PREPARATION, CHARACTERIZATION, AND IMMUNOGENICITY OF *HAEMOPHILUS INFLUENZAE* TYPE b POLYSACCHARIDE-PROTEIN CONJUGATES

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Serum anticapsular polysaccharide antibodies confer immunity to invasive diseases caused by *Haemophilus influenzae* type b (HIB)[1] (1–10). These anti-type b antibodies exert their protective effect by initiating complement-mediated activities including opsonization and bacterial lysis (2–5, 11). A protective level of ~0.15 μg of anti-type b antibodies/ml serum was estimated based upon analyses of patients with X-linked hypogammaglobulinemia who were passively immunized with immunoglobulin and on analyses of vaccinees injected with the purified HIB capsular polysaccharide (HIB Ps) (6–8, 11–13). In two field trials the HIB Ps induced a protective immune response only in children older than 18–24 mo (6–8). Reinjection of the HIB Ps did not induce a booster effect at any age (8, 13, 14). This age-related immunological behavior to HIB Ps in humans is similar to that observed with other purified bacterial capsular polysaccharides (14–17) and has been characterized as "thymic-independent" (12, 18, 19). To achieve immunoprophylactic control of meningitis caused by HIB and other encapsulated bacteria, a more effective vaccine for infants, perhaps with "thymic-dependent" properties, must be developed.

Goebel (20) and Avery and Goebel (21, 22) prepared protein conjugates of the pneumococcus type 3 polysaccharide as well as of its repeating dissacharide unit, cellobiuronic acid. In contrast to the poor immunogenicity of the purified type 3 polysaccharide in rabbits, these protein-carbohydrate conjugates induced high levels of serum antibodies that increased in concentration with reinjection and were protective against challenge with the live organisms (23, 24). Other investigators, using carbohydrates derived from pathogenic bacteria, have prepared covalently bound conjugates with proteins, noncovalently bound protein-polysaccharide complexes, or polysaccharide-erythrocyte conjugates. These products revealed enhanced immunogenicity of the carbohydrate moiety (25–36). In some cases, the conjugate-induced anti-carbohydrate antibodies were shown to have a protective effect against the bacteria from which they were derived. Many investigators injected these conjugates

[1] *Abbreviations used in this paper* Ab, antibody, ADH, adipic acid dihydrazide, AH, adipic hydrazide, BCG, Calmette-Guérin bacillus, BSA, bovine serum albumin CFA, complete Freund's adjuvant, CNBr, cyanogen bromide, EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HIB, *Haemophilus influenza* type b; HIB ps, purified HIB capsular polysaccharide, HSA, human serum albumin, Johnson, Johnson-Yale strain of HIB; Kd, partition coefficient; LPS, lipopolysaccharide, Mad, Madigan strain of HIB, PBS, phosphate-buffered saline, Pn 3, pneumococcus type 3 capsular polysaccharide, TNBS trinitrobenzene sulfonic acid

in complete Freund's adjuvant (CFA) and/or by the intravenous or intraperitoneal routes, factors that limit their applicability for use in humans.

In this study, conjugates of HIB Ps and serum albumins, HIB Ps and *Limulus polyphemus* hemocyanin, and HIB Ps and diphtheria toxin were prepared. Their composition, molecular size, and immunogenicity, both as saline solutions and in CFA, were characterized in several laboratory animals. The effect of the carrier molecule upon the immunogenicity of the HIB Ps was studied by comparing the serum anti-HIB Ps response to conjugates prepared with these proteins to those prepared with two polysaccharides: HIB Ps itself and pneumococcus type 3 polysaccharide.

Materials and Methods

Chemicals. The following reagents were used: Adipic acid dihydrazide (ADH) (lot A8X), trinitrobenzene sulfonic acid (TNBS) (lot 8746), and cyanogen bromide (CNBr) (lot KL23X), all from Eastman Kodak Co., Rochester, N. Y., 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDAC) (lot 18733) and sodium dodecyl sulfate, both from Bio-Rad Laboratories, Richmond, Calif.; hexadecyltrimethylammonium bromide (Cetavlon), NAD (grade AA; lot 36C01097), RNAase A (type II-A; lot 16C-8055), pronase (protease type VI) (lot 56C-0914), and bovine serum albumin (ESA), (lot 58C-8040), all from Sigma Chemical Co., St Louis, Mo.); human serum albumin (HSA) (lot NC602), from Cutter Laboratories, Inc., Berkley, Calif.; DNase I (lot D-56M631), from Worthington Biochemical Corp., Freehold, N J.; CL-4B Sepharose (lot 12985), from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J., brain-heart infusion, from Difco Laboratories, Detroit, Mich.) The HSA was treated with activated charcoal (Norit A; Fisher Scientific Co., Pittsburgh, Pa.) as described elsewhere (37) The diphtheria toxin was prepared by Dr. John Robinson, Vanderbilt University, Nashville, Tenn. (Food and Drug Administration contract 223-73-12367) according to a published method (38) The hemocyanin was prepared from the cell-free hemolymph of the horseshoe crab (*L. polyphemus*) (39) The fluid was centrifuged three times at 150,000 *g*, for 5 h at 4°C The dark blue pellet was redissolved in phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 16,000 *g*, for 1 h at 4°C, then centrifuged at 150,000 *g*, for 5 h at 4°C The pellet, resuspended after the third ultracentrifugation, was passed through a sterile 0.45-nm membrane, (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) and stored at 4°C Acid-cleaned glassware and double-distilled pyrogen-free water were used.

