PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **184**, 154–161 (1987)

# *Haemophilus influenzae* Type b Polysaccharide–Protein Conjugate Vaccine (42460)

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*Abstract.* An *Haemophilus influenzae* type b capsular polysaccharide–protein conjugate has been prepared. The polysaccharide was coupled to the serotype II protein of group B meningococcus through the spacer 6-aminocaproic acid using cyanogen bromide and water soluble carbodiimide. The conjugate can be shown to be reproducible and is stable and highly immunogenic in mice and African green monkeys. Clinical evaluation of this conjugate in children 3 months to 4 years of age showed that it elicited an antibody titer to the polysaccharide moiety greater than 1000 ng/ml in children 8 months of age or older. 

*Haemophilus influenzae* type b is the most common cause of bacterial meningitis in the United States (1, 2). Despite appropriate therapy, 7–8% of the children who develop *H. influenzae* type b meningitis die. In addition, many survivors suffer from serious sequelae, such as hearing and/or visual impairment, seizures, motor abnormalities, and mental retardation. (3) *H. influenzae* meningitis is a leading cause of acquired mental retardation (4, 5).

Results from a large clinical trial demonstrate that vaccination with purified capsular polysaccharide from the *H. influenzae* type b organism can protect children older than 18 months (6). Results from that trial and other data (7) indicate a possible correlation between protection from *H. influenzae* type b disease and the amount of serum antibody to the capsular polysaccharide; it has been proposed that a level of 1000 ng/ml of vaccine-induced antibody may be protective (8). Unfortunately, about 95% of the meningitis due to *H. influenzae* and two-thirds of all *H. influenzae* type b disease occur in children less than 2 years of age, and children less than 2 often fail to respond to the capsular polysaccharide vaccine (6, 9, 10).

Recent progress in developing a more efficacious vaccine has centered on conjugating the *H. influenzae* type b polysaccharide to a protein carrier. Anderson (11) has conjugated oligosaccharides derived from acid-hydrolyzed polysaccharide to a nontoxic diphtheria ($CRM_{197}$) protein. The conjugate elicited a strongly enhanced anti-capsular polysaccharide antibody in weanling rabbits. Schneerson *et al.* (12, 19) have conjugated the polysaccharide to various proteins, including BSA, limulus polyphemus hemocyanin, and diphtheria toxin through the spacer adipic acid dihydrazide. All conjugates showed greatly enhanced immune response in mice. These authors suggested that by coupling to a protein carrier, the polysaccharide was converted from thymic-independent to thymic-dependent immunogen.

In the present study, we report the conjugate of *H. influenzae* type b capsular polysaccharide to the serotype II protein of group B meningococcus through the spacer 6-aminocaproic acid (ACA). Results have shown this conjugate to be highly immunogenic in mice and African green monkeys. Clinical evaluation has demonstrated the immunogenic activity of the conjugate in children 8 months of age or older.

**Materials and Methods.** *Preparation of polysaccharide. H. Influenzae* type b strain Ross 768 (obtained from State University of New York) was grown in heart infusion broth (25 g/liter) supplemented with NAD (5 mg/liter), yeast extract dialysate (3 g/liter), and dextrose (5 g/liter). The culture was grown at 37°C to the late logarithmic-growth phase. The cells were inactivated with thimerosal (1:10,000, w/v) and removed from the culture medium by centrifugation; the polysaccharide was isolated from the culture medium. Approximately 180 liters of medium was concentrated to 4.6 liters using an Amicon DC-30 unit with XM-50 hollow fiber cartridges. Stepwise, precipitations were performed at 10, 20, and 50% ethanol concentrations. The pellet from 50% ethanol precipitation was sus-



