

## Priming and induction of *Haemophilus influenzae* type b capsular antibodies in early infancy by Dpo20, an oligosaccharide-protein conjugate vaccine

Porter Anderson, PhD, Michael Pichichero, MD, Kathryn Edwards, MD, Carol Ray Porch, MD, and Richard Insel, MD

From the Departments of Pediatrics, University of Rochester and Vanderbilt University

**A conjugate vaccine (Dpo20) was made by direct coupling of diphtheria toxoid and oligosaccharides obtained by periodate oxidation of *Haemophilus influenzae* type b capsular polysaccharide. This approach gave a higher multiplicity of saccharides per protein and greater immunogenicity in infancy than our previously studied conjugates. Thirty-three healthy infants received three sequential injections, and no serious side effects were observed. When Dpo20 was given with diphtheria-tetanus-pertussis vaccine at ages 2, 4, and 6 months, the geometric mean titer of anticapsular antibody rose to 5.9  $\mu\text{g/ml}$  at age 7 months. Dpo20 given at 3, 5, and 7 months raised the mean to 3.2  $\mu\text{g/ml}$  at 7 months (after two injections) and 15.4  $\mu\text{g/ml}$  at 10 months. The antibodies included IgG and were bactericidal in vitro. Thus, antibody activities potentially protective against invasive *H. influenzae* b infections were induced in the most susceptible age range. The infants also became primed for mature-for-age responses to (unconjugated) polysaccharide vaccine given as a booster at age 12 months. (J PEDIATR 1987;111:644-50)**

Antibody to the capsular polysaccharide is protective against invasive infections by *Haemophilus influenzae* type b. Humans can be immunized by injection of the purified polysaccharide (PRP). Competence for a response to PRP vaccine matures slowly and becomes adequate for protection under field conditions at about 2 years of age. Since the maximal incidence of *H. influenzae* b meningitis occurs at about 9 months of age, several investigators have attempted to induce PRP antibody earlier in infancy by

coupling the antigen to a protein carrier.<sup>1</sup> Whereas other laboratories have used highly polymerized PRP as a component of such "conjugate" vaccines, we have examined the use of oligosaccharide haptens. These were coupled by reductive amination directly to amino groups of

PRP	Linear polymer of [3-D ribose (1-1) ribitol-5-phosphate]
DTP	Diphtheria-tetanus-pertussis vaccine
GM	Geometric mean
ELISA	Enzyme-linked immunosorbent assay

Supported by U.S. Public Health Service grants AI 17938, AI 17217, and AI 02645.

Submitted for publication Aug. 23, 1986; received July 10, 1987.

Reprint requests: Porter Anderson, Ph.D., University of Rochester Medical Center, Box 690, 601 Elmwood Ave., Rochester, NY 14642.

diphtheria toxoid or the related nontoxic protein CRM197.<sup>2</sup> Using oligosaccharides with hemiacetal termini (which coupled slowly, giving a low hapten multiplicity), a conjugate (Dcr-VS) was prepared that induced PRP anti-

bodies in early infancy and also primed for a more mature response to PRP vaccine given at ages 9 to 11 months; however, the quantity of antibody produced by the primary course (given at ages 2, 4, and 6 months) was quite variable.<sup>3</sup> Using oligosaccharides with free aldehyde ends, conjugates could be made with a higher multiplicity of haptens. One of these, made with an average saccharide length of 20 repeats (Dpo20), was highly and consistently immunogenic in 1-year-old infants, eliciting PRP antibody bactericidal to *H. influenzae b*, as well as antibody to the toxoid carrier.<sup>4</sup>

In the present study, to examine the immunogenicity earlier in infancy, Dpo20 has been studied in alternative protocols in which a three-injection sequence was given at approximate ages 2, 4, and 6 months (simultaneous with conventional DTP) or at 3, 5, and 7 months (following DTP by 1 month). Total serum PRP antibodies and their Ig class distribution and bactericidal activity were determined. To examine the question of the persistence of immunity, antibodies were measured through age 12 months. At this point, to examine the most appropriate means for "boosting" the Ab level, the subjects were divided into three groups and given injections of Dpo20 or PRP or no booster.