Polysaccharides. HIB strains 1482, Madigan (Mad), Eagen (kindly provided by Dr Porter Anderson and Dr David H. Smith of the Department of Pediatrics, University of Rochester, Rochester, N. Y.), Johnson-Yale (Johnson), and Rab were grown in Levinthal media supplemented with 10 mg/liter of NAD and 0.5 g/liter each of $CaCl_2$, $MgSO_4$, and citric acid. The culture was grown at 37°C with high aeration and was stirred up to the late logarithmic-growth phase The cells were harvested by centrifugation, and the paste was suspended in 1.0% Cetavlon, 0.5 M NaCl (~50 g wet weight of cells/liter) and stirred vigorously at 37°C for 2 h (E. C Gotschlich. Unpublished observations.) The suspension was centrifuged at 16,000 *g* for 2 h at 4°C, and the pellet discarded. The supernate was brought to 25% ethanol, stored overnight at 3–8°C, and then centrifuged at 16,000 *g* for 1 h at 4°C The pellet was discarded, and the supernate brought to 70% ethanol and stored overnight at 3–8°C The precipitate was removed by centrifugation at 2,500 *g*, at 4°C and washed two times with 95% ethanol. The precipitate was treated first with DNase I and RNAase A followed by pronase and was extracted with cold phenol, pH 7.0, to remove residual protein (40–42). The resultant product was dialyzed against 0.1 M $CaCl_2$ and then ultracentrifuged to remove lipopolysaccharide (LPS), dialyzed exhaustively against double-distilled water at 3–8°C, and freeze-dried as described (41). The final yield was ~50 mg of HIB Ps/liter culture fluid Pneumococcus type 3 capsular polysaccharide (Pn 3), manufactured for the National Institutes of Allergy and Infectious Diseases (NIAID) by the Eli Lilly & Co., Indianapolis, Ind. (lot 812346), was kindly provided by Dr James C. Hill of the NIAID, National Institutes of Health (NIH). This Pn 3 preparation contained 50.0% wt/wt uronic acid and had <2.0% protein and nucleic acids

Escherichia coli K13 Ps was kindly donated by Dr Willie Vann, Bureau of Biologics, Food and Drug Administration

Analytical Methods The protein, nucleic acid, moisture, endotoxin (LPS) content of the HIB Ps preparations were assayed as described (42–44) The polysaccharide contents of the HIB Ps solutions and of the conjugates were measured by the Bial reaction using an HIB Ps sample dried to constant weight over P_2O_5 in vacuo at 37°C (45) The hydrazide content of the derivatized proteins and polysaccharides was estimated by the TNBS method and is expressed as mol adipic hydrazide (AH)/mol protein (46, 47) shown as the numerical designation after the carrier (e.g.; Johnson-BSA-33). Briefly, 0.5 ml of the ADH standards or of the unknowns was added to 0 5 ml of a saturated solution of sodium borate equilibrated at room temperature A 0 15-ml aliquot of 3 0% TNBS solution was added to each tube, mixed, and the absorbance was read within 5 min at 500 nm. The readings obtained with the native protein (usually <10% of the derivatized protein) were subtracted. The sensitivity of the assay was 5×10^{-6} mol of ADH The number of AH residues/mol protein is shown as the numerical designation after the carrier.

Hyperimmune Antisera. Burro and rabbit HIB typing antisera were prepared in our laboratory and were also obtained from Ms. Leslie Wetterlow, Massachusetts Public Health Biologics Laboratories, Jamaica Plains, Mass and Dr. Lucille Greenwood, Michigan State Public Health Laboratories, East Lansing, Mich. (10).

Serologic Serum HIB anticapsular antibodies were measured by a radioimmunoassay having a sensitivity of 0.03 μg antibody (Ab)/ml and a reproducibility of ± 10% (12). Serum complement-dependent bactericidal antibodies were assayed as described using precolostral calf serum as a complement source and the Eagen strain as the target organism (10). Immunodiffusion analysis employed 1.0% agarose in saline under previously described conditions (41). Quantitative precipitin analysis employed 0 2-ml aliquots of the HIB Ps and 0 2 ml of burro 132 HIB immunoglobulin (14.1 mg anti-type b Ab/ml).

HIB PS-Protein Conjugates. AH derivatives of BSA, HSA, hemocyanin, and diphtheria toxin (collectively designated AH-protein) with varying degrees of derivatization were prepared (47–49). Protein solutions (25 mg/ml) and ADH (3.45 mg/mg protein) were reacted with three different concentrations of EDAC (0 1, 0.3, and 0.6 mg/mg protein) The pH of the reaction mixture was maintained at 4.7 ± 0.2 with 0.1 N HCl The reaction proceeded at room temperature for 3 h and the reaction mixtures were dialyzed at 3–8°C with two changes/d against 6 liter of 0 2 M NaCl The albumin and Pn 3 polysaccharide derivatives were then dialyzed against two 6-liter changes of deionized water and freeze-dried. The diphtheria toxin and hemocyanin derivatives were then dialyzed against two changes/d of PBS, sterile-filtered, and stored at 3–8°C. The HIB Ps was activated with CNBr as described (12, 49, 50) Briefly, a solution of HIB Ps (5.0 mg/ml), equilibrated at 4°C, was rapidly brought to pH 10.5 with 0.1 N NaOH. 100 mg/ml CNBr was added to a final concentration of 0.4 mg/mg polysaccharide, and the pH maintained at 10.5 for 6 min. Then the reaction mixture was brought to pH 8 5 with 0.5 M $NaHCO_3$, and the CNBr-activated HIB Ps added to an equal weight of AH-protein or Pn 3 (50 mg/ml dissolved in 0 5 M $NaHCO_3$). The reaction mixture was tumbled gently overnight at 3–8°C and then centrifuged at 16,000 *g*, 4°C for 20 min. The supernate was passed through a CL-4B Sepharose column, 1 5 × 90 cm, that was equilibrated with 0 2 M ammonium acetate. The void-volume fractions were pooled, dialyzed against 0.01 M phosphate-buffered 0.145 M NaCl, pH 7.0, at 3–8°C, and passed through a 0 45-nm membrane (Nagle Co., Nalgene Labware Div.) and stored at 3–8°C.

