Evaluation of the Protective Efficacy of *Haemophilus influenzae* Type b Vaccines in an Animal Model

NEAL A. HALSEY,^{1*} TERRI LYNN JOHANSEN,² LINDA C. BOWMAN,¹ AND MARY P. GLODE²

Departments of Tropical Medicine* and Pediatrics, Tulane University, New Orleans, Louisiana 70112,¹ and
Department of Pediatrics, The University of Colorado Center for Health Sciences and The Children's
Hospital, Denver, Colorado 80218²

Received 25 October 1982/Accepted 21 December 1982

The infant rat model for *Haemophilus influenzae* type b (Hib) disease was modified to test the protective efficacy of candidate vaccines. The administration of 0.4 µg of polyribosylribitol phosphate (PRP) combined with pertussis vaccine or with diphtheria toxoid-tetanus toxoid-pertussis vaccine (DTP) to infant rats at 5, 10, and 15 days of age resulted in significant ($P < 0.001$) protection against Hib bacteremia after challenge at 20 days of age. A dose-response effect was demonstrated, and suppression of the protective effect was noted with high doses of PRP. The administration of pertussis vaccine alone or DTP alone did not protect against Hib bacteremia. The degree of protection against Hib bacteremia correlated ($P < 0.005$) with the serum anti-PRP antibody response measured before bacterial challenge. High-molecular-weight PRP preparations administered alone or in combination with pertussis vaccine were less effective than standard PRP combined with pertussis vaccine. These data compare favorably with the antibody response in human infants receiving the same vaccines. The observations in this animal model should facilitate the development and testing of other vaccine preparations for human infants.

Experimental *Haemophilus influenzae* type b (Hib) vaccines have been tested in children as rats, rabbits, and other animals have not responded to purified polyribosylribitol phosphate (PRP) preparations (2, 3, 11, 15-17, 20). An efficient and reliable animal model for testing immunogenicity would aid in the development of successful vaccines.

The infant rat model for Hib meningitis developed by Smith et al. (21) and refined by Moxon et al. (14) simulates human Hib infections in several respects. Modification of the inoculation technique has demonstrated that nasal colonization is followed by bacteremia and meningitis and that transmission from animal to animal occurs within litters (7). The immune response in Hib-infected infant rats is similar to that observed in human infants (6). Modification of this animal model has allowed us to evaluate the protective efficacy of purified Hib PRP combined with pertussis vaccine alone or with diphtheria and tetanus toxoids and pertussis vaccine (DTP).

MATERIALS AND METHODS

Animals. Pathogen-free, outbred, timed pregnant Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). After birth, animals were housed in litters of 10 pups each

with mothers in vivariums at the respective institutions.

Vaccines. All vaccine preparations were provided by Lederle Laboratories Division of American Cyanamid Co. (Pearl River, N.Y.). The physicochemical and biological characteristics of these PRP preparations have been described previously by Kuo et al. (9). The following preparations were tested. PRP-P1, Purified PRP combined with pertussis vaccine (4.0 opacity units per 0.5 ml of vaccine); PRP-P2, purified PRP combined with a second preparation of pertussis vaccine (4.0 opacity units per 0.5 ml of vaccine); PRPee, a high-molecular-weight PRP preparation; PRPee-P, high-molecular-weight PRP combined with pertussis vaccine; DTP, diphtheria and tetanus toxoids and pertussis vaccine (Tri-Immunol); PRP-DTP, PRP combined with DTP; P, pertussis vaccine (4.0 opacity units per 0.5 ml of vaccine). Vaccines were stored at 2 to 8°C before use and administered subcutaneously in a fixed volume of 0.2 ml. All PRP preparations contained 20 µg of PRP per ml. When less than 4 µg of PRP was administered, appropriate dilutions were prepared with phosphate-buffered saline (PBS) immediately before administration.

Study design. Infant rats were randomized, reasigned to nursing females, and enrolled into the vaccine studies at 4 to 5 days of age. Four to eight litters of 10 to 11 pups each were included in each study. Three or four animals (controls) within each litter received PBS, and the remaining animals were divided into two study groups.