## METHODS

The preparation and properties of vaccine Dpo20 have been described.<sup>4</sup> In brief, PRP was partially cleaved by periodate, and oligosaccharide chains of mean length about 20 repeat units were isolated and coupled to purified diphtheria toxoid. The resulting conjugate contained 0.24  $\mu\text{g}$  saccharide/ $\mu\text{g}$  protein, which is equivalent to about 2 oligosaccharide chains per protein molecule; for injection it was dissolved in phosphate-buffered saline solution with 0.01% thimerosal at 50  $\mu\text{g}$  protein/ml, and 0.5 ml doses were injected intramuscularly in the upper arm. The DTP vaccine was lot NDC 142-5297 from Lederle Laboratories, Pearl River, N.Y.; the conventional 0.5 ml doses were injected intramuscularly in the thigh.

Infants were recruited at a private pediatric practice in Rochester, N.Y., and at the Vaccine Clinic at Vanderbilt University, Nashville, Tenn. The protocol and informed consent agreement with the parents were approved by human studies committees at the University of Rochester, Vanderbilt University, and the National Institutes of Health. The infants were healthy and had had no known exposure to *H. influenzae b* antigens. The subjects in Rochester included 16 in the 2-4-6 and two in the 3-5-7 protocol; in Nashville the distribution was eight and seven, respectively. After injection the infants were observed for 20 minutes for indications of immediate hypersensitivity

**Table 1.** Incidence of adverse signs and symptoms within three days after vaccination

Sign or symptom	Incidence after vaccination with (%)			
	Dpo20	DTP	Dpo20 + DTP	
Local				
Erythema	4	*	29	
Swelling	11	*	52	
Tenderness	24	*	57	
Temperature >100.4° F	0	*	35	44
Unusual behavior				
Crying	0	*	50	52
Screaming	5		15	16
Fretfulness	30		50	32
Sleepiness	5	*	45	48
Anorexia	0		16	4
Nausea	0		5	0

\* $P < 0.05$  by  $\chi^2$  tests ( $2 \times 2$ ) between the two groups indicated; other pairwise differences were not significant.

and then released to their parents for observation and recording of possible local or systemic adverse reactions. Signs and symptoms were recorded for both Dpo20 and DTP.

Total serum antibodies to PRP were assayed by radioantigen binding: 25  $\mu\text{l}$  serum or serum diluted in antibody-free fetal bovine serum was equilibrated at 4° C with 50  $\mu\text{l}$   $^3\text{H}$ -PRP at 10 ng/ml, and the assay was completed as described; apparent antibody concentration was estimated using a dilution of the test serum binding <33% of the  $^3\text{H}$ -PRP and a standard curve with reference antiserum SK furnished by Rachel Schneerson of the National Institutes of Health. The minimal detectable binding of 3% in undiluted serum corresponded to 0.02  $\mu\text{g}$  antibodies/ml; sera binding below the limit were assigned the value of 0.02 for calculation of GMs and 95% confidence intervals.<sup>4</sup> Statistical methods were performed as described<sup>5</sup>; calculations were done with the logs of the antibody values. For the sera obtained at Vanderbilt University, the radioassay was done independently both there and in Rochester. The values agreed with a correlation of  $R = 0.89$ . Only the latter data are given below.

IgM, IgG, and IgA antibodies to PRP were determined by ELISA as described.<sup>4</sup> The results of the ELISA (like the radioassay) depend on the affinities, as well as concentrations of the antibody populations. This version of ELISA is calibrated after the step in which antibody bind to the immunosorbent and so probably fails to detect low-affinity antibodies; thus the combined values for IgM, IgG, and IgA typically are less than the radioassay

**Table II.** Total serum polysaccharide (PRP) antibody estimated by radioantigen binding in infants given three injections of conjugate vaccine Dpo20

Vaccination schedule	Approximate age (mo)	No. of sera assayed (No. with <0.02 µg/ml)	µg Antibody/ml serum	
			GM*	95% CI†
2-4-6 mo‡	2	24 (6)§	0.16	0.08-0.31
	4	24 (6)§	0.10	0.06-0.19
	6	24	0.56	0.25-1.23
	9	24	3.1	1.3 -7.2
	12	22	1.4	0.67-3.0
	6	12	0.36	0.15-0.85
	7	12	5.9	2.4 -14
	9	12	3.0	1.0 -8.5
	3-5-7 mo¶	3	9 (2)§	0.11
5		9 (1)§	0.23	0.09-0.56
7		9	3.2	0.72-14
10		9	15.4	5.8 -41
12		7	7.4	2.6 -21

\*Antilog of the mean log of the values.

