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Antibody Responses to *Haemophilus influenzae* Type b and Diphtheria Toxin Induced by Conjugates of Oligosaccharides of the Type b Capsule with the Nontoxic Protein CRM_{197}

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Oligosaccharides were made from *Haemophilus influenzae* type b capsular polysaccharide and conjugated to CRM_{197} by reductive amination. Conjugates were made with a range of lengths and multiplicities of saccharide chains. All elicited a strongly enhanced anti-*H. influenzae* type b capsular polysaccharide response when injected into weanling rabbits. One series of conjugates also elicited antibodies to diphtheria toxin.

It is established that passively administered serum antibody to the capsular polysaccharide can be protective against invasive infections by *Haemophilus influenzae* type b (1). The realization that children with *H. influenzae* b meningitis often undergo neurological damage despite appropriate therapy (28) has prompted efforts to immunize actively with the purified polysaccharide, a linear polymer with the repeating unit [—3-β-D-ribosyl(1-1)ribitol(5-phosphate)—] (PRP) (5). Injection of PRP elicits a copious and long-lived antibody response in mature humans (26). Susceptibility to *H. influenzae* b meningitis increases with the disappearance of maternal antibody, to a maximum in the first year of life (8). Unfortunately, maturation of the antibody response to purified PRP is slow; titer rises in the first year are infrequent, small, and transient (23, 29). Vaccination has appeared to protect children ≥18 months of age (22), indicating that a modest anti-PRP response can confer resistance to *H. influenzae* b under field conditions. Protection against the majority of cases will require creating such responsiveness in the first months of life.

Enhancement of immunogenicity of pneumococcal capsular polysaccharide determinants in the rabbit was achieved by covalent linking of polymer (11) or disaccharide hapten (10) to a protein carrier. This approach, heretofore used in immunochemical research (13), may have value in vaccinating humans against bacteria with poorly immunogenic capsules. The coupling methods generally have linked the carbohydrates to the proteins through aromatic or heterocyclic residues (13), whose biological safety would be difficult to assure. Development of conjugates for human vaccination will require consideration of toxic potential as well as immunogenicity. Stability of the linkage should be an additional prerequisite.

For immunization against *H. influenzae* b, PRP covalently linked to several different carrier proteins has been shown to induce an enhanced anti-PRP response in mice, rabbits, and monkeys (25). The conjugates were made by first coupling the protein with adipic dihydrazide as a "spacer" and then coupling to PRP activated by cyanogen bromide. Antibodies to one of the proteins employed, diphtheria toxin, are desirable per se in humans; however, the effect of the coupling procedures upon the antigenicity of the carrier proteins was not reported (25).

The present report describes an alternative approach in which oligosaccharides derived from PRP are coupled directly to a carrier protein. Fragments with reducing termini were created by selective cleavage and coupled by reductive amination. Differing size ranges were employed to explore for an optimal hapten size. CRM_{197}, a nontoxic but antigenically identical variant of diphtheria toxin (20, 30), was used as the carrier. Conjugates were tested for immunogenicity in weanling rabbits, which are unresponsive to purified polysaccharides in general, including PRP. Serum antibodies both to PRP and to diphtheria toxin were examined.

MATERIALS AND METHODS

Diphtheria toxin (DTx) (the reagent supplied for Schick testing) and formalized diphtheria toxoid (DTd) were obtained from the Massachusetts Department of Public Health (Jamaica Plain, Mass.); the latter was made from a relatively pure toxin preparation such that 1 limit of flocculation (Lf) ≅ 2.5 μg. CRM_{197} was prepared from *Corynebacterium diphtheriae* C7 (β197) by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose as described (20). In polyacrylamide gel electrophoresis with staining by Coomassie blue,

