INFECTION AND IMMUNITY, Jan. 1994, p. 9–14
0019-9567/94/$04.00+0
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Vol. 62, No. 1

Opposite Effects of Actively and Passively Acquired Immunity to the Carrier on Responses of Human Infants to a *Haemophilus influenzae* Type b Conjugate Vaccine

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Received 30 June 1993/Returned for modification 24 September 1993/Accepted 6 October 1993

Vaccination of infants with *Haemophilus influenzae* type b (Hib) capsular polysaccharide (HibCP) coupled to carrier proteins has proven protective against invasive Hib diseases in several trials. However, insufficient immunogenicity has been noted in certain populations. Therefore, studies analyzing factors influencing the antibody response to conjugate vaccines are needed. In this study, the response to HibCP coupled to tetanus toxoid (TT) was examined in relation to (i) priming with or coadministration of the carrier protein and (ii) the levels of passively acquired maternal TT antibodies. One hundred forty-four infants were vaccinated with HibCP-TT at 5 and 6 months. They were randomized into three groups that received TT as part of a diphtheria-tetanus-polio vaccine at either 6 and 7 months (group A), 5 and 6 months (group B), or 4 and 5 months (group C). Maternally acquired TT antibodies inhibited the anti-HibCP response to the first HibCP-TT dose in groups A and B ($r = -0.5$ and -0.4, respectively; $P < 0.005$). In these groups, infants with prevaccination anti-TT levels above the median failed to reach the defined long-term protective level of HibCP antibodies (1 μg/ml) more often than infants with low prevaccination levels after the first ($P = 0.0001$) and the second ($P = 0.01$) doses of HibCP-TT. In contrast, active priming with TT at 4 months resulted in a threefold-higher median level of anti-HibCP (group C; 1.34 μg/ml) than in the unprimed group (group A; 0.40 μg/ml) after the first dose of HibCP-TT ($P = 0.01$). Coadministration of TT had no enhancing effect (group B; 0.58 μg/ml). No significant differences between the median anti-HibCP levels were seen after the second HibCP-TT dose (6.72, 9.63, and 11.44 μg/ml in groups A, B, and C, respectively; $P = 0.25$).

Invasive infections with *Haemophilus influenzae* type b (Hib) in children represent a major problem throughout the world. In most areas Hib is the predominant cause of bacterial meningitis in the first and second years of life. Antibody to Hib capsular polysaccharide (HibCP) is protective, but for children younger than 18 months, purified HibCP has proven inefficient as a vaccine because it fails to induce protective antibody levels (24). However, conjugate vaccines, in which HibCP is covalently linked to a protein, have proven immunogenic in the first year of life (2, 7, 10, 12), and efficacy rates of close to 100% have recently been found after two or three doses in large-scale field trials in Finland and the United States (6, 12). The antibody levels induced by the first vaccination, however, are often low (9, 14, 19), and as a consequence, cases of invasive Hib infections have occurred after one or two immunizations with conjugate Hib vaccines (6, 12). Furthermore, some conjugates have proven inefficient in certain populations, such as Alaskan Eskimos (32) and American Indians (26). These populations are characterized by a very early debut of Hib infections (8, 33). A similar epidemiology is found among Australian aboriginals (17) and Gambians (5), and it is likely that at least some of the currently available conjugate HibCP vaccines will prove inefficient in these populations and in others in developing areas.

One factor that may affect the immunogenicity of conjugate HibCP vaccines is the administration of other vaccines. Tetanus toxoid (TT) and diphtheria toxoid (DT) are used as carriers in several HibCP conjugates, and since these antigens are also part of the existing immunization programs, an interaction is especially likely to occur. It is well known that priming with the carrier can increase the antibody response to Hib conjugates in rodents (1, 28), but this has not been convincingly demonstrated in humans. In fact, we have recently shown that in adults preimmunization with the carrier protein suppresses the response to HibCP conjugates (4). We therefore found it important to address the possible interactions in infants between preexisting immunity to TT and the antibody response to an HibCP-TT conjugate vaccine.