†Ninety-five percent confidence interval of the GM = antilog of the mean log  $\pm$  (t  $\times$  SEM log), where t is the value of Student's t for P < 0.05, two tailed (i.e., 2.1 to 2.3).

‡Twenty-five micrograms of conjugate protein injected simultaneously with DTP vaccine but in a separate site.

§The number of sera in parentheses had less than the detectable limit of 0.02 µg/ml but were assigned this value for calculation of the GM and confidence interval.

||A subgroup of infants whose parent consented to an extra serum sampling at age 7 months, which was estimated to be nearer the peak antibody response.

¶Twenty-five micrograms of conjugate protein injected about 1 month after DTP vaccine.

estimate for total serum antibodies. Serum bactericidal activity was assayed with *H. influenzae* b strain Eag as described.<sup>6</sup>

## RESULTS

**Side effects.** Signs and symptoms of possible adverse reactions were recorded for 3 days after each vaccination (Table I). At the injection sites, erythema, swelling, or tenderness was observed after a minority of the injections of Dpo20; there was no significant difference in the incidences among first, second, or third injections. These three indexes of local inflammation were observed much more frequently (P < 0.05) at the site of DTP injections (Table I). Fever was observed after 0% of injections of Dpo20 given alone but after 44% of instances in which both Dpo20 and DTP were given (P < 0.05), which does not differ significantly from the incidence after DTP given alone (35%). Likewise, the incidences of unusual behavior were low after Dpo20 alone but higher after DTP or both (Table I). Several minutes after a second injection of Dpo20, one infant became pale; she immediately was examined and found to have normal heart rate and blood pressure. The pallor resolved spontaneously after a short time.

**Total serum anti-PRP antibodies determined by radioassay.** Within the two schedules (2-4-6 month or 3-5-7

month) the antibody values of infants from Nashville and Rochester did not differ significantly and have been combined (Table II). In the 2-4-6-month schedule, between the first injection at age 2 months and the next serum sample at age 4 months, three of 24 infants had a measurable rise but most had a decline; the GM value thus declined from 0.16 to 0.10 µg/ml. Between the second injection at 4 months and the next sample at 6 months, 18 of the 24 had a rise, and the GM rose to 0.56 µg/ml. Between the third injection at 6 months and the sample at 9 months, one infant (W) with no previous rise had no rise, two infants with very high 6-month levels had no further rise, and the rest had first-time or secondary rises; the GM rose to 3.1 µg/ml. Of the 22 infants with sera available at 12 months, the levels of all had declined since age 9 months and the GM was 1.4 µg/ml. For a more detailed evaluation of the kinetics of the response to the third injection at age 6 months, a subgroup of 12 Rochester infants were bled at age 7 months, as well as at 9 months. All of these infants had a rise between 6 and 7 months and all had a decline between 7 and 9 months; the respective GMs were 0.36, 5.9, and 3.0 µg/ml (Table II).

In the 3-5-7-month schedule, between the first injection at 3 months and the next serum sample at 5 months, four of the nine infants had a rise and the GM rose from 0.11 to 0.23 µg/ml (not a statistically significant rise by paired t



**Table III.** Effect of "booster" reimmunization at age 12 months with conjugate vaccine Dpo20 or unconjugated polysaccharide (PRP) vaccine on PRP antibody in Dpo20-primed infants

Booster vaccine	No. with rise*: No. in group	Total PRP antibody ( $\mu\text{g/ml}$ serum) GM (95% confidence interval)		
		Age 12 mo (preboost)	Age 13 mo	P value
None (controls)	1:6	4.4 (1.1-18)	3.9 (1.0-15)	
PRP	9:10	0.95 (0.28-3.2)	7.9 (3.4-18)	<0.05†
Dpo20	6:6	0.73 (0.16-3.3)	28 (3.1-250)	<0.05†

\*An increase of >33% over the previously measured value.