the preparation appeared ≥98% as a single band of molecular weight about 60,000; when electrophoresed after reduction with β-mercaptoethanol, about 20% of the protein appeared as A and B subunits ("nicked"). PRP lot 19 was isolated as the sodium salt from *H. influenzae* b strain Eag and was analyzed as previously described (4). The preparation contained 39% pentose, 16% organic phosphate (calculated as $—PO_2—$), 0.40% protein, and 0.36% nucleic acids; it eluted as a single peak of K_{av} 0.57 on a column of Sepharose 2B. Reducing oligosaccharides were generated by controlled acidic hydrolysis and separated into three size ranges as follows. A 72-mg portion of the PRP was heated for 6 min at 100°C in 0.01 N H_2SO_4, chilled, and neutralized with NaOH. The ratio of total pentose to reducing pentose, T/R, was thereby lowered from 490 to 21. The hydrolysate was fractionated on a column of BioGel P10 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.1 M triethylammonium acetate. Three fractions defined by ratio of elution volume to void volume (Ve/Vo) were retained and designated small (S; Ve/Vo, 1.39 to 2.00, T/R = 9.1), medium (M; Ve/Vo, 1.09 to 1.38, T/R = 19), and large (L; Ve/Vo, ≤1.08, T/R = 31). To the extent that cleavage of the ribosyl-ribitol linkage is the only mode of hydrolysis, the T/R ratio is an estimate of the number average *n* for fragments of the general formula (ribitolyl-phosphate-ribose)$_n$.

For the four preparations designated no. 1, CRM_{197} was incubated with fractions S, M, L, or—as control—no oligosaccharide as follows. Each 1.5-ml reaction tube received, in order: (i) 4.0 μmol (reducing pentose) of S, 2.7 μmol of M, or 1.6 μmol of L added as aqueous solutions and lyophilized, or no oligosaccharide; (ii) 2.7 mg of CRM_{197} dissolved in 160 μl of 2.5% $NaCNBH_3$ solution; (iii) 18 μl of 2 M potassium phosphate buffer, pH 8. After 18 days of incubation at 37°C, the protein (with or without conjugated oligosaccharides) was precipitated with ammonium sulfate at 80% saturation, dissolved with 8 M urea, reprecipitated with ammonium sulfate, dissolved, and dialyzed against saline. Thus, in the procedure CRM_{197} was exposed to sodium cyanoborohydride and oligosaccharides before the introduction of phosphate buffer. The preparations, designated C-P_S no. 1, C-P_M no. 1, C-P_L no. 1, and C-control no. 1, respectively, were colloidal suspensions that spontaneously sedimented. In the two preparations designated no. 2, CRM_{197} strongly buffered from the onset was incubated with equal amounts of fraction S in two different reaction volumes as follows: (i) tubes a and b received 1 μmol of S, lyophilized; (ii) tube a received 15 μl of the 2 M buffer and 510 μg of CRM_{197} in 100 μl of water; tube b received 2 μl of the 2 M buffer and 510 μg of CRM_{197}, and the contents were relyophilized to reduce volume; (iii) tube a received 15 μl of 20% $NaCNBH_3$, and tube b received 15 μl of water and 2 μl of $NaCNBH_3$ solution. Estimated final volumes were 140 μl for a and 25 μl for b. Incubation and isolation of protein were as described for no. 1 except that dialysis was against 0.01 M sodium phosphate buffer, pH 7.2. The preparations (designated C-P_S no. 2a and C-P_S no. 2b) were not sedimented by 3-min centrifugation at 8,000 × *g*.

Total pentose was determined by the orcinol method and reducing activity by the alkaline ferricyanide method (15); D-ribose was the standard for both assays. Protein was determined by the Folin phenol method (15), with bovine albumin as standard. Anti-PRP antibody was assayed by binding of [^{3}H]PRP, and PRP antigenic equivalence was assayed by inhibition of this assay exactly as described; changes of ±50% in this assay were statistically significant (2). Complement-dependent bactericidal antibody was assayed as described (3). Antibody binding to DTd was assayed by an adaptation of an enzyme-linked immunoadsorbent assay (ELISA) (6). Polystyrene microtiter wells were coated by incubation for 1 h with DTd at 1 μg/ml, washed, and incubated 16 to 18 h with the test serum. The DTd antigenic equivalence of CRM_{197} and its conjugates were estimated by inhibition of the ELISA. A standard rabbit antiserum was preincubated for 1 h with test solutions or various known concentrations of DTd and then incubated in the washed and coated wells for 16 to 18 h. Neutralizing antibody to DTx was titrated in the skin of a nonimmune adult rabbit (9); calibration was with horse antitoxin obtained from Merrell-National Laboratories (Swiftwater, Pa.).