0037-9727/87 $1.50

pended in 150 ml of $H_2O$ at 4°C and extracted with 200 ml of cold 2 *M* $CaCl_2$ for 30 min in an Omnimixer. The $CaCl_2$ extract was adjusted to 20% ethanol and allowed to stand for 18 hr. The mixture was centrifuged at 11,000*g* for 30 min and the pellet was discarded. The supernate was brought to 75% ethanol at 4°C and allowed to stand for 18 hr to precipitate the polysaccharide. The precipitate was washed three times with 100% ethanol and acetone. The polysaccharide then was resuspended in 0.49 *M* sodium acetate, pH 6.9, and extracted four times with phenol to remove the proteins. The phenol extract was centrifuged for 20 min at 11,000*g* at 4°C to break the emulsion. The aqueous phase was extracted three additional times with phenol and then dialyzed vs $H_2O$ overnight. The dialysate was brought to 0.05 *M* $CaCl_2$ and 75% ethanol and stored overnight at 4°C. The mixture was centrifuged at 27,000*g* for 30 min.

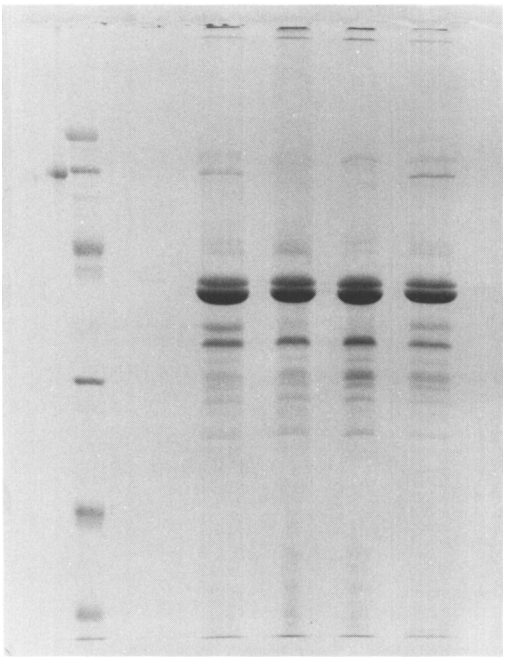

FIG. 1. Dodecyl sulfate–polyacrylamide gel electrophoresis (12.5%) of meningococcal group B serotype II protein preparations. Lanes 2–5 are different preparation lots. Molecular weight markers (lane 1) are phosphorylase b ($M_r$ = 94,000), bovine serum albumin ($M_r$ = 68,000), ovalbumin ($M_r$ = 45,000), carbonic anhydrase ($M_r$ = 30,000), soybean trypsin inhibitor ($M_r$ = 21,000), lysozyme ($M_r$ = 14,300).

The polysaccharide pellet was washed with absolute ethanol and acetone and dried over anhydrous $CaCl_2$ *in vacuo* at 4°C for 18–24 hr. The dried polysaccharide was resuspended in 200 ml of 0.05 *M* $CaCl_2$ and 20% ethanol. The mixture was centrifuged at 100,000*g* for 2 hr to remove lipopolysaccharide. The pellet was discarded and the supernate was brought to 75% ethanol and stored overnight. The polysaccharide was recovered by centrifugation and washed again with absolute ethanol and acetone. The final product was dried *in vacuo* as described above. The yield was 20 mg of polysaccharide/liter culture fluid. Several lots of polysaccharide were prepared for this study. They generally had size distributions between 0.31 and 0.38 kDa (Sepharose 4B); pentose, content 28.6–38.3%; phosphorous, 5.3–7.9%; nucleic acid, 0.1–0.5%; protein, 0.1–1.4%. All polysaccharide lots were also tested and passed the rabbit pyrogen test using specifications set for meningococcal polysaccharides (0.025 μg polysaccharide/ml/kg).

*Preparation of protein carrier.* Serotype II protein of group B meningococcus was used as the protein carrier in this study. The protein was prepared according to the procedure described by Helting *et al.* (13) As shown in Fig. 1, the protein pattern was consistent from preparation to preparation. All protein lots were tested and passed the rabbit pyrogen test as described above.