For a three-dose schedule, vaccine (or placebo) was administered at 5, 10, and 15 days of age. When four

TABLE 1. Rates of bacteremia and protective efficacy in infant rats given Hib PRP vaccines

Vaccine	Vaccine dosage (μ g of PRP)				No. bacteremic/ no. vaccinated	Bacteremia (%)	Efficacy (%)	P value ^a
	Dose no. 1	Dose no. 2	Dose no. 3	Dose no. 4				
Placebo	— ^b	—	—	—	308/363	84.8		
PRP-P1	0.04	0.04	0.04	0.04	10/11	90.9	0	NS ^c
	0.4	0.4	0.4	—	37/68	54.4	35.9	<0.001
	0.4	0.4	0.4	0.4	6/10	60.0	29.3	NS
	0.4	0.4	4.0	—	32/77	41.6	51.0	<0.001
	—	4.0	4.0	—	13/24	57.0	32.7	<0.001
	4.0	4.0	—	—	19/23	82.6	2.6	NS
	4.0	4.0	4.0	—	20/33	60.6	28.6	<0.001
	4.0	4.0	4.0	4.0	19/22	86.4	0	NS
PRP-P2	—	0.4	0.4	—	14/29	48.3	43.0	<0.001
	0.4	0.4	0.4	—	13/37	35.1	58.6	<0.001
	0.4	0.4	4.0	—	45/81	55.6	34.4	<0.001
	4.0	4.0	4.0	4.0	10/15	66.7	21.4	NS
PRPee	0.4	0.4	0.4	0.4	19/25	76.0	10.4	NS
	0.4	0.4	4.0	—	14/22	63.6	25.0	<0.01
	4.0	4.0	4.0	—	8/10	80.0	5.7	NS
PRPee-P	0.4	0.4	0.4	—	8/12	66.7	21.4	NS
	0.4	0.4	4.0	—	39/49	79.6	6.2	NS
	4.0	4.0	4.0	—	15/18	83.3	1.8	NS
PRP-DTP	—	0.4	0.4	—	4/14	28.6	66.3	<0.001
	0.4	0.4	0.4	—	32/125	25.6	69.8	<0.001
	0.4	0.4	0.4	0.4	4/9	44.4	47.7	<0.005
	0.4	0.4	4.0	—	14/26	53.8	36.6	<0.001
DTP ^d	0.02 cc	0.02 cc	0.02 cc	—	145/166	87.3	0	NS
P	0.02 cc	0.02 cc	0.02 cc	—	22/23	95.6	0	NS

^a Values determined by the chi-square test for the rate of bacteremia in vaccinated versus placebo animals.

^b —, PBS only; no vaccine administered.

^c NS, Not significant ($P < 0.01$).

^d 0.2 cc of a 1:10 dilution was administered. This is equivalent to the same amount of P administered when 0.4 μ g of PRP is given in PRP-P vaccine.

doses were administered, vaccine was given at 4, 8, 12, and 16 days of age. Tail vein blood was obtained for serological studies on day 20, before challenge with bacteria. Sera from several animals receiving the same vaccine schedule were combined to provide sufficient quantity for antibody analysis. Exceptions to the routine schedules are noted in the tables.

Organisms. A streptomycin-resistant mutant of Hib strain Eagan (11) was grown to mid-log phase, centrifuged, and suspended in PBS. An inoculum of 20 μ l containing 10^6 to 10^7 CFU was dropped atraumatically into the nares of all rats twice a day on days 20 and 21 of age.

Cultures. Cultures of the nasopharynx and of tail vein blood (20 μ l) from individual animals were performed on days 23, 25, and 27 of age by previously described methods (7). In preliminary experiments, cisternal punctures for cerebral spinal fluid cultures were performed on day 27. Cerebral spinal fluid analyses were discontinued after blood cultures proved to be more reliable in detecting disease and the effect of vaccine. All specimens for culture were plated on brain heart infusion agar supplemented with Levinthal

base and streptomycin (200 μ g/ml). Colonies of questionable morphology were observed in fewer than 2% of the cultures. These organisms were Gram stained and evaluated for halo production on antiserum agar plates (12) to confirm the presence of Hib.

Antibody assay. Serum was stored at -70°C before analysis. Anti-PRP antibody was determined by radioimmunoassay with tritium-labeled PRP at Lederle Laboratories (10). The sensitivity of this assay is 20 to 40 ng of antibody per ml of serum.

Data analysis. Standard chi-square analyses were used. Since the rates of bacteremia observed in animals given placebo were consistent, the results from all placebo-vaccinated animals were combined for statistical testing. A P value of <0.05 was considered significant. Calculation of correlation coefficients and one-way analysis of variance were performed with a standard statistical package (Minitab) on a DEC 2060 computer.