Immunogenicity was tested in 7-week-old New Zealand White rabbits by subcutaneous injection three times at 1-week intervals of the conjugates or control preparations in saline containing 0.0125 M aluminum phosphate suspension, pH 7.

RESULTS

Compositional data and antigenicity (i.e., binding of antibody) are summarized in Table 1. In series no. 1, increasing proportions of pentose were incorporated in the conjugates C-P_L, C-P_M, and C-P_S; PRP-antigenic equivalence per unit of protein increased in the same order. No DTd antigenic equivalence was detected in the conjugates of series no. 1; however, there was no DTd-antigenicity in the control preparation C-control no. 1, indicating that the antigenic denaturation of CRM_{197} was caused by the conditions of preparation rather than by the oligosaccharide residues per se. Conjugates C-P_S no. 2a and no. 2b were made in such small quantities that colorimetric assay of pentose incorporation was not done. The PRP-antigenicity of no. 2b (reacted at sixfold greater concentration) was 13-fold greater than 2a. The DTd-antigenicity of no. 2a and no. 2b per unit of protein were roughly equivalent to unreacted CRM_{197} and to DTd itself.

The serum anti-PRP responses of rabbits injected with the series no. 1 conjugates or control antigens beginning at age 7 weeks are shown in Table 2. Antibody concentrations as measured by radioantigen binding were generally <20 ng/ml before vaccination. Responses were barely detectable in the animals given (high-molecular-weight) PRP and undetectable in those given nonconjugated CRM_{197} (C-control no. 1). Likewise, in a separate experiment, animals given simple mixtures of CRM_{197} and PRP oligosaccharides made little or no response (data not tabulated). In contrast, strong anti-PRP responses were elicited by the conjugates (doses of 25

TABLE 1. Pentose content and antigenic properties of conjugates of CRM_{197} with PRP oligosaccharides and of control preparations

Preparation	Ratio of nmol of pentose/nmol of protein[a]	Antigenic equivalence	
		ng of PRP/μg of protein	μg of DTd/μg of protein
C-control no. 1	<0.4	<0.003	0.004
$C\text{-}P_S$ no. 1	6.2	0.1	<0.002
$C\text{-}P_M$ no. 1	2.9	0.08	<0.002
$C\text{-}P_L$ no. 1	0.80	0.006	<0.002
$C\text{-}P_S$ no. 2a	NT[b]	0.04	0.5
$C\text{-}P_S$ no. 2b	NT[b]	0.5	2
unreacted CRM_{197}	<0.4	<0.003	1.2

[a] The reducing terminal ribose upon attachment becomes a ribitol residue, which is not detected by the orcinol method.

[b] NT, Not tested, due to insufficient quantity.

μg adsorbed to aluminum phosphate and injected subcutaneously). The antibody levels increased with succeeding injections and after the third were over 1,000-fold the prevaccination levels. (Previous experience with noncovalent PRP-protein complexes had shown that the response would be maximal at about 1 week postinjection.) Responses to the conjugates made with oligosaccharides of the three different size ranges S, M, and L were roughly equal. Complement-dependent bactericidal activity was assayed with *H. influenzae* b strain Eag, a meningeal isolate (Table 2). None of the rabbits had measurable bactericidal activity before vaccination; after three injections of any of the three conjugates, but not the controls, substantial titers were found. Neither the conjugates nor the controls raised the level of antibody to DTd (not tabulated).