Animals. BALB/c, C57BL/6, and general-purpose mice from the NIH colony, weighing ~20 g, were injected subcutaneously with saline solutions of the conjugates or of the controls Female white albino rabbits, weighing ~1.0 kg, were injected with 35 μg of the Johnson-BSA-33 conjugate or with one of the controls emulsified in 3 0 ml of CFA prepared with 1 volume of the antigen in PBS, 0.7 volume of Arlacel A (Atlas Chemical Co., Miami, Fla.), 1 4 volumes Bayol 92 (Exxon Chemical Co., N Y), and 10 mg of dried Calmette-Guérin bacillus(BCG)/animal (prepared by Dr. Quentin Myrvik, Bowman Gray College of Medicine, Winston-Salem, N C.)

Mutagenesis Assay Three compounds were assayed for their mutagenic and toxicity potential using the assay of Ames et al (51) and McCann et al (52). Five *Salmonella typhimurium* strains,

TA1535, TA1537, TA1538, TA98, and TA100, were used with and without mouse liver S-9 fraction Undiluted ADH (500 μg/plate), Johnson-BSA-33 (68.3 μg/plate) and BSA-33 (300 μg/plate), and fourfold dilutions of each showed no toxicity or mutagenicity at the highest concentrations tested. These assays were kindly performed by Dr E. J. Lazear, Division of Mutagenesis Research, National Center for Toxicological Research, Food and Drug administration

Results

Characterization of HIB Ps-Protein Conjugates. The molecular sizes of the HIB Ps, the AH-protein derivatives, and a typical HIB Ps-albumin conjugate, Johnson-BSA-33, is illustrated in a gel filtration using CL-4B Sepharose (Fig. 1). The AH-BSA showed a major symmetrical component with a partition coefficient (Kd) of 0.71 and a small peak (~5% of the preparation) with a Kd of 0.60. The HIB Ps Johnson showed a symmetrical peak with a Kd of 0.33. Gel filtration of the HIB Ps-protein conjugate, Johnson-BSA-33, showed a new component, with a larger molecular size than either the HIB Ps or the AH-protein derivative, passing unretarded through the column (Kd = 0.00) with both protein and polysaccharide. There was second peak containing ~15% of the protein and ~50% of the Hib Ps.

Not shown are the elution profiles obtained with the same materials passed through a CL-4B Sepharose column equilibrated with 1.0% SDS. The HIB Ps had a smaller molecular size with the SDS solvent yielding a Kd of 0.60. The AH-BSA had a Kd of 0.70 with a slight trail. The mixture of these two showed the same patterns. The Johnson-BSA-33 conjugate showed a void-volume peak and a component (~40%) with a Kd of 0.35. All the fractions obtained with the HIB Ps-protein conjugate showed both protein- and pentose-active material.

Immunodiffusion analysis of the HIB Ps-protein conjugates with antisera, prepared either by intravenous injections of whole formalinized organisms (typing antiserum) or by immunization with the Johnson-BSA-33 conjugates in CFA, are shown in Fig. 2. With the HIB typing antisera, a single precipitin line was observed with HIB Ps

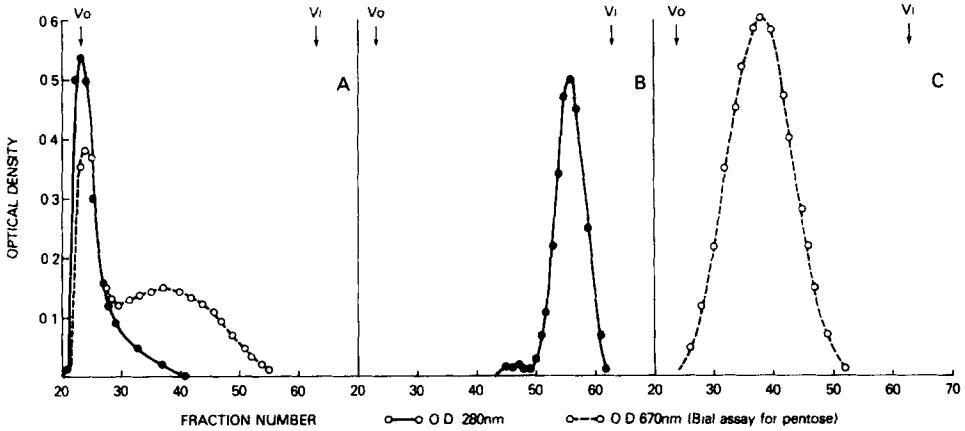

FIG 1 Gel filtration through CL-4B Sepharose of. (A) HIB Ps-Johnson-BSA-33 conjugate, (B) AH-BSA-33, and (C) Johnson HIB Ps The protein content of the fractions was measured by recording the absorbance at 280 nm and the polysaccharide was measured by the Bial reaction (absorbance at 670 nm) (44) The fractions 22-28, containing both protein and polysaccharide components, were pooled, dialyzed against saline, and sterile-filtered for further studies and have been designated as Johnson-BSA-33 Vi, total volume of column, Vo, void volume

