib-TT	0.20 (6/9)	2.32 (10/10)	5.17 (9/9)
3	K100-HCH	0.12 (4/10)	1.43 (10/10)	7.72 (10/10)
4	K100-TT	0.06 (2/10)	0.27 (5/10)	0.69 (8/8)
5	Pn6A-HCH	0.03 (0/10)	0.06 (1/10)	0.04 (0/10)
6	Pn6A-TT	0.04 (1/10)	0.13 (4/10)	0.45 (7/10)
7	Hib-HCH + K100-HCH	0.40 (8/10)	4.64 (10/10)	8.47 (10/10)
8	Hib-HCH + K100-TT	0.19 (7/10)	1.66 (9/9)	3.93 (11/11)
9	Hib-HCH + Hib-TT	0.24 (6/10)	4.53 (10/10)	12.01 (9/9)
10	Hib-HCH + Pn6A-HCH	1.70 (10/10)	4.09 (9/9)	5.31 (10/10)
11	Hib-HCH + Pn6A-TT	1.86 (10/10)	3.83 (10/10)	8.79 (10/10)
12	Hib-TT + DTP	1.74 (10/10)	9.36 (10/10)	18.10 (10/10)
13	Hib-HCH + DTP	0.39 (7/9)	7.62 (10/10)	10.37 (10/10)
14	Hib-HCH + K100-HCH + Pn6A-HCH + DTP	0.36 (10/10)	4.72 (10/10)	Not done

[a] GMs are ranked in increasing order and grouped by the multiple comparison method (59).
First injection: 6, 4, 3, 8, 2, 9, 14, 13, 7, 1, 10, 12, 11.
Second injection: 6, 4, 1, 3, 8, 2, 11, 10, 9, 7, 14, 13, 12. Third injection: 6, 4, 8, 2, 10, 1, 3, 7, 11, 13, 9, 12.

Anti-Hib antibody of 6-week-old female NIH mice injected subcutaneously with Hib, K100, and Pn6A conjugates 1, 2, and 3 times is shown.

second injection, the GM was higher in the mice receiving the Pn6A conjugates along with the Hib-HCH than in mice receiving monovalent Hib-HCH ($P < 0.05$); this difference was no longer detectable after the third immunization. The combination of the Pn6A conjugate with Hib-HCH elicited a 100% response after the second and third injections. In these groups, the dose of Hib polysaccharide was 2.5 μg as compared with 1.25 μg of Hib polysaccharide in the groups receiving the combination of Hib and K100 polysaccharides.

Anti-Pn6A antibody response. In general, the Pn6A polysaccharide conjugates were less immunogenic than the Hib conjugates (Table 3). The more immunogenic of the two Pn6A conjugates, Pn6A-TT, induced only 2 of 10 responders after the first injection (GM, 13.5 ng of AbN per ml). This progressively increased to 6 of 10 responders (GM, 159.3 ng of AbN per ml) after the third immunization. The Pn6A-HCH conjugate induced only 2 of 10 responders after the third immunization with a GM of 21.0 ng of AbN per ml.

When Hib-HCH and Pn6A-HCH were injected simultaneously, they induced an anti-Pn6A response only after the second and third injections. This combination elicited anti-Pn6A antibodies in 4 of 10 mice with a GM of 54.4 ng of AbN per ml. The combination of Pn6A-TT and Hib-HCH elicited 9 responders out of 10 mice after the third injection with a GM of 418 ng of AbN per ml.

Anti-tetanus toxin response. The anti-TT titers are expressed as units of antitoxin with the U.S. standard anti-tetanus toxin as the reference. These units were derived from the ELISA assays of the individual sera compared with the mouse reference serum (200 U/ml).

Table 4 shows the relation between the amount of antitoxin and anti-TT as obtained by ELISA for four representative mouse sera. There is only a fair correlation, as two of the values for anti-TT and ELISA are compatible. Accordingly, the protective level of anti-TT (0.01 U/ml) in sera assayed by ELISA can only be estimated. Table 5 shows the anti-TT induced by the conjugates alone or in combination with DTP. There was no anti-TT induced by the Hib and K100 conjugates prepared with HCH. All three conjugates prepared with TT induced an antitoxin response. All animals that received any conjugate or combination of conjugates prepared with TT responded with protective levels (≧0.01 U/ml) of anti-TT after the second and third immunizations. There was no difference in the antitoxin response induced by the three TT conjugates after any of the three immunizations. As would be expected, the addition of DTP yielded the highest anti-TT of all the groups. After the third immunization, the mice receiving Hib-TT conjugate responded with the highest anti-TT (108.9 U/ml) as compared to those receiving DTP and conjugates prepared with HCH ($P < 0.05$).