The conjugates $C\text{-}P_S$ no. 2a and no. 2b were tested for immunogenicity in a lower dose (8 μg of protein) (Table 3). In the animals given only conjugates (rabbits 1 through 4), no significant rises in anti-PRP were found 1 week after the first injection, but sharp rises were found after the second and third. Despite the sixfold difference in PRP antigenic equivalence, the responses to no. 2a and no. 2b were very similar. To test for a carrier priming effect, rabbits 5 and 6 were given 100-μg injections of DTd at age 8 weeks, and had made strong anti-DTd responses by the time of their first injection of conjugate no. 2b at age 9 weeks. These two animals showed small but significant anti-PRP rises 1 week after their first injection of conjugate no. 2b. Their anti-PRP titers after the second and third injections, however, were not appreciably greater than in the unprimed rabbits receiving no. 2b.

In the anti-DTd antibody determined by ELISA, rabbits 1 through 4 had no significant increase after the first injection of conjugate; there were rises in three of four after the second and in all four after the third injection. The sera of these animals were also tested for ability to neutralize diphtheria toxin. Antitoxic activity was not detected (<0.01 U/ml) in any of the preimmunization sera or in three of the four 12-week sera; however, the serum of rabbit 1 acquired approximately 0.05 antitoxic U/ml by age 12 weeks.

DISCUSSION

This study was undertaken with the following rationale. (i) Oligosaccharide haptens coupled to protein at an optimal multiplicity (19) might be more likely to engage a T lymphocyte helper effect (21) than conjugates made with the corresponding polymer. (ii) The use of linkers or spacers may cause an unnecessary immunization against linker determinants (19). Ideally, as long as the antigenicity of the hapten were not compromised, direct coupling would be desirable. (iii) The protein toxoids used as vaccines in infancy would be good candidates as carriers. Conservation of antigenicity of the carrier might be important, particularly if a carrier-specific helper effect (21) were to be gained from prior vaccination with the toxoid.

Reductive amination (27) seemed worthwhile to evaluate. In this method reducing carbohydrates are directly joined to the amino groups of lysyl residues, producing a stable secondary amine linkage. Conjugation may proceed with minimal modification or denaturation of the protein; e.g., enzymes have been conjugated with good retention of enzymatic activity (18). The first synthetic glycoproteins made by reductive amination were well-defined conjugates with mono- and disaccharides (27). Quite recently,

TABLE 2. Serum antibody responses of weanling rabbits to control vaccines or to conjugates of CRM_{197} with PRP oligosaccharides of series no. 1

Vaccine injected[a]	Rabbit no.	Antibody response at age of animal					
		Anti-PRP (ng/ml) by radioantigen binding				Bactericidal titer[b]	
		7 wk	8 wk	9 wk	10 wk	7 wk	10 wk
PRP lot 19	1	<10	12	28	40	<2	<2
	2	<10	<10	27	26	<2	<2
C-control no. 1	3	35	25	31	36	<2	<2
	4	16	34	40	48	<2	<2
Conjugate C-P_S no. 1	5	19	980	26,000	49,000	<2	128
	6	<10	84	23,000	31,000	<2	256
Conjugate C-P_M no. 1	7	<10	37	2,500	11,000	<2	16
	8	23	11,000	49,000	150,000	<2	64
Conjugate C-P_L no. 1	9	14	73	3,700	26,000	<2	64
	10	<10	340	9,800	76,000	<2	32

[a] Injections of 25 μg of the indicated vaccine were given immediately after the bleedings at ages 7, 8, and 9 weeks.