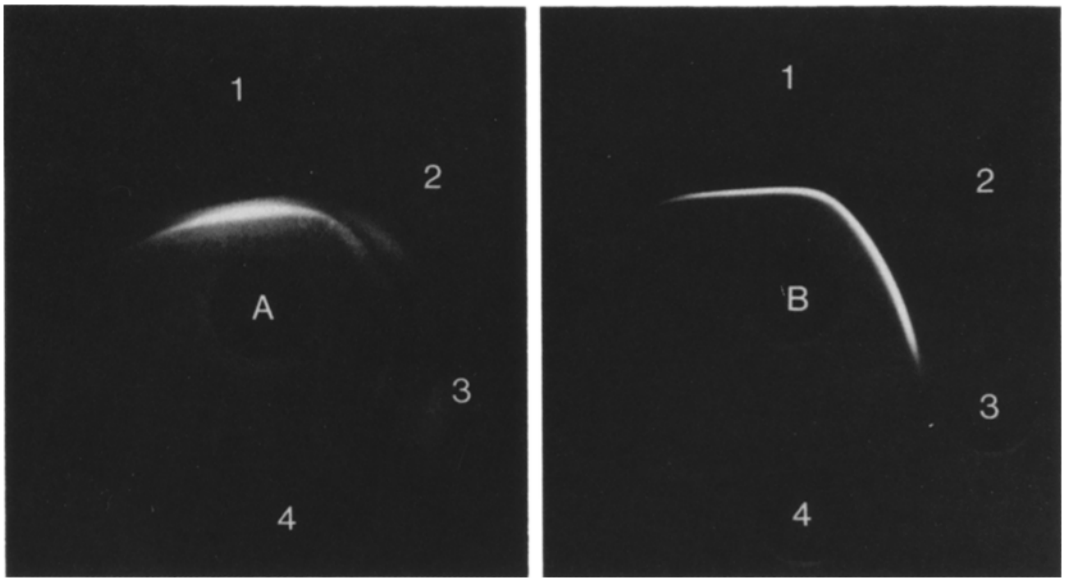

FIG 2 Immunodiffusion of HIB Ps, HIB Ps-BSA conjugate, *E coli* K13-BSA conjugate, and *E coli* K13 Ps with antiserum from rabbit No 2 injected with Johnson-BSA-33 in CFA (center well A) (See Table VII) and B132 HIB typing serum (center well B) For (A) and (B) well 1 HIB Ps, 0 2 mg/ml, well 2 Johnson-BSA-33, 0.5 mg/ml; well 3. *E coli* K13-BSA-29 conjugate, well 4 *E coli* K13 polysaccharide, 0 5 mg/ml

and the HIB Ps-BSA conjugate (Fig. 2 B). There was no precipitin reaction with the AH-BSA alone or with the *E. coli* K13-BSA-33 conjugate. A single precipitin reaction was observed with the rabbit antisera raised against the Johnson-BSA-33 conjugate, and the HIB Ps, Mad-BSA-33, and *E. coli* K13-BSA conjugates (Fig. 2 A). No reaction with the *E. coli* polysaccharide was observed with either antisera. These observations, using gel filtration in aqueous and dissociating solvents and immunodiffusion, indicate that the HIB Ps and protein are covalently bound.

Table I shows the Kd values for the various HIB Ps preparations. The molecular size of the HIB Ps conjugates exceeded that of either component. The ratio of the HIB Ps. to protein in the various conjugates varied from 1.15 (Rab-BSA-29) to 0.16 (Mad-hemocyanin-29). All the HIB Ps-protein conjugates induced serum anti-type b antibodies in general-purpose mice, although a rigorous analysis of their comparative immunogenicity was not carried out. The approximate yield of the conjugate was estimated by comparing the total amount of HIB Ps covalently bound to the protein carrier in the void-volume fractions to that in the lower-molecular-weight fractions after gel filtration through CL-4B Sepharose.

Two conjugates were prepared using AH derivatives of Pn 3 and HIB Ps (Mad-Pn 3-33 and Mad-Mad-12). Both polysaccharide-polysaccharide conjugates passed unretarded through the CL-4B Sepharose column.