Anti-HCH response. Serum anti-HCH antibodies were detected in all groups injected with an HCH conjugate alone or in combination of an HCH with a TT conjugate. As expected, no anti-HCH antibodies were detected in groups injected with TT conjugates (Table 6). We did not list the number of responders since there is no known protective activity for anti-HCH antibodies.

The level of anti-HCH antibodies increased with the number of injections in all groups. The K100-HCH and Pn6A-HCH conjugates induced similar levels of anti-HCH antibodies after each

TABLE 3. Serum anti-Pn6A antibody response of mice injected with HiB or Pn6A polysaccharides conjugated to HCH or TT alone or with DTP adsorbed[a]

Group	Conjugates	GM ng of AbN per ml of anti-Pn6A (no. of responders/total) after injection		
		1	2	3
5	Pn6A-HCH	13.7 (0/10)	7.1 (1/9)	21.0 (2/10)
6	Pn6A-TT	13.5 (2/10)	3.1 (3/10)	159.0 (6/10)
10	Hib-HCH + Pn6A-HCH	4.2 (0/10)	52.9 (3/9)	54.4 (4/10)
11	Hib-HCH + Pn6A-TT	14.6 (0/10)	59.1 (4/10)	418.0 (9/10)
12	Hib-TT + DTP	5.9 (0/10)	1.2 (0/10)	2.5 (0/10)
13	Hib-HCH + DTP	Not done	Not done	53.0 (3/9)

[a] The GMs are ranked in increasing order and grouped by the multiple comparison method (59).
First injection: 10, 12, 6, 5, 11.
Second injection: 12, 6, 5, 10, 11.
Third injection: 12, 5, 13, 10, 6, 11.

Anti-Pn6A antibody of 6-week-old female NIH mice injected subcutaneously with conjugates containing 2.5 μg of polysaccharides one, two, and three times is shown.

TABLE 4. Serum anti-tetanus toxin antibodies, measured by neutralization and ELISA, in mice immunized with Hib-TT conjugates with and without DTP[a]

Conjugate	No. of injections	Anti-TT (U/ml)	
		Neutralization	ELISA
Hib-TT	2	<0.02	0.025
Hib-TT	3	>0.2-<2.0	0.144
Hib-TT + DTP	2	>0.2-<2.0	22.69
Hib-TT + DTP	3	>20-<200	168.0

[a] Individual sera taken from mice injected two or three times with Hib-TT conjugates with and without simultaneous injection of 0.1 ml of DTP at a different site, were assayed for their anti-TT antibody by both ELISA and toxin neutralization.

injection. The addition of DTP elicited higher levels of anti-HCH antibodies than the Hib-HCH conjugate alone ($P < 0.01$). The anti-HCH antibodies elicited by the combination of Hib-HCH plus K100-HCH plus Pn6A-HCH plus DTP (group 14), containing approximately 8.0 μg of HCH for each injection, were the highest ($P < 0.05$).

Cross-reaction between Hib and Pn6A polysaccharides. Hib-TT and Hib-HCH injected alone did not induce anti Pn6A antibodies. The anti-Pn6A response elicited by the Pn6A-TT conjugates after the third immunization was enhanced by the addition of the Hib-HCH after the third injection (Table 3). Injection of the combination resulted in a higher GM and number of responders than did the injection of Pn6A-TT alone (GM of 418 versus 159 and 9 versus 6 responders, respectively, out of 10 mice). Unexpectedly, the more immunogenic of the two Pn6A conjugates, Pn6A-TT, induced anti-Hib especially after the third injection (GM of anti-Hib, 0.45; 7 responders out of 10 mice) (Table 2). This same conjugate, Pn6A-TT, given in combination with Hib-HCH enhanced the anti-Hib antibodies after the first injection as compared with Hib-HCH alone (GM of 1.8 and 10 responders out of 10 mice versus GM of 0.42 and 7 responders out of 10 mice; $P < 0.05$).