[b] Reciprocal of highest dilution of serum giving >90% killing of 3×10^2 colony-forming units of *H. influenzae* type b strain Eag in the presence of 6% complement (antibody-free calf serum).

reductive amination has been used with the intent of enhancing the immunogenicity of bacterial capsular polysaccharides. Conjugates of tetanus toxoid with oligosaccharides made from groups A and C meningococcal capsules were found to induce anticapsular antibodies in rabbits and mice. Although the effect of conjugation upon the carrier was not directly examined, some induction of anti-tetanus antibody was inferred from immunodiffusion patterns (14). Intact pneumococcal type 19 polysaccharide was conjugated to several carrier proteins, producing an enhanced anti-capsular response in mice; the anti-carrier responses were not examined, but the conjugates retained some reactivity with antisera to the carrier proteins (16).

In series no. 2 of the present study, reductive amination gave preparations having the desired properties of enhanced immunogenicity for the saccharide component with retention of immunogenicity of the carrier protein. Formation of a covalent linkage has not been demonstrated chemically, but can be inferred from the fact that the PRP antigenicity became both $(NH_4)_2SO_4$ precipitable and nondialyzable in the presence of 8 M urea. Since the isolation method after the conjugation reaction would not separate conjugate from unconjugated CRM_{197}, it remains to be demonstrated that the very CRM_{197} molecules bearing immunogenic amounts of PRP oligosaccharides have conserved the antigenicity of the protein. However, since the ELISA reactivity of preparations C-P no. 2a and no. 2b were essentially equal to that of unreacted CRM_{197}, it would be unlikely that all this activity was provided by unconjugated protein molecules. Planned future work includes separation of conjugates from any unconjugated protein that might remain, to test the point directly.

The (routine) vaccination of infants with DTd constitutes a potential priming with carrier that could enhance the subsequent response to a hapten conjugated to the carrier (21). (The concept has recently been challenged [12]). The possibility of a carrier priming effect was examined in a limited fashion by administering conjugate no. 2b to rabbits without or with a prior immunization with DTd (Table 3). The anti-PRP response appeared to begin after the first injection of conjugate only in the two primed animals; however, after the second and third, there was no obvious advantage. Clear demonstration of a priming effect (or its absence) in this system would require exploring the relevant variables with a large number of animals.

CRM_{197} results from a missense mutation in the gene for the A chain of DTx, rendering the molecule nontoxic, although antigenically indistinguishable from the toxin (20, 30). It has advantages as a starting material for a conjugate: relative to native toxin there is no biohazard in working with it; relative to ordinary toxoid, in which some amino groups are formalized, more of the lysyl sidechains are available for conjugation. It is a reasonable assumption that conjugation of diphtheria toxin (or toxoid) with PRP oligosaccharides would tend to reduce toxicity. It is conceivable, however, that the tendency for certain preparations of formalized purified diphtheria toxin to spontaneously regain toxicity (17)

TABLE 3. Antibodies to PRP and to DTd in unprimed or DTd-primed rabbits injected with conjugates of CRM_{197} with oligosaccharide S of series no. 2

Rabbit no.	Primed with DTd[a]	Conjugate[b]	Assayed for antibody to:	Antibody value at age of animal				
				8 wk	9 wk	10 wk	11 wk	12 wk
1	−	$C\text{-}P_S$ no. 2a	PRP[c]	NT[d]	47	60	210	14,000
2	−	$C\text{-}P_S$ no. 2a	PRP[c]	NT[d]	<20	20	2,000	11,000
3	−	$C\text{-}P_S$ no. 2b	PRP[c]	NT[d]	<20	27	1,600	4,900
4	−	$C\text{-}P_S$ no. 2b	PRP[c]	NT[d]	23	<20	2,900	26,000
5	+	$C\text{-}P_S$ no. 2b	PRP[c]	NT[d]	<20	48	380	1,000
6	+	$C\text{-}P_S$ no. 2b	PRP[c]	NT[d]	22	180	3,500	11,000
1	−	$C\text{-}P_S$ no. 2a	DTd[e]	NT[d]	27	34	260	720[f]
2	−	$C\text{-}P_S$ no. 2a	DTd[e]	NT[d]	31	27	31	140[g]
3	−	$C\text{-}P_S$ no. 2b	DTd[e]	NT[d]	15	12	45	590[g]
4	−	$C\text{-}P_S$ no. 2b	DTd[e]	NT[d]	13	5	46	410[g]
5	+	$C\text{-}P_S$ no. 2b	DTd[e]	52	670	1,100	2,300	>3,000
6	+	$C\text{-}P_S$ no. 2b	DTd[e]	39	950	1,600	2,800	>3,000

[a] Primed by subcutaneous injection at age 8 wk with 100 μg of DTd in 0.5 ml of 0.0125 M aluminum phosphate suspension.