†P <0.05 by paired t test. The difference within the control group is not significant.

contains >1  $\mu\text{g}$  antibody/ml. Tested under the same conditions, the standard (adult) PRP antiserum SK maintains detectable activity through dilutions down to about 1  $\mu\text{g/ml}$ .

**Booster at age 12 months with Dpo20 or unconjugated PRP vaccine.** In the hope of having three experimental groups with equivalent distributions of antibody, the subjects were ordered according to the total antibody level at age 9 to 10 months and sequentially distributed into three groups for a controlled study of boosting at age 12 months. (There were numerous "dropouts," however, so that of those completing this phase of the study, the control group by chance had a GM about fivefold higher than the other two, although the difference was not statistically significant; Table III.) The groups received Dpo20, PRP vaccine, or no booster; they were bled again after 1 month to estimate the peak response to the two types of reimmunization (Table III). In the no-booster control group, only one of the six infants who completed the sequence had a rise in the 1-month interval. In the PRP group, nine of the 10 infants had rises. Among those who responded was the infant (W) who previously had no IgG response; her level rose from 0.06 to 0.7  $\mu\text{g/ml}$ . All six infants in the Dpo20 group had rises. The GM titers after PRP (7.9  $\mu\text{g/ml}$ ) and Dpo20 (28  $\mu\text{g/ml}$ ) were not significantly different ( $T = 1.50$ ;  $P = 0.1$  to  $0.2$ ). IgG antibody rose in all subjects who responded to the PRP, as well as Dpo20, vaccines.

## DISCUSSION

From the viewpoint of side effects, Dpo20 appeared acceptable for use in infancy. When it occurred, local inflammation was of mild degree. When used alone, Dpo20 gave little sign of systemic upset; when used simultaneously with DTP, systemic indexes were no greater than with DTP alone. The one incident of postvaccination pallor appears to have been a mild vasovagal reaction.

Interpretation of the antibody response data should include the concept that infants begin life with varying

levels of maternally derived IgG PRP antibody, which is catabolized with a half-life of about 1 month.<sup>1</sup> Particularly in infants with higher prevaccination levels, measurements of total PRP antibody at 2-month intervals will tend to show a decline unless vaccine-induced antibody formation exceeds the decline caused by catabolism. Indeed, the isotype-specific ELISA showed (as in the previous study<sup>3</sup>) that increases of IgM antibody were detectable after first injections even though IgG and total antibody might decline during the 2-month interval.

The immunogenicity of Dpo20 in the 2-4-6-month schedule can be compared with the previously studied conjugate Dcr-VS and PRP polysaccharide vaccine used in identical protocols.<sup>3</sup> With PRP vaccine, antibody levels declined through age 7 months (and were not significantly different from those of unvaccinated infants). Dcr-VS gave no statistically significant rise after the first injection but gave GMs of 0.19  $\mu\text{g/ml}$  after the second and 0.52 after the third. With Dpo20 there was a trend to higher levels, which became significant after the third injection ( $t = 3.14$ ;  $P < 0.01$ ). Dpo20 differs from Dcr-VS not only in having higher multiplicity and length of saccharides but also in using formalin-treated toxoid rather than the mutant diphtheria toxin (CRM197 (which is difficult to produce). Previously, when toxoid and CRM197 were compared as carriers in conjugates with low hapten multiplicity, the toxoid was much less active when 1-year-old infants were studied.<sup>2</sup> Here the same preparation of toxoid was satisfactory as a carrier, a difference we attribute to the higher multiplicity or length of the periodate-cleaved saccharide chains in Dpo20. CRM197 also may have greater potency than toxoid in high-multiplicity conjugates, but this variable remains to be evaluated.