*Preparation of polysaccharide–protein conjugate.* Purified *H. influenzae* type b polysaccharide (10 mg) was dissolved in 2 ml of $H_2O$ and the pH was adjusted to 10.5 with 0.01 *N* NaOH at 4°C. The polysaccharide was activated with 7.5 ng of CNBr (in 75 μl of $H_2O$ for 6 min at 4°C) and the pH was maintained at 10.5. At the end of activation, the polysaccharide solution was mixed with 50 mg of 6-aminocaproic acid in 0.5 ml of 0.5 *M* borate buffer, pH 8.5, and incubated for 18 hr at 4°C. The mixture was dialyzed extensively against $H_2O$ and lyophilized. Lyophilized polysaccharide–ACA complex (10 mg) was dissolved in 2 ml of protein solution (5 mg/ml in $H_2O$) and the pH was adjusted to 5.0 with 0.01 *N* HCl. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide–HCl (10 mg) was added to the mixture which then was stirred for 120 min at pH 7.5. At the end of reaction, the solution

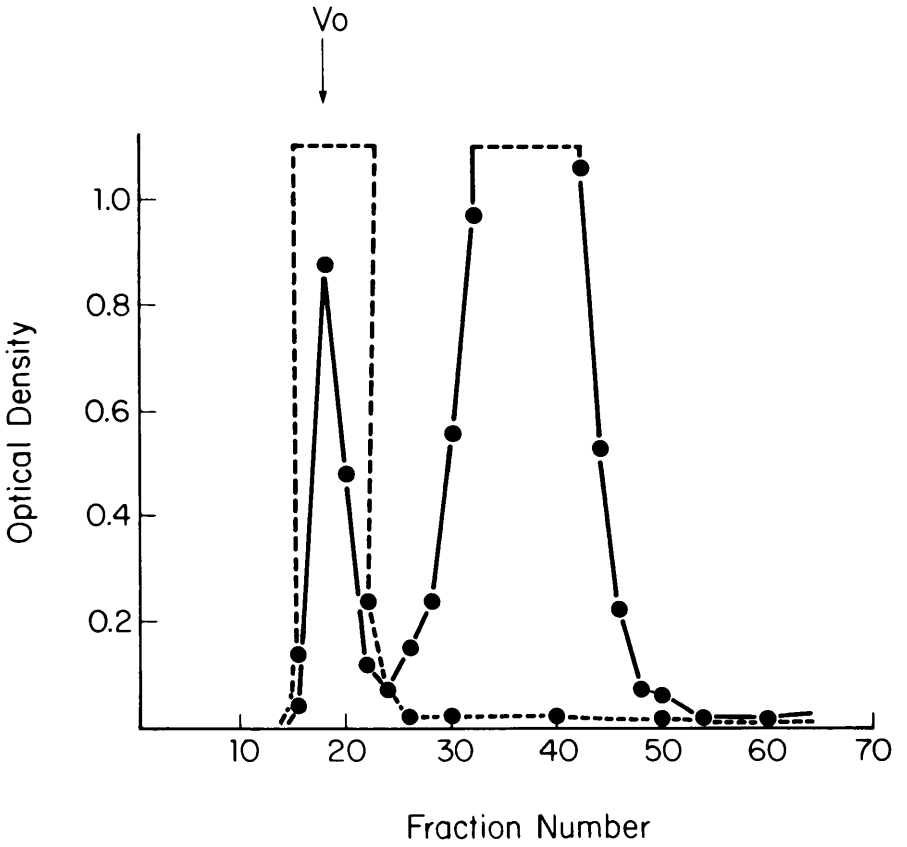


FIG. 2. Purification of *H. influenzae* type b polysaccharide–protein conjugate on Sepharose CL-4B column (1.5 × 90 cm) in 0.2 *M* ammonium acetate, pH 7.0. (—) OD 670 nm (Bial assay for pentose); (---) OD 280 nm.