Effect of Dose upon the Immunogenicity of HIB PS-Protein Conjugate. In many preliminary experiments involving the three mouse strains used in the following experiments, only an occasional animal responded to injection of purified HIB Ps (R. Schneerson, O. Barrera, C. M. Hardegee, W. B. Habig, and J. B. Robbins. Unpublished observations.). The immunogenicity of the conjugates was characterized by comparing

TABLE I

Physicochemical Characteristics of Haemophilus influenzae Type b Conjugates

Preparation	Molecular size		Approximate yield
	HIB Ps	HIB Ps-protein conjugate	
			%
Johnson-BSA-33	0 33	0 77	33
Mad-BSA-29	0 30	1 0	25
Rab-BSA-29	0 38	1 15	20
1482 BSA-33	0 45	0.84	25
Mad-HSA-17	0 30	0 43	10
Mad-diphtheria toxin-5	0 30	0 89	25
Mad-diphtheria toxin-10	0 30	1.68	21
Mad-hemocyanin-29	0.30	0.16	26
Mad-Pn 3-33	0 30	—	18
Mad-Mad-12	0 30	—	25

The molecular size of the HIB Ps and HIB Ps-protein conjugates is characterized by their Kd through CL-4B Sepharose (41). The main component of the conjugates passed through the column in the void volume and, therefore, had a Kd of 0.00. The ratio of HIB Ps carrier in the conjugates was determined by measurement of the HIB Ps using the Bial reaction and the measurement of protein was determined by the method of Lowry (41, 42). The yield of conjugate is expressed as the percent of HIB Ps in the conjugate passing unretarded through the CL-4B Sepharose column The number of mol AH residues/mol protein, as estimated by the TNBS reaction (43), is shown as the numerical designation after the carrier

both the percentage of responding recipients and the level of the response compared to the controls. Animals were considered responders if they reacted to immunization with serum anti-type b Ab $\geq$0.15 μg Ab/ml (estimated to be the protective level in humans) (6–8, 11–13). The outbred general-purpose mice were chosen for these experiments. In a representative experiment, the effect of increasing doses of an HIB Ps-AH-BSA conjugate, Rab-BSA-29, injected 2 wk apart, upon the level of serum anti-type b antibodies 7 d after the last injection was studied (Table II). Doses of 0 01 and 0.1 μg of the Rab-BSA-29 conjugate failed to induce a detectable antibody response. At the 1.0 μg dose, 8/10 animals responded and the geometric mean of the responding animals was 1.5 μg Ab/ml. A slight increase in immunogenicity was observed with doses of 2.5 and 10 μg. With 2.5 μg, 9/10 of the animals responded with a geometric mean of 2.3 μg Ab/ml; with 10 μg, 9/10 responded with a geometric mean of 4.0 μg Ab/ml. Based upon these experiments and in consideration of the dosage of HIB Ps used in human infants (6–8, 12, 13, 16), 2.5 μg was chosen for further studies in mice

Effects of Repeated Injections of HIB Ps-Protein Conjugates in Mice Table III shows a representative experiment illustrating the effect of repeated immunizations with Mad-hemocyanin-29 upon the level of serum anti-type b A. Groups of 10 mice were injected with 2.5 μg of the HIB Ps-protein conjugate every 2 wk for a total of three doses. Groups of 10 mice were bled 2 wk after the first immunization and 1 wk after the subsequent immunizations and their sera assayed for HIB Ps antibodies Controls were unimmunized animals or animals injected with HIB Ps alone (data not shown) that were bled concurrently. There was an immune response after the first immuni-

TABLE II

Effect of Dose Upon Immunogenicity of HIB Ps-AH-BSA Conjugates

Micrograms conjugate injected*	Responders/total		Anti-type b antibodies	
			Geometric mean of responders	Range of all mice
		%	μg/ml	
0 01	1/10	10	—	<0 03–0 46
0.10	0/11	0	—	<0.03
1 0	8/10	80	1.50	<0 03–8 5
2.5	9/10	90	2.30	<0.03–31 0
10.0	9/10	90	4 00	0 05–33 0

General-purpose mice, weighing ~20.0 g, were injected subcutaneously twice (second injection two wk after the first) with varying doses of the HIB Ps-protein conjugate Rab-BSA-29. 1 wk after the second injection, the mice were bled and their sera assayed for HIB Ps Ab by radioimmunoassay (12) Responders are defined as those animals responding with serum HIB Ps Ab ≥0.15 μg/ml serum. Animals injected with comparable doses of HIB Ps or the AH-BSA derivative alone did not have detectable serum Ab.

* Calculated as micrograms HIB Ps

TABLE III

Effect of the Number of HIB Ps-Protein Conjugate Immunizations Upon the Level of Serum Anti-Type b Antibodies in Mice

Number of injections	Responders/total		Anti-type b antibodies	
			Geometric mean of responders	Range of all mice
		%	μg/ml	
1	5/10	50	1 40	<0 03–4.00
Control	0/10	0	—	<0.03
2	8/10	80	2 72	<0.03–17 0
Control	0/10	0	—	<0.03
3	8/10	80	3 26	0 04–33.0
Control	1/10	0	—	<0.03–0.48

General-purpose mice, weighing ~20 0 g, were injected subcutaneously with 2 5 μg of Mad-hemocyanin-29 conjugate one, two, or three times (mice that received a total of two or three injections received the subsequent injection[s] at 2-wk interval[s]). Controls were injected with 2.5 μg of Mad HIB Ps alone. The animals were bled 14 d after the first injection and 7 d after the second and third injections and their sera assayed for HIB Ps Ab by radioimmunoassay (12) Responders are defined as those animals responding with serum HIB Ps Ab ≥0.15 μg/ml

zation with the HIB Ps-protein conjugate (5/10 responders). The number of responders increased to 8/10 after the second and third injections. The geometric mean Ab level progressively increased from 1.40 μg/ml after the first injection to 2.72 μg/ml and then to 3.26 μg/ml after the second and third immunizations, respectively. There was no detectable serum antibody response among both control groups (saline or HIB Ps alone).