Experiments in weanling rabbits and inference from the response of 1-year-old infants had indicated that the anti-PRP antibody level maximizes within 2 weeks after injection of Dpo20.<sup>4</sup> The present observation, in which the GM rose sixteen-fold between 6 and 7 months and then declined about 50% from age 7 to 9 months (Table II), is

consistent with this expectation. Thus, increases in total antibody levels after first and second injections may have been more apparent if serum samples had been taken more frequently.

Dpo20 raised higher titers in the 3-5-7-month protocol (Table II). This difference might be attributed to the carrier-priming effect<sup>1</sup> of the D component of the preceding DTP vaccine or to the extra month of maturation. Such a schedule in practice would require additional office visits, but two rather than three injections might be sufficient.

The antibodies elicited in infancy by the coupled oligosaccharides appear to have functional potential. Passive animal protection studies are not feasible with the limited volumes of sera available; however, complement-dependent killing of encapsulated *H. influenzae b* in vitro increased in 32 of 33 infants, and the relation of bactericidal titer to radioassay value was similar to that observed with older children or PRP-vaccinated adults.<sup>3</sup> Without immunosorption data, some component of the bactericidal activity in these infants hypothetically could be attributed to naturally acquired antibodies to *H. influenzae* somatic antigens,<sup>6</sup> but such seems unlikely on the basis of experience with sera of unimmunized healthy infants. It should not be assumed that serum bactericidal activity is required for protection against *H. influenzae b*. Minimum protective levels of PRP antibody assayed in infant rats<sup>7</sup> or estimated in humans<sup>1</sup> are about ten-fold lower than that required for bacteriolysis in vitro, presumably because protection in vivo is accomplished to a large degree by the opsonic activity of the antibodies.

Persistence of the PRP antibodies will be an important factor in deciding how conjugate vaccines might be used in a program of prevention of *H. influenzae b* disease. There may be a limit to the number of injections of a conjugate that could be used before hypersensitivity to the carrier protein component becomes a problem. In the previous study, infants "primed" with Dcr-VS conjugate at 2, 4, and 6 months had a mature-for-age response to (uncoupled) PRP vaccine given at 9 to 11 months.<sup>3,8</sup> This indicated that the subjects would also have been primed for an anamnestic response on encounter with the antigen in its natural (bacterial) form and suggested that PRP could be used as a booster, which would have potential advantages of economy, safety, and possible longevity of antibody elevation. Likewise, in the present study PRP induced responses at a much higher frequency and level than would be expected of unprimed 12-month old infants.<sup>3</sup> Notably, two infants with barely detectable antibody rises during the primary series and infant W (who had no measurable rise) responded well to the booster injection of PRP: levels at age 13 months rose to 2.0, 1.8, and 0.7  $\mu\text{g/ml}$ , respectively. Thus the conjugate may have exerted a

priming action not apparent in the immediate antibody response. As a booster at age 12 months, the conjugate induced a three and a half-fold higher GM than PRP, but the difference was not statistically significant. More of interest will be the persistence of antibody levels and the isotype distribution and functional properties of the antibodies during the second and third years of life in these subjects and in the unboosted control group. High-for-age responses to PRP after a priming course with other conjugate vaccines have recently been reported.<sup>9,10</sup>

When studied in adults and 1-year-old infants not receiving DTP vaccine, Dpo20 also induced IgG antibodies to diphtheria toxoid.<sup>4</sup> Toxoid antibodies rose in the present study, but the concurrent use of DTP makes the results uninterpretable.

In the past it had been feared that there might be an insurmountable maturational delay of PRP antibody responsiveness in infancy. This and similar studies with oligosaccharide<sup>11</sup> and polymeric PRP-protein conjugate vaccines<sup>12,13</sup> suggest that the lag can be circumvented by appropriate presentation of the antigen. However, it should not be assumed that all conjugate vaccines will be equivalent in potency or even in mechanism. For example, a PRP-membrane protein conjugate vaccine was recently reported to induce primary responses very effectively in early infancy, whereas the secondary responses were relatively modest.<sup>13</sup>

Further studies are required to better define the relation of structure to the immunogenicity of protein-saccharide conjugates and to work out problems of consistency of synthesis, stability, etc. Nevertheless, the prevention of the bulk of systemic *H. influenzae b* disease by vaccination is a realistic prospect.