was brought to pH 7.0 and centrifuged at 12,000*g* for 5 min. The supernate was placed on a Sepharose CL-4B column (1.5 × 100 cm), equilibrated, and eluted with 0.2 *M* ammonium acetate. The void-volume fractions were pooled and dialyzed against $H_2O$ overnight. Water and lactose were added to the conjugate solution to give a final concentration of 20 mg of polysaccharide conjugate and 4 mg of lactose/ml conjugate. The conjugate was lyophilized and stored at −70°C. All conjugate lots were tested and passed the rabbit pyrogen test as described above.

*Analytical methods.* Analysis of 6-aminocaproic acid was performed on a Beckman 120 amino acid analyzer by hydrolyzing the polysaccharide–ACA complex with 6 *N* HCl at 110°C for 20 hr. Polysaccharide was determined by the Orcinol reaction (14) using ribose as a standard. Protein was determined by Lowry using BSA as a standard. Antibody titers from mouse potency testing and human clinical trials were determined by radioimmunoassay. (15)

*Mouse potency test.* Groups of 10 female ICR/Ha strain mice, 5 weeks of age, were injected subcutaneously on Days 0 and 14 with 0.5 ml of capsular polysaccharide–protein-conjugated vaccine containing 2.5, 0.6, or 0.15 μg of polysaccharide. Control groups receiving either saline solution or unconjugated polysaccharide vaccine were treated similarly. Twenty-one days after initiation of the test, all animals had blood samples removed from the suborbital plexus; all resultant serum samples were assayed for antibodies to capsular polysaccharide by a radioimmunoassay procedure. (15)

TABLE I. PROFILES OF *H. influenzae* TYPE b POLYSACCHARIDE–PROTEIN CONJUGATES

| Conjugate | PS concentration (μg/ml) | Protein concentration (μg/ml) | PS/protein | Yield (%) | Mouse potency (ICR/Ha) RIA, ng ab/ml |
|---|---|---|---|---|---|
| HICT-53 | 88 | 324 | 0.27 | 11 | ≥4000 |
| HICT-108 | 49 | 224 | 0.21 | 11 | 8682 |
| HICT-121 | 98 | 500 | 0.19 | 12 | 8116 |
| HICT-124 | 60 | 310 | 0.19 | 10 | 4918 |
| HICT-125 | 99 | 440 | 0.22 | 14 | 19067 |
| HICT-136 | 20 | 844 | 0.23 | 10 | 6277 |
| Lot 940 | 20 | 133 | 0.15 | 9 | 3233 |
| Lot 941 | 20 | 143 | 0.14 | 8 | 9691 |
| Lot 942 | 20 | 125 | 0.16 | 12 | 6261 |
| Lot 949 | 20 | 125 | 0.16 | 14 | ≥8405 |
| Lot 950 | 20 | 111 | 0.18 | 17 | ≥3584 |

*Note.* PS, polysaccharide.

*Monkey potency test.* Groups of six African green monkeys of mixed sex and weighing 2–3 kg were injected subcutaneously with 1.0 ml of conjugate or polysaccharide vaccines at 0 time and 1 month later. Blood samples were removed immediately before each injection and 2 weeks after the second injection; the sera were assayed for antibodies to capsular polysaccharide by radioimmunoassay (15).

*Clinical study.* Conjugate vaccine prepared according to the described procedure was administered initially to 10 healthy adults without adverse effect (unpublished results). Following the trial in adults, 31 children in apparent good health residing in suburban Philadelphia were enrolled in the clinical trial. Informed written consent was obtained from the parent or guardian of each child. Sixteen children received two 10-μg doses of vaccine Lot 941 administered 1 month apart; four of these children received an additional 20-μg dose of vaccine about 2 months later. Ten children received a single 20-μg dose of vaccine and five received two 20-μg doses of the vaccine administered 1 month apart. Temperature readings were taken approximately 4 hr after vaccination and at daily intervals for 10 days. Observation for local and systemic reactions were carried out by physicians and qualified nurses aided by record forms provided to parents and guardians. Children were bled prior to and approximately 1 month after each vaccination. Sera were assayed for antibody to *H. influenzae* type b polysaccharide by radioimmunoassay (15) and to the meningococcal carrier protein by FIAX (16).