Vaccine efficacy was calculated from the standard formula: efficacy (%) = [(rate of bacteremia in placebo animals - rate of bacteremia in vaccinated animals)/rate of bacteremia in placebo animals] $\times$ 100.

RESULTS

Nasal colonization. Vaccination with any of the PRP preparations did not affect the rate of nasal colonization (90 to 97%) by Hib. In 6 of 105 litters, less than 40% nasal colonization was observed, including the placebo-inoculated animals. The explanation for the low levels of colonization in these litters was not elicited, and blood culture data from these animals are not included in the following tables.

Prevention of bacteremia. Bacteremia was detected in 84.8% (308 of 363) of the control animals given PBS. This rate remained constant throughout the study. The rate of bacteremia in vaccinated animals varied from 15 to 100% with different dosages and types of vaccines (Table 1).

The PRP-P2 and PRP-DTP preparations were significantly ($P < 0.05$) more efficacious than the PRPee preparations, and the highest protective efficacy was induced by PRP-DTP. No protection was induced with either P alone or DTP alone.

Of the 308 control animals that developed bacteremia, 191 (62%) had blood bacterial densities of $\geq 10^4$ CFU/ml on one or more of the three blood cultures obtained. Quantitative cultures from 148 animals that became bacteremic despite receiving effective doses of PRP-P vaccine revealed that 84 (56.8%) developed blood bacterial densities of $\geq 10^4$ CFU/ml. These rates of high density bacteremia in vaccine failures were not significantly different than the 62% rate observed in placebo animals. Thus, there was no detectable evidence of partial protection against Hib bacteremia when vaccine failure occurred.

Dose-response curve. A bell-shaped dose-response curve was demonstrated for PRP-P1 and PRP-P2 vaccines (Fig. 1). When the optimal dose of vaccine was exceeded, progressively lower levels of protection against bacteremia were observed. The optimal total dose of PRP-P1 vaccine was 4.8 μ g (0.4, 0.4, and 4.0 μ g at 5, 10, and 15 days, respectively). The optimal total dose for both PRP-P2 and PRP-DTP was 1.2 μ g (0.4, 0.4, and 0.4 μ g, respectively).

The mean weights of infant rats were 9.9 g at 5 days of age, 17.8 g at 10 days, and 24.1 g at 15 days of age. Therefore, on a weight basis, the dose of PRP administered varied between 16.7 and 40 μ g/kg when 0.4 μ g of vaccine was administered.

Effect of age and vaccine schedule. The age of the animal as well as the dose was important in determining the protection induced by PRP-P vaccines. When two 4- μ g doses of PRP-P1 were administered to animals at 5 and 10 days of age, 19 of 23 animals developed bacteremia after challenge at 20 days of age (Table 1). However, when the same amount of vaccine was adminis-

FIG. 1. Protective efficacy of Hib PRP combined with P by dose administered.

tered at 10 and 15 days of age, only 13 of 24 animals developed bacteremia ($P < 0.05$). The optimal protection with each candidate vaccine was observed after three doses of vaccine. A fourth dose of vaccine usually had a detrimental effect on protective efficacy.

Meningitis. Meningitis was detected in 48 (40.7%) of 118 control animals that had been bacteremic on days 23, 25, or 27 of age. Similarly, the rate of detectable meningitis in animals that had received PRP-P vaccine and became bacteremic was 26 of 54 (48.1%). Although the rate of meningitis appeared to parallel the rate of bacteremia, cultures from 10 to 20% of the animals in each experiment were uninterpretable because of inadequate quantity of cerebral spinal fluid or difficulty in interpreting low colony counts. When high levels ($\geq 10^4$ CFU/ml) of bacteria were present in the blood, low colony counts in the cerebral spinal fluid could be secondary to small hemorrhages at the time of cisternal puncture.

Correlation of protective efficacy and antibody response in rats. The optimal doses of PRP-P1, PRP-P2, and PRP-DTP were associated with detectable radioimmunoassay antibody responses in pooled sera collected at 20 days of age (Fig. 2). No detectable antibody response was observed after administration of the high-molecular-weight preparations (PRPee and PRPee-P) or P alone. No antibody response was detected in PBS-inoculated animals in 14 of 15 experiments. In the remaining experiment, the postvaccination titer in placebo animals was 0.40 μ g/ml. The rate of bacteremia after immunization correlated inversely ($r = 0.56$; $P < 0.005$) with the anticapsular antibody response measured in pooled sera.