[b] Conjugate was injected subcutaneously at ages 9, 10, and 11 weeks at a dosage of 8 μg of protein in 0.0125 M aluminum phosphate suspension.

[c] Expressed as nanograms of anti-PRP antibody per milliliter of serum as determined by radioantigen binding. Two rabbits receiving no vaccine had no significant rises.

[d] NT, Not tested (irrelevant).

[e] Expressed as absorbance at 400 nm (1-cm light path) × 1,000 per hour generated in ELISA by a 1:100 dilution of the serum. Two rabbits receiving no vaccine had no significant rises.

[f] Contained approximately 0.05 U of antitoxic activity per ml.

[g] Contained <0.01 U of antitoxic activity per ml.

might be increased by saccharide substitution. Thus, with toxin or ordinary toxoid as carrier, there is at least a hypothetical possibility of toxicity that is circumvented by the use of CRM_{197}. A disadvantage at present is the low yield of CRM_{197} in cultures. The available strains of *C. diphtheriae* containing phage β197 produce much less of the protein than the strains developed for production of native toxin. Increased yields of CRM_{197}, which may be possible through genetic manipulation of the structural gene, would facilitate the synthesis of sufficient amounts of conjugates for biochemical analysis.

In conjugate series no. 1 the CRM_{197} (inadvertently) was antigenically denatured, apparently from exposure to cyanoborohydride before the addition of buffer. Nonetheless, there was a highly enhanced anti-PRP response, implying that the enhancement may be due as much to general physicochemical properties of the conjugate as to the particular antigenic specificity of the carrier. In this series, the chain lengths of oligosaccharides S, M, and L put into the conjugation reactions differed greatly. (The lengths and multiplicities actually attached were not defined.) A differential in anti-PRP response might have been expected on the basis of several hypotheses, e.g., terminal and nonterminal determinants in a saccharide chain can differ sharply in antibody elicitation (24), and the ratio of these would be quite different between the S and L preparations. Moreover, in one well-defined synthetic antigen model system, the intramolecular distance between the antigenic determinant and the T lymphocyte-dependent carrier could not exceed a discrete limit without loss of helper activity (7); if the terminal sugars were immunodominant in PRP, such a distance would vary considerably with haptens S, M, and L. Within the limitations of the single dose size and the few animals examined, however, comparable anti-PRP responses, determined by radioassay or by bactericidal activity, were found to preparations S, M, and L. Likewise, conjugates appearing to differ considerably in the ratios of oligosaccharide S to CRM_{197} (no. 2a and no. 2b) produced similarly enhanced anti-PRP responses. It may not be worthwhile to investigate the variables of chain length and multiplicity in great detail in the rabbit, for the conclusions may not hold for the immature human lymphoid system. Rather, these variables will be more thoroughly explored as experimental immunization of humans is approached. Such experiments are encouraged by the immunogenic potency of these conjugates in the weanling rabbit. Preparations no. 2a and no. 2b were used in a dosage (<3 μg of protein per kg of body weight) and with an adjuvant ($AlPO_4$) quite acceptable for the vaccination of human infants. In one rabbit the diphtheria antitoxin approxi-

mated a level considered protective in humans (0.03 U/ml, by Schick testing). The anti-PRP antibodies reached levels 7- to 170-fold that estimated to be protective against systemic *H. influenzae* b diseases under field conditions (150 ng/ml) (22).

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