**Results.** *Conjugation of H. influenzae type b polysaccharide to protein carrier. H. influ-*

TABLE II. STABILITY OF *H. influenzae* TYPE b POLYSACCHARIDE–PROTEIN CONJUGATES (ANTIBODY RESPONSE OF MICE)

| | RIA Titer ng ab/ml (GMT) | | | | |
|---|---|---|---|---|---|
| Conjugate | Months: 0 | 3 | 6 | 9 | 13 |
| HICT-121 | 8116 | 13,429 | 8,436 | 11,585 | 6711 |
| HICT-124 | 4918 | 3,057 | 1,256 | 11,486 | |
| HICT-136 | 6277 | 8,316 | | | |
| Lot 940 | 3233 | 12,035 (55 days) | 8,964 | 15,670 | |
| Lot 941 | 9691 | 3,536 | 5,308 | 8,065 (7 months) | |
| Lot 942 | 6261 | 5,917 (50 days) | 13,759 (5 mo.) | 10,007 (7 months) | |

TABLE III. ANTIBODY RESPONSE OF MICE IMMUNIZED WITH *H. influenzae* TYPE b POLYSACCHARIDE–PROTEIN CONJUGATE VACCINE OR POLYSACCHARIDE VACCINE ALONE

| Sample | No. doses | Geometric mean antibody titer[a] to indicated vaccine dose: 2.5 mcg | 0.6 mcg | 0.15 mcg |
|---|---|---|---|---|
| Lot 941 conjugate | 1 | 2671 | 512 | 112 |
| Polysaccharide | 1 | <100 | — | — |
| Lot 941 conjugate | 2 | 9691 | 1646 | 624 |
| Polysaccharide | 2 | 129 | — | — |

[a] RIA titer (ng ab/ml).

*enzae* type b polysaccharide was coupled to the serotype II protein of group B meningococcus through the spacer 6-aminocaproic acid by CNBr and carbodiimide. Amino acid analysis showed that each milligram of polysaccharide–ACA complex contained 30–50 nmole of 6-aminocaproic acid. When purified on a Sepharose CL-4B column (Fig. 2), the conjugate was eluted at void volume. The conjugate retained its reactivity toward anti-polysaccharide antibody as shown by rate nephelometric assay (results not shown). Using the coupling procedure described here, the conjugate can be prepared reproducibly with a polysaccharide–protein ratio between 0.14 and 0.27 (Table I). The conjugate has been shown to be stable either at 4 or −70°C (Table II) when lyophilized in the presence of lactose.

*Mouse potency studies.* Conjugate vaccine (Table III) prepared according to the coupling procedure described above was shown to be highly immunogenic in ICR/Ha mice. Lot 941 was chosen as an example because it was used subsequently for the human clinical trial. After one injection of conjugate vaccine containing 2.5 μg of polysaccharide, all mice responded with antibody production while none of the mice receiving polysaccharide vaccine alone showed an antibody response. After the second injection, this difference was even more pronounced. The same conjugate also was tested in African green monkeys (Table IV). After one injection of conjugate vaccine containing 16.9 μg of polysaccharide, all monkeys (six of six) responded with a 20-fold increase in antibody titer. After the second injection, the GMT antibody titer increased another 2-fold; animals that received polysaccharide vaccine alone remained immunologically nonresponsive after two injections.