Pn6A-HCH was less immunogenic than Pn6A-TT, yet both Pn6A conjugates gave a similar enhancement of response to anti-Hib when given with Hib-HCH after the first injection as compared with Hib-HCH alone. There was no demonstrable effect of these Pn6A conjugates added to Hib-HCH on the anti-Hib antibodies after the second and third injections; all mice responded in these groups with high levels of anti-Hib antibodies.

Effect on anti-Hib response of simultaneous injection of conjugates with DTP adsorbed. DTP (0.1 ml) was injected simultaneously at another site with Hib-TT or Hib-HCH to study the effect of this common pediatric vaccine on the anti-Hib response to the conjugates (Table 2). DTP enhanced the anti-Hib response to the Hib-TT, but not to the Hib-HCH, after the first injection; the number of responders was increased from 6 to 10 responders out of 10 mice, and the GM increased from 0.20 to 1.74 μg/ml ($P < 0.05$).

TABLE 5. Serum anti-TT responses in mice immunized with Hib, K100 or Pn6A conjugated to TT or HCH alone or in combination with DTP

Group	Conjugates	GM anti-TT U/ml (no. of responders/total) after injection		
		1	2	3
2	Hib-TT	0.018 (6/6)	0.049 (6/6)	0.172 (4/4)
3	K100-HCH	0	0	0
4	K100-TT	0.034 (4/8)	0.201 (10/10)	0.290 (9/9)
6	Pn6A-TT	0.020 (7/8)	0.093 (9/9)	0.53 (9/9)
8	Hib-HCH + K100-TT	0.023 (10/10)	0.177 (9/9)	0.064 (11/11)
9	Hib-HCH + Hib-TT	0.009 (6/10)	0.027 (9/10)	0.099 (7/7)
11	Hib-HCH + Pn6A-TT	0.051 (10/10)	0.128 (9/9)	0.407 (9/9)
12	Hib-TT + DTP	0.331 (10/10)	6.92 (10/10)	108.9 (10/10)
13	Hib-HCH + DTP	0.623 (9/9)	4.85 (10/10)	36.5 (10/10)
14	Hib-HCH + K100-HCH + Pn6A-HCH + DTP	0.485 (10/10)	2.98 (8/8)	Not done

[a] The GMs are ranked in increasing order and grouped by the multiple comparison method (59).

First injection: 9, 2, 6, 8, 4, 11, 12, 14, 13.

Second injection: 9, 2, 6, 11, 8, 4, 14, 13, 12.

Third injection: 8, 9, 2, 4, 11, 6, 13, 12.

Groups of 10 mice were injected with conjugates containing about 3.0 to 4.0 μg of either TT or HCH. Their sera were assayed for anti-TT by ELISA. These results were converted to anti-TT units with the use of a reference antiserum.

TABLE 6. Serum anti-HCH antibodies in mice immunized with Hib, K100, or polysaccharides conjugated to HCH or TT with and without DTP adsorbed[a]

Group	Conjugates	GM anti-HCH (μg of antibody per ml of serum) after injection		
		1	2	3
1	Hib-HCH	0.08	0.19	0.62
2	Hib-TT	Not done	0	Not done
3	K100-HCH	0.12	0.58	0.90
4	K100-TT	Not done	0	Not done
5	Pn6A-HCH	0.09	0.29	0.99
7	Hib-HCH + K100-HCH	0.10	0.33	0.78
8	Hib-HCH + K100-TT	0.09	0.36	0.69
9	Hib-HCH + Hib-TT	0.07	0.18	0.45
10	Hib-HCH + Pn6A-HCH	0.13	0.36	0.99
11	Hib-HCH + Pn6A-TT	0.11	0.16	0.43
13	Hib-HCH + DTP	0.11	0.46	1.5
14	Hib-HCH + K100-HCH + Pn6A-HCH + DTP	0.17	1.05	Not done

[a] The GMs are ranked in increasing order and grouped by the multiple comparison method (59).
First immunization: 14, 10, 3, 13, 11, 7, 8, 5, 1, 9.
Second immunization: 14, 3, 13, 10, 8, 7, 5, 1, 9, 11.
Third immunization: 13, 10, 5, 3, 7, 8, 1, 9, 11.