DISCUSSION

Appropriate doses of PRP combined with P or DTP induce significant protection against Hib

FIG. 2. Anticapsular antibody response in infant rats vaccinated with Hib PRP vaccines combined with P or DTP.

bacteremia in infant rats. This protection is not due to nonspecific responses to either P or DTP since no protection was induced with either of these preparations given alone. Purified PRP alone has not induced immunity in human infants, rats, or rabbits (2, 15, 17, 20). The addition of P or DTP results in an adjuvant effect similar to that observed with other bacterial vaccine preparations (5, 23). The mechanism of the adjuvant effect is not known, but pertussis and DTP may provide carrier proteins which convert the normally T-cell-independent PRP antigen to a T-cell-dependent antigen (1, 2, 20). The protection against Hib bacteremia in rats was dose related. At low doses (total dose, 0.16 μg of PRP), little or no protection was observed. Intermediate doses (total dose, 1.2 to 4.8 μg of PRP) provided optimal levels of protection, and higher doses (total dose, 8 to 16 μg of PRP) resulted in reduced efficacy. The mechanism of the apparent immunological blockade after high doses of PRP has not been explained, but may be related to the induction of suppressor T-cells (23). Schneerson et al. (20) were unable to induce tolerance in mice with 10 μg of PRP linked to bovine serum albumin. However, the same investigators (19) and Smith et al. (22) observed lower antibody responses in children vaccinated with 50 and 67 μg of PRP than in children vaccinated with lower doses. The optimal dose for young children of Hib PRP vaccine combined with P or DTP is not known.

The high-molecular-weight PRPee preparations were less effective in the animal model than the standard lower-molecular-weight PRP preparations. This contrasts with studies of meningococcal polysaccharide in which increased molecular size was associated with increased immunogenicity (4).

The induction of protective efficacy in infant rats compares favorably with the development of anti-PRP antibody in human infants vaccinated with the same preparations. Pincus et al. (18) reported that one 10- μ g dose of PRP-P resulted in an enhanced anti-PRP antibody response as

compared with PRP alone. King et al. (8) administered three doses of PRP-P vaccine to human infants at 2, 4, and 6 months of age. Seventy percent of these infants developed anti-PRP antibody titers of $\geq 0.15 \mu\text{g/ml}$, a level estimated to correlate with protection against disease (17). Other human trials (M. P. Glode and R. S. Daum, personal communication) revealed that 15 (32%) of 47 infants who received three doses of the PRP-P preparations developed antibody titers of $\geq 0.15 \mu\text{g/ml}$. This response was significantly ($P < 0.05$) better than that observed in infants receiving DTP alone (1 of 31) or PRPee preparations (3 of 47). Although the response to PRP-P vaccines in humans has been variable, in all studies PRP-P has been more immunogenic than PRP alone.

The failure of Hib vaccination to prevent nasal colonization is not surprising and has been observed in older children who responded to PRP vaccines (17). The factors associated with vaccine-induced immunity in humans and rats are apparently unrelated to colonization of Hib in the upper respiratory tract.

Differences in pertussis antigens or factors related to the methods of combining DTP with PRP may result in variable responses to PRP. Moxon et al. (13) observed a decreased anti-Hib antibody response in a small number of malnourished Peruvian children when DTP was mixed (at the time of administration) with 5 µg of PRP. They urged caution in interpreting their results because of the small numbers and the malnourished state of the children. Anderson et al. (3) observed no effect on the antibody response to PRP in infants given multiple doses of PRP mixed with DTP or when DTP was administered simultaneously at separate sites. The DTP and PRP preparations used by these investigators were produced by different manufacturers. Interpretation of all human and animal studies involving PRP combined with P is hampered by the lack of knowledge regarding the component of P responsible for the adjuvant effect.

We believe that this animal model will prove useful for the testing of *H. influenzae* vaccines. Additional data are needed to establish the degree of correlation between human antibody responses and the prevention of bacteremia in the animal model.

ACKNOWLEDGMENTS

This work was partially supported by American Cyanamid Company.

The authors thank Hani Zaki for technical assistance, Barbara Mellert for secretarial assistance, Julia Temple for editorial assistance, and Robert Daum for guidance and support.

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