*Clinical study.* Children aged ≥2 years responded with good levels of antibody after one 10-μg dose of the vaccine (Table V) and all children ≥ 1 year showed protective levels of

TABLE IV. ANTIBODY RESPONSE OF AFRICAN GREEN MONKEYS IMMUNIZED WITH *H. influenzae* TYPE b POLYSACCHARIDE–PROTEIN CONJUGATE VACCINE, LOT 941, OR POLYSACCHARIDE CONTROL VACCINE

| | Monkey No. | Dose[a] (μg) | RIA titer (ng ab/ml): Individual Months: 0 | ~1 | ~1½ | GMT 0 | ~1 | ~1½ |
|---|---|---|---|---|---|---|---|---|
| Vaccine conjugate | 5 | 16.9 | <100 | 11750 | 18125 | 93 | 1852 | ≥3237 |
| | 6 | | <100 | 187 | 210 | | | |
| | 7 | | 192 | 12750 | ≥20000 | | | |
| | 8 | | 162 | 4400 | 9000 | | | |
| | 10 | | 170 | 1925 | 4800 | | | |
| | 11 | | <100 | 170 | 350 | | | |
| Polysaccharide control | 12 | 20.0 | <100 | <100 | 125 | 121 | 139 | 113 |
| | 13 | | 132 | 115 | 110 | | | |
| | 16 | | <100 | <100 | <100 | | | |
| | 17 | | 384 | 440 | 228 | | | |
| | 18 | | <100 | 120 | <100 | | | |
| | 19 | | 500 | 480 | 272 | | | |

[a] Of polysaccharide.

TABLE V. SERUM ANTIBODY RESPONSE OF CHILDREN INJECTED WITH TWO 10-μg DOSES OF *H. influenzae* TYPE b POLYSACCHARIDE–PROTEIN CONJUGATE VACCINE

| Age (months) | Radioimmunoassay antibody (ng ab/ml) | | |
|---|---|---|---|
| | Pre | Post dose 1 | Post dose 2 |
| 54 | 2,350 | 66000 | 46000 |
| 54 | 750 | >70000 | >77000 |
| 49 | 105 | 54000 | 62000 |
| 48 | 11,750 | 30000 | 44000 |
| 38 | 56,000 | 74000 | 102000 |
| 30 | 5875 | 74000 | 37000 |
| 25 | 135 | 7125 | 3900 |
| 23 | <100 | 830 | 1200 |
| 21 | <100 | 10750 | 10000 |
| 19 | 112 | 190 | 1025 |
| 13 | <100 | 155 | 1100 |
| 10 | 590 | >2200 | 3550 |
| 10 | <100 | <100 | <100 |
| 4 | <100 | <100 | <100 |
| 4 | 380 | 128 | 190 |
| 3 | <100 | <100 | <100 |

antibody (≥ 1000 ng Ab/ml) after two 10-μg doses. In children <1 year of age, only one of five responded with protective levels of antibody after two 10-μg doses of vaccine. Approximately one-fourth of the children had local reactions (soreness, erythema, swelling, or induration) after either the first or the second injection (Table VI). Other complaints reported were irritability, fatigue, gastrointestinal illness, and upper respiratory infections and temperature elevation. Of these, only irritability and fatigue appeared to be related to the vaccination. The two children with temperature elevation had physician-diagnosed viral illnesses. The four children less than 1 year of age who failed to respond to two 10-μg doses of vaccine received an additional 20 μg dose to which only one of these four responded. Following this third dose, one of the four children had a local reaction; no other reactions were noted.

Based on these results and unpublished animal data, a second cohort of 15 children < 18 months were enrolled and received 20-μg dose(s). Sera from 11 of these 15 were assayed for antibody to the polysaccharide (Table VII). Of the 11 tested, 4 showed a good response to this higher dose. Of the 7 poor responders, 4 received a second 20-μg dose. The other 3 children did not receive a booster dose because of high temperatures (2 children, oral temperature 101–103°F) or withdrawal from the study (1 child). All children > 8 months of age and 1 of 2 children 8 months old responded to either one or two 20-μg doses. Children were more apt to have local reactions and possibly more apt to have temperature elevations following a 20-μg dose than following a 10-μg dose.