Immunogenicity of HIB Ps-Serum Albumin Conjugates in Three Mouse Strains. One outbred strain of mice, general-purpose mice from the NIH colony, and two inbred strains, BALB/c and C57BL/6, were injected with various HIB Ps-protein conjugates using a total of two 2.5-μg doses injected subcutaneously at a 2-wk interval. The mice

were bled 1 wk after the last injection. All three mouse strains showed at least 70% responders with geometric mean antibody levels from 0.50 to 2.5 μg/ml; individual levels of responders ranged from 0.16 to 19.0 μg/ml (Table IV). We could not detect any significant difference between the overall type b antibody response in the three mouse strains. Table V shows the anti-type b antibody response induced by the HIB Ps-diptheria toxin conjugates. Both lightly and heavily derivatized diphtheria toxin preparations formed immunogenic conjugates with 80 and 90% percent of the mice responding with geometric mean level of antibodies of 0.72 and 0.80 μg/ml, respectively. Controls showed only 1/20 responding.

Bactericidal Activity of HIB Ps-Protein Conjugates-induced Antibodies. In a representative experiment, a group of nine general-purpose mice were injected two times with 2.5 μg of Mad-BSA-29 2 wk apart and then bled 1 wk after the last injection. The bactericidal titer to HIB organisms and anti-type b Ab in the preimmune animals were 1/20 and <0.03 μg/ml, respectively. Their postimmune bactericidal titers to HIB organisms and anti-type b Ab levels were 1/525 and 2.50 μg/ml, respectively, in the immunized group and 1/62 and <0.03, respectively, in the controls ($P < 0.001$).

Immunogenicity of HIB Ps-Capsular Polysaccharide Conjugates. Two different prepara-

TABLE IV

Immunogenicity of HIB-Ps AH-Albumin Conjugates in Three Mouse Strains

Mouse strain	Conjugate injected	Responders/ total		Anti-type b antibodies	
				Geometric mean of responders	Range of all mice
			%	μg/ml	
GPM*	Johnson-BSA-33	7/10	70	0.50	<0.03–1.3
BALB/c	Mad-HSA-17	8/10	80	1.50	0.05–3.4
BALB/c	Rab-BSA-29	7/9	78	1.80	<0.03–19.0
C57BL/6	Rab-BSA-29	9/10	90	2.50	0.06–19.0

Mice were injected subcutaneously with 2.5 μg HIB Ps-albumin conjugate in 0.1 ml saline twice (second injection 2 wk after the first) The animals were bled 1 wk after the last injection and their serum anti-type b antibodies assayed Responders are those animals with a serum anti-type b antibody content ≥0.15 μg/ml

* General-purpose mice.

TABLE V

Immunogenicity of HIB Ps-Diphtheria Toxin Conjugates in BALB/c Mice

Conjugate	Responders/ total		Anti-type b antibodies	
			Geometric mean of responders	Range of all mice
		%	μg/ml	
Mad-diphtheria toxin-5	8/10	80	0.72	0.06–2.40
Mad-diphtheria toxin-18	9/10	90	0.80	0.06–5.05
Controls	1/10	10	—	<0.03–0.12

BALB/c mice were subcutaneously injected with one of the two diphtheria toxin–HIB Ps-protein conjugates or saline (controls) twice (second injection 2 wk after the first) and then bled 7 d after the second injection. Their sera was assayed for anti-type b antibodies by radioimmunoassay (12)

TABLE VI

Effect of Carrier Priming upon Serum Anti-Type b Antibodies in Mice Injected with HIB Ps-AH-BSA Conjugates

Initial injection	Experiment 1			Experiment 2			Total percentage of responders
	Responders/total		Geometric mean of responders*	Responders/total		Geometric mean of responders*	
		%			%		
BSA	4/10	40	0.56	12/20	60	0.40	53.3
AH-BSA-20	6/10	60	1.54	10/20	50	0.61	53 3
AH-BSA-33	7/11	64	0 69	6/20	30	0.28	41 9‡
Controls	2/9	23	0.36	2/30	7	0 41	10.3§

General-purpose mice were injected subcutaneously with 2.5 μg of BSA, or the AH-BSA derivatives used for preparation of the HIB Ps-protein conjugates, or saline. 2 wk later, they were injected with Mad-BSA-29 conjugates (2 5 μg HIB Ps-protein conjugate) and then bled 1 wk later Their sera were assayed for anti-type b antibodies by radioimmunoassay (12).

* Expressed as micrograms/milliliter anti-type b antibodies.

‡ $P < 0.001$ compared to each of the above percentages (53 3%)

§ $P < 0.01$ compared to the above percentage (41 9%)

TABLE VII

Serum Anti-Type b Antibodies in Rabbits Injected with HIB Ps-BSA Conjugate with CFA

Days after immunization	Immunogen											
	HIB-Ps-BSA conjugate			AH-BSA			Controls					
							HIB Ps			AH-BSA + HIB Ps		
	1*	2	3	4	5	6	7	8	9	10	11	12
							μg Ab/ml					
0	<0.03	0.06	0.40	0.09	0 06	0 07	0.12	<0 03	0 10	0.48	<0.03	0 08
10	280	320	88	0.05	0.11	0.13	0 18	0.05	0.90	0 15	<0 03	<0 03
30*	500	640	140	0.07	0.11	0.17	0 20	<0 03	0 45	0 16	<0.03	0 16
9	1480	4800	500	0.15	2.30	0.34	0.25	0 08	1 90	0 54	0.05	0 18
17	1480	4600	520	0 10	1 60	0.78	0.23	0.06	3 10	0.35	0 07	0 23