Groups of 10 mice were injected with conjugates containing about 3.0 to 4.0 μg of either HCH or TT. The animals were bled as described in the text, and their sera were assayed for anti-HCH by ELISA.

After the second and third immunizations, all mice in both groups responded with higher levels of anti-Hib antibodies, but there was no statistically significant difference between the Hib-TT groups with and without DTP. In the Hib-HCH groups, DTP enhanced the anti-Hib response from 8 out of 10 mice and a GM of 0.74 μg/ml to 10 out of 10 and a GM of 7.62 ($P < 0.05$). After the third immunization, all mice responded with high levels of anti-Hib antibodies, and there was no significant difference caused by the addition of DTP.

DTP was also injected simultaneously with three conjugates: Hib-HCH, K100-HCH, and Pn6A-HCH. All animals responded after the first and second injection, although the anti-Hib level was similar to that elicited by the Hib conjugates alone.

Effect of adsorption of conjugates on alum. Not shown are the anti-Hib antibody responses of mice that received the Hib-TT conjugate adsorbed on alum. There was no difference in the mice receiving the alum-adsorbed conjugate compared to the response elicited by the conjugate in saline.

DISCUSSION

The serum anti-Hib antibody response, elicited by the conjugates prepared by derivatization of the Hib polysaccharide rather than the protein carrier, was similar to those reported for the original method (47, 53). The attachment of the spacer (ADH) to the polysaccharide did not adversely affect its solubility or reaction with typing antiserum and eliminated the problems of solubility encountered with some of the AH protein derivatives. There was no difference between the anti-Hib antibody response induced by conjugates prepared with the "useful" carrier, TT, over that prepared with a " nonsense" carrier, HCH. Therefore, to avoid injection of adventitious antigens into infants, we suggest that conjugates should be prepared with "useful carriers." This method of synthesis seems to be a general one as AH polysaccharides have been coupled to several "useful" carrier proteins (R. Schneerson, C. Chu, M. C. Hardegree, J. and B. Robbins, manuscript in preparation). Beuvery et al. have reported on the antibody response elicited by conjugates composed of group A meningococcal polysaccharide and TT prepared without a spacer and with spacers containing two- and six-carbon chains (Beuvery et al., Abstr. Conference on Pathogenic *Neisseria*). These three conjugates elicited a similar primary anti-group A response. However, there was a graded anti-group A secondary response related to the length of the carbon spacer; the highest response was elicited by the conjugate with the six-carbon spacer. The conjugate prepared with the six-carbon spacer also elicited the highest serum antibodies to the carrier protein (TT) after both primary and secondary immuni-

zations. They postulated that the six-carbon spacer allowed more interaction of the two macromolecules comprising the conjugate with lymphoid cells and thus a greater immunogenicity than the conjugates prepared with no spacer or a two-carbon spacer.

The combination of the Hib-TT conjugate and DTP, which elicited the highest levels of anti-Hib, did not induce anti-Pn6A antibodies after three injections. The combination of Hib-HCH and DTP induced a slight anti-Pn6A response after the third injection. Similarly, Pn6A-TT induced a slight anti-Hib response, which increased with the number of injections. When Hib-HCH was injected with either Pn6A-HCH or Pn6A-TT, both the anti-Hib and anti-Pn6A responses were increased over that induced by either conjugate alone. Therefore, the injection of two conjugates did not exert a negative effect, and it was encouraging to see that the combination of Hib-HCH and Pn6A conjugates induced 10 of 10 responders after only one injection. One explanation for this enhanced anti-Hib and anti-Pn6A response was that the total amount of the cross-reacting polysaccharides was two times (5.0 μg/dose) than that injected with either conjugate alone. The steep dose-response curve observed for the Hib conjugates makes this a possibility (2, 47, 52). Another explanation for the enhancement in the antibody responses to both polysaccharides elicited by their simultaneous administration as conjugates may be due to the cross-immunogenicity between the Hib and Pn6A polysaccharides reported in hyperimmune animal sera (36), but hitherto unreported in humans. The structural basis for this cross-reactivity is that both the Hib and Pn6A contain ribitol 5-phosphate in their repeat unit (12, 42). The cross-reactivity between the Hib and Pn6A polysaccharides in mice, elicited by the single conjugate, was only observed after two and three injections; in the aforementioned clinical study, the adult volunteers received only one injection of the Pn6A polysaccharides (43, 55). A similar enhancement of both anti-Hib and anti-Pn6A antibodies has been observed in primates injected with these Hib and Pn6A conjugates (Chu, Schneerson, Robbins, and Hardegree, unpublished data).