Children tended to show significant antibody increases to the carrier protein after a single injection with a boost in titer following the second injection. This increase was related to the dose for the first injection only; response to the second injection was similar regardless of whether children received a 10- or 20-μg dose. The four children who received a third injection had no further increase in antibody to the protein.

TABLE VI. CLINICAL COMPLAINTS AMONG CHILDREN WHO RECEIVED *H. influenzae* CONJUGATE VACCINE

| Complaint | Percent reporting reaction within 5 days after vaccination | | | | |
|---|---|---|---|---|---|
| | First (10 μg) ($n$ = 16) | Second (10 μg) ($n$ = 15) | Third (20 μg) ($n$ = 4) | First (20 μg) ($n$ = 15) | Second (20 μg) ($n$ = 5) |
| Local (arm) | 19 | 33 | 25 | 73 | 0 |
| Fever (oral temperature <101° F) | 0 | 13 | 0 | 20 | 0 |
| Irritability | 19 | 13 | 0 | 33 | 20 |
| Fatigue | 6 | 0 | 0 | 0 | 20 |
| Gastrointestinal illness | 19 | 0 | 0 | 7 | 0 |
| Upper respiratory infection | 6 | 0 | 0 | 0 | 0 |
| Disturbed sleep | 0 | 0 | 0 | 0 | 20 |

TABLE VII. SERUM ANTIBODY RESPONSE OF CHILDREN INJECTED WITH TWO 20-μg DOSES OF *H. influenzae* TYPE b POLYSACCHARIDE–PROTEIN CONJUGATE VACCINE

| Age (months) | Radioimmunoassay antibody (ng/ml) | | |
|---|---|---|---|
| | Pre | Post dose 1 | Post dose 2 |
| 17 | 1450 | 4500 | NG* |
| 16 | <100 | <100 | 1224 |
| 14 | <100 | 300 | 2050 |
| 13 | <100 | 2500 | 5875 |
| 11 | 1175 | 3100 | NG* |
| 9 | <100 | 550 | 2500 |
| 8 | <100 | <100 | NG** |
| 8 | <100 | 2750 | NG* |
| 6 | 177 | 134 | 197 |
| 6 | <100 | <100 | NG*** |
| 5 | 105 | <100 | NG** |

*Note.* NG*, not given; protective antibodies obtained after first dose. NG**, not given, child had fever (oral temp 101–103°F) after first dose. NG***, not given, child withdrew from study.

**Discussion.** Serotype II protein of group B meningococcus and 6-aminocaproic acid previously have been used in humans without significant adverse effect (17, 18). A conjugate of *H. influenzae* type b capsular polysaccharide and serotype II protein using 6-aminocaproic acid as a spacer can be made reproducibly with good stability for at least 13 months of storage. The conjugate is highly immunogenic in both mice and African green monkeys.

Previous studies indicated that an antibody titer of >1000 ng Ab/ml is capable of protecting human children and infants from *H. influenzae* disease. The unconjugated type b polysaccharide has been shown to induce these levels only in children ≥ 18 months of age. In contrast, clinical experience with the present conjugate vaccine in children showed that it induces protective antibody levels in children ≥ 8 months of age after one or two doses. However, it remains necessary to examine persistence. The vaccine is well tolerated at 10 μg and has acceptable reactogenicity at 20 μg. Investigation continues in the laboratory to prepare a conjugate vaccine immunogenic in children of all ages.

We gratefully acknowledge the assistance of Dr. R. Maigetter for fermentation, P. J. Kniskern for polysaccharide preparation, and L. Lukacs for clinical trial analysis.

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Received January 28, 1985. P.S.E.B.M. 1987, Vol. 184.
Accepted October 14, 1986.