Animals reimmunized with original preparation in incomplete Freund's adjuvant. Young adult female rabbits were injected with 35 μg of Johnson-BSA-33 conjugate dissolved in 1.0 ml of saline, 2 ml of CFA, and 5 mg of dried, heat-killed BCG at multiple intradermal sites They were reinjected with the same material without the BCG 30 d later. Controls included the AH-BSA derivative alone, or the AH-BSA derivative mixed with the HIB-Ps, or the HIB Ps alone The anti-type b antibodies were measured by radioimmunoassay (14)

* Rabbit No

tions of conjugates formed by the reaction of CNBr-activated HIB Ps with AH derivatives of Pn 3 or HIB Ps itself (Table I) were injected into general purpose mice with doses of 0.5 and 2.5 μg HIB Ps/conjugate. The same dose was injected twice into 10 animals 2 wk apart. The mice were bled 1 wk after the last injection. There were no responders detected in all the experimental or the control mice (saline or HIB Ps alone).

Effect of Priming with Carrier Proteins upon Anti-Type b Antibodies Induced by HIB Ps-Protein Conjugates. Groups of 10 general-purpose mice were injected with 2.5 μg of BSA, AH-BSA-20 or AH-BSA-33, or saline. 2 wk later, they were injected with 2.5

μg of Johnson-BSA-33 conjugate in one experiment, and Mad-BSA-20 in another. The animals were bled 1 wk later. Table VI shows that a priming effect, as defined by an enhanced serum anti-type b A response after injection of the conjugate, was induced by the lightly ($P < 0.001$) and heavily AH-derivatized preparations ($P < 0.01$) and by the native BSA ($P < 0.001$). There was no effect upon the HIB Ps Ab response induced by saline injection.

Immunogenicity of HIB Ps-Protein Conjugates in Rabbits. Groups of three rabbits each were injected each with 35 μg of Johnson-BSA-33 conjugate emusified in CFA. The controls included the HIB-Ps or the AH-BSA-33 derivative alone, or the two physically mixed together. The animals were reinjected 30 d later with the same preparations emulsified in incomplete Freund's adjuvant. Table VII shows the serum anti-type b Ab at various intervals after the injections. The controls, injected with either HIB Ps, the AH-BSA derivative, or both mixed together yielded little or no increase in serum anti-type b Ab. The three rabbits that received the conjugate in CFA reacted with a 1,300- to 76,000-fold increase in anti-type b Ab with postimmune levels ranging from 520 to 4,600 μg anti-type b Ab/ml ($P < 0.001$).

Not shown are the results of the bactericidal Ab titers of the rabbit sera with the HIB strain Eagen. All preimmune sera had low bactericidal antibody titers ranging from 1/2 to 1/32. The HIB Ps-AH-BSA conjugate in CFA induced post-immune bactericidal titers of 1/1,024 to 1/8,192, whereas, the controls had levels ranging from 1/20 to 1/512 (rabbit 7). Absorption of the postimmune sera with the HIB Ps removed most or all of the bactericidal activity.

Discussion

HIB Ps is a polymer composed of the following repeating subunit·

$$\rightarrow 3)\text{-}\beta\text{-}D\text{-Rib}f\text{-}(1 \rightarrow 1)\text{-ribitol-5-}(PO_4 \rightarrow \quad (53).$$

There are no carboxylic acids or as-yet-detectable terminal aldehyde moieties to form direct covalent linkages with the amino groups of proteins or other potential carriers Further, the phosphodiester bond linking ribitol and ribose is labile at both acidic and basic conditions and at temperatures higher than 37°. Accordingly, we used CNBr activation under previously described conditions to create an electrophilic group on the HIB Ps (12, 49, 50). Although this intermediate HIB Ps product reacts efficiently to form a covalent bond with amino groups such as tyramine (12, 50), we were unable to form conjugates with several proteins including BSA. It has been reported that some proteins and other macromolecules require a spacer to quantitatively bind to another moiety such as an insoluble carbohydrate (48). Of the six-carbon moieties we studied as a spacer, only the ADH consistently provided relatively high yields of convalently bound HIB Ps-protein conjugates. Our initial experiments indicate that ~20 mol of AH/mol of carrier protein provide the most effective substrate for CNBr-activated HIB Ps The AH derivatization procedure caused some of the diphtheria toxin and hemocyanin to become insoluble. AH derivatives of bovine and human serum albumins, hemocyanin, diphtheria toxin, and Pn 3, prepared by the water-soluble carbodiimide condensation method, are stable for at least 6 mo and may be stored at 3–8°C. The reaction between these AH derivatives and the CNBr-activated HIB Ps is completed at pH 8.5 at 4°C in 15 h. We estimate that at least 90 percent of the protein is covalently bound to the HIB Ps and that the

average HIB Ps:protein ratio of immunogenic conjugates was 0.5–1.5:1. All seven HIB Ps-protein conjugates with these characteristics had similar immunogenicity in three mouse strains. Not shown were the results obtained with one HIB Ps-protein conjugate preparation, Johnson-BSA-33, that had depolymerization of the HIB Ps component during the CNBr-activation step. This conjugate had a Kd of 0.45 (smaller size than original HIB Ps) and an 8.33 ratio of HIB Ps:BSA. This conjugate had similar immunogenicity as the other higher-molecular weight-preparations. This finding suggests that there may be a wide range of molecular sizes and polysaccharide ratios that yield immunogenic polysaccharide-protein conjugates. This method of preparing protein-polysaccharide conjugates has been extended to *E. coli* K_1 and K_{13} capsular polysaccharides, which have carboxylic acid residues and do not contain phosphodiester bonds (41, 54; and R. Schneerson, O. Barrera, and J. B. Robbins. Unpublished observations.).