No advantage upon anti-Hib antibody formation was achieved by using the K100 conjugates alone or in combination with Hib-HCH. However, in contrast to the studies with combinations of Pn6A and Hib conjugates, the total amount of polysaccharide in the experiments when K100 and Hib conjugates were combined was the same (2.5 μg per dose) as that administered with the monovalent preparations. Further experiments are planned to study the effect of various doses and combinations of Hib, Pn6A, and K100 conjugates upon the enhancement and acceleration of the anti-polysaccharide response.

DTP-adsorbed vaccines contain two components, *Bordetella pertussis* cells and aluminium salts, known to modify the human serum antibody response (18). Moxon et al. reported that simultaneous injection of purified Hib polysaccharide and DTP, mixed together, into 2- to 5-year-old children depressed the anti-Hib response (35). King et al. reported that the immunogenicity of Hib polysaccharide, previously incubated wtih *B. pertussis* cells, was increased in infants (28, 55). The nature of the association between the Hib polysaccharide and the *B. pertussis* was not reported. The effect of *B. pertussis* upon the response to another capsular polysaccharide, pneumococcal type 3, in mice has been studied. When *B. pertussis* was injected intraperitoneally, there was a depressant effect upon the anti-type 3 response (29). However, *B. pertussis* exerted an enhanced anti-type 3 response when it was injected subcutaneously (60). The simultaneous administration of DTP with the Hib conjugates appeared to have two effects. The first was an accelerated anti-Hib response after the first injection of Hib-TT with DTP; both the GM and number of responders were greater than those elicited by Hib-TT alone. This accelerated anti-Hib response was not noted when DTP was added to the Hib-HCH conjugate, despite the fact that a similar response was elicited by these two Hib conjugates injected singly. The enhancement may have been due to the additional tetanus component in the DTP exerting a "carrier" effect. Accordingly, further experiments are planned to study the effect of simultaneously injecting diphtheria toxoid and TT along with polysaccharide conjugates prepared with these two pediatric vaccine components. The second effect of DTP was an enhancement of both the anti-polysaccharide and anti-carrier antibody levels after the second and third injections. This enhancement may have been due to the adjuvant effect of the lymphocytosis-promoting factor from the *B. pertussis* cells or the aluminium salts (or both) (16, 29, 31). This adjuvant effect, described for the lymphocytosis-promoting factor and similar to that reported for cholera toxin (11), may have been responsible for the enhancement of the anti-Hib response reported by King et al. (28). It was not possible to decide whether the DTP exerted an adjuvant effect upon the anti-TT antibodies in the groups receiving TT conjugates. We failed to detect an adjuvant effect upon anti-Hib levels in animals injected with an Hib conjugate adsorbed with aluminium salts.

Mitani et al. reported that conjugates, prepared with pullalan (linear copolymer of maltotriose) and TT elicited considerably lower levels

of immunoglobulin E (IgE) anti-TT antibodies than a similar dose of Formalin-treated, aluminium-precipitated TT (34). Both the conjugate and the formalinized, precipitated TT elicited similar amounts of IgM and IgG anti-TT antibodies. The conjugation procedure reduced the toxicity of the TT to satisfactory levels. The pullalan-TT conjugate also elicited less IgE antitetanus antibodies than the formalinized, aluminium-precipitated TT (20, 31). This provides supportive information that conjugates such as those described in this paper may be considered for clinical trials.

Our experiments have shown that a "useful" carrier is as effective as a "nonsense" carrier in mice. Therefore, it would seem that a "useful" carrier would be preferred for human use provided it does not interfere with established immunization programs or the acquisition of "natural" immunity. Additional possible candidates for carrier proteins would be outer membrane proteins of Hib that induce bactericidal antibodies (19, 22, 31a).

As yet we have no explanation for the lesser immunogenicity of K100-TT as compared to K100-HCH or Pn6A-HCH as compared with Pn6A-TT. There was no demonstrable difference in the molecular size characteristics or chemical composition of these less immunogenic conjugates. This is an important problem, as it will be necessary to accurately predict the immunogenicity of these conjugates if they are to be considered for clinical studies.

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