We thank Susan Porcelli, Ann Kittelberger, and Pam Palmer for technical assistance.

## REFERENCES

1. Robbins JR, Schneerson R, Pittman M. *Hemophilus influenzae* type b infections. In: Germanier R, ed. Bacterial vaccines. New York: Academic Press, 1984;289-316.
2. Anderson P, Pichichero ME, Insel RA. Immunogens consisting of oligosaccharides from the capsule of *Hemophilus influenzae* type b coupled to diphtheria toxoid or CRM 197. *J Clin Invest* 1985;76:52-9.
3. Anderson P, Pichichero ME, Insel RA. Immunization of two-month old infants with protein-coupled oligosaccharides derived from the capsule of *Hemophilus influenzae* type b. *J PEDIATR* 1985;107:346-51.
4. Anderson P, Pichichero ME, Insel RA, et al. Vaccines consisting of periodate-cleaved oligosaccharides from the capsule of *Hemophilus influenzae* type b coupled to a protein carrier: Structural and temporal requirements for priming in the human infant. *J Immunol* 1986;137:1181-6.

5. Colton T. Statistics in medicine. Boston: Little, Brown & Co, 1974;1-372.
6. Anderson P, Flesher A, Shaw S, et al. Phenotypic and genetic variation in the susceptibility of *Hemophilus influenzae* type b to antibodies to somatic antigens. J Clin Invest 1980;65:885-91.
7. Schreiber JR, Barrus V, Cates L, et al. Functional characterization of human IgG, IgM, and IgA antibody directed to the capsule of *Hemophilus influenzae* type b. J Infect Dis 1986;153:8-16.
8. Insel RA, Anderson PW. Oligosaccharide-protein conjugate vaccines induce and prime for oligoclonal IgG antibody responses to the *Hemophilus influenzae* b capsular polysaccharide in human infants. J Exp Med 1986;163:262-9.
9. Berkowitz CD, Ward JI, Hendley JO, et al. Persistence of antibody to *Hemophilus influenzae* type b and response to PRP and PRP-D booster immunization in children initially immunized with either vaccine at 15 to 24 months (abstr). Pediatr Res 1987;21:321.
10. Weinberg GA, Einhorn MS, Lenoir AA, Granoff PD, Granoff DM. Immunization of infants with *Hemophilus influenzae* type b polysaccharide-outer membrane protein conjugate vaccine primes for IgG1 booster responses to conventional polysaccharide vaccine (abstr). Pediatr Res 1987;21:320.
11. Eby RJ, Madore D, Johnson C, et al. A new stable vaccine for *Hemophilus influenzae* b highly immunogenic for human infants composed of oligosaccharides of capsular polymer (PRP) linked to CRM197 (abstr). Pediatr Res 1986; 20:899.
12. Kayhty H, Eskola J, Peltola H, et al. Immunogenicity in infants of a vaccine composed of *Hemophilus influenzae* type b capsular polysaccharide mixed with DPT or conjugated to diphtheria toxoid. J Infect Dis 1987;155:100-6.
13. Einhorn MS, Weinberg GA, Anderson EL, et al. Immunogenicity in infants of *Hemophilus influenzae* type b polysaccharide in a conjugate vaccine with *Neisseria meningitidis* outer-membrane protein. Lancet 1986;2:299-302.

#### FELLOWSHIPS

Available fellowships in pediatric subspecialties and those for general academic pediatric training are listed once a year, in May, in The Journal of Pediatrics. Each October, forms for listing such fellowships are sent to the Chairman of the Department of Pediatrics at most major hospitals in the United States and Canada. Should you desire to list fellowships, a separate application must be made each year for each position. All applications must be returned to The C. V. Mosby Company by February 15 of the listing year to ensure publication. Additional forms will be supplied on request from the Journal Editing Department, The C. V. Mosby Company, 11830 Westline Industrial Drive, St. Louis, MO 63146/314-872-8370.