Our experiments offer some evidence that the HIB Ps has been converted from a thymic-independent to a thymic-dependent immunogen. This evidence may be summarized as follows: (*a*) The immunogenicity of the HIB Ps was increased when presented as a covalently bound protein-polysaccharide conjugate. The covalent bond seems to be necessary because the HIB Ps mixed with negatively charged macromolecules to form multiple electrostatic interactions, such as with poly-L-lysine, poly-L-Lys,Tyr,Ala, or methylated serum albumins, or methylated diphtheria toxin, does not invariably induce serum antibodies after subcutaneous injection of saline solutions into mice or rabbits (41, 54; and R. Schneerson, O. Barrera, C. M. Hardegree, W. B. Habig, and J. B. Robbins. Unpublished observations.). (*b*) The immunogenicity of the HIB Ps component of the conjugate is related to the carrier molecule. Carrier molecules composed of thymic-dependent immunogens such as serum albumins, hemocyanin, and diphtheria toxin, served to enhance the immunogenicity of HIB Ps. In contrast, conjugates prepared with the thymic-independent Pn 3 or the HIB Ps itself, though of comparatively high molecular size, failed to induce a serum anti-type b response. This finding is consistent with studies that show that covalent binding of haptens to thymic-independent molecules, such as pneumococcal capsular polysaccharide type 3, confer the immunogenic properties of the carrier molecule to the antigenic components of the conjugate (55, 56). Although a negative finding, such as the lack of immunogenicity, is difficult to interpret, the experiments with both the Pn 3-HIB Ps and the HIB Ps-HIB conjugates suggest that the molecular size of the conjugates, shown to be directly related to the immunogenicity of polysaccharides, may not be a critical variable of the immunogenicity of these hybrid molecules (40, 42, 43). Both of these polysaccharide-polysaccharide conjugates had considerably higher molecular-sizing characteristics than the parent molecule, yet they failed to induce serum antibodies. (*c*) Reinjection of HIB-Ps-protein conjugates induced an increase in both the number of responders and in the total level of anti-type b Ab (booster effect) (57–59). (*d*) Carrier priming, induced by the BSA and AH-BSA derivatives, resulted in an enhanced serum anti-type b antibody response to initial immunization with the conjugate (59–62). Further experiments to study the cellular basis for the enhanced immungenicity of the HIB Ps-protein conjugates and the molecular characteristics of the serum antibodies are planned (60, 61).

In preliminary experiments, the HIB Ps-protein conjugate induced an eightfold increase in anti-type b antibodies with bactericidal activity in young adult primates

(*Macaca fascicularis*). Injection of the purified HIB Ps into this, as well as other, primate species yielded a lesser serum anti-type b antibody response (62; L. Martin and P Anderson. Unpublished data.). Furthermore, the *E. coli* K1- and K13-protein conjugates induced serum anticapsular polysaccharide antibodies in rabbits and rats, respectively. Experiments are planned to study the comparative immunogenicity of these capsular polysaccharides bonded to different carriers as well as to characterize the reactivity to the carrier protein (60, 61).

The serum antibodies induced by the HIB Ps-protein conjugates exerted a complement-dependent bactericidal activity that has been associated with immunity against HIB type b diseases (1–12). The ADH, the AH derivative of BSA, and the HIB Ps-BSA conjugate showed no toxicity or mutagenic activity using an in vitro bacterial assay system (51, 52). Toxicity tests showed that the lethal dose was >100 mg/22- to 25-g laboratory mouse (63). Accordingly, this synthetic approach to prepare capsular polysaccharide-protein conjugates may be considered for clinical investigation.

Summary

A method is presented for covalently bonding *Haemophilus influenzae* type b capsular polysaccharide (HIB Ps) to several proteins. The method is efficient and relies upon the use of adipic dihydrazide as a spacer between the capsular polysaccharide and the carrier protein. In contrast to the poor immunogenicity of the purified HIB Ps in mice and rabbits, the HIB Ps-protein conjugates induced serum anti-type b antibodies having bactericidal activity at levels shown to be protective in humans when low doses were injected subcutaneously in a saline solution. The antibody response in mice was related to the dose of the conjugates, increased with the number of injections, and could be primed by the previous injection of the carrier protein. The HIB Ps-protein conjugates were immunogenic in three different mouse strains. The importance of the carrier molecule for the enhanced immunogenicity of the HIB Ps-protein conjugates was shown by the failure of HIB Ps hybrids prepared with either the homologous polysaccharide or pneumococcus type 3 polysaccharide to induce antibodies in mice Rabbits injected with the HIB Ps-protein conjugates emulsified in Freund's adjuvant produced high levels of serum anti-type b antibodies which induced a bactericidal effect upon *H. influenzae* type b organisms. It is proposed that the HIB Ps component of the polysaccharide protein conjugates has been converted to a thymic-dependent immunogen. This method may be used to prepare protein-polysaccharide conjugates with HIB Ps and other polysaccharides to be considered for human use.

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