MATERIALS AND METHODS

Infants. One hundred forty-seven healthy Danish infants with gestational ages of ≥37 weeks and birth weights of ≥2,500 g were recruited from the maternity ward of Hvidovre Hospital, Copenhagen, Denmark, shortly after birth. One to three months after the initial contact, informed consent was obtained from the parents, and the infants were randomized into three groups. Infants with a history of Hib disease or exposure to Hib or with known allergy to the

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TABLE 1. Median levels of HibCP and TT antibodies in 144 healthy Danish infants vaccinated with HibCP-TT at 5 and 6 months and differing with respect to priming with TT as part of a DT-TT-polio (DT-IPV) vaccine

Group	Vaccination(s) given at age[a] (mo):				No. of individuals	Anti-TT[b] (IU/ml) at 5 mo.	Anti-HibCP[b] (μg/ml) at age (mo):		
	4	5	6	7			5	6	7
A		HibCP-TT	HibCP-TT DT-IPV	DT-IPV	43	0.13 (0.08–0.27)	0.13 (0.08–0.19)	0.40 (0.32–1.1)	6.72 (3.9–11)
B		HibCP-TT DT-IPV	HibCP-TT DT-IPV		48	0.12 (0.07–0.17)	0.13 (0.10–0.17)	0.58 (0.38–1.4)	9.63 (6.6–13)
C	DT-IPV	HibCP-TT DT-IPV	HibCP-TT		53	0.51[c] (0.39–0.76)	0.11 (0.09–0.18)	1.34[d] (0.87–2.6)	11.44 (7.6–16)

[a] The indicated ages are approximate. The median ages at the first and the second HibCP-TT vaccinations, respectively, were as follows: group A, 22.0 and 26.3 weeks; group B, 22.1 and 26.6 weeks; and group C, 22.4 and 27.0 weeks.
[b] Median, 95% confidence limits of the median (based on the binomial distribution) are in parentheses.
[c] Higher than the levels in groups A and B; $P < 0.000001$.
[d] Higher than the levels in group A; $P = 0.01$.

components of the vaccines were excluded from the study. All except three infants were Caucasian. The protocol was approved by the regional ethical committee.

Vaccines. Hib conjugate vaccine containing 10 μg of HibCP coupled to 24 μg of TT (HibCP-TT) (ActHib, lots S2181 and S2428) was supplied by Institut Pasteur Mérieux (Lyon, France). Diphtheria-tetanus-polio (DT-IPV) vaccine, containing $Al(OH)_3$-adsorbed TT (12.5 Lf, equal to approximately 45 μg), DT, and formalin-inactivated polio virus, was obtained from Statens Seruminstitut (Copenhagen, Denmark). This vaccine was sometimes given by the general practitioner, and several lots were used (lots 77, 78, 79, 80, 81, 82, and 83).

Study design. The schedule for vaccination and blood sampling is given in Table 1. The infants were randomized into three groups to receive DT-IPV at 6 and 7 months (group A), 5 and 6 months (group B), or 4 and 5 months (group C). All infants were immunized with HibCP-TT at 5 and 6 months, and blood was obtained by vein puncture before and 1 month after these vaccinations. Serum was prepared immediately and frozen at −20°C until testing. Both vaccines were given subcutaneously, laterally in the thigh, but on opposite sites. The same side and lot were used for both HibCP-TT immunizations.

Antibody determinations. Infant sera were tested for total antibody against HibCP and TT by enzyme-linked immunosorbent assays (ELISA) as described in detail elsewhere (4). In brief, plastic microtiter plates were coated with either HibCP coupled to poly-L-lysine (a gift from Connaught Laboratories, Swiftwater, Pa.) or purified TT (Statens Seruminstitut). Serum samples were added as six twofold dilutions. Only dilution buffer was added to four wells to allow determination of the background optical density (OD). After overnight incubation, the plates were washed and peroxidase-coupled rabbit anti-human total immunoglobulin antibodies (Dako, Glostrup, Denmark) were added (diluted 1/1,000). After 2 h, the washing was repeated and the plates were developed with 1,2-phenylenediamine dihydrochloride. The chromogenic reaction was stopped after 15 min by addition of 2 M H_2SO_4, and the ODs were read at 492 nm.

The results were expressed in micrograms per milliliter for HibCP antibodies and in international units per milliliter for TT antibodies by the use of two reference serum pools. The reference used in the anti-HibCP ELISA was calibrated against the Food and Drug Administration standard (lot 1983, assigned 70 μg/ml), while that used in the anti-TT ELISA was calibrated against a serum pool with a known content of TT antibody supplied by Iver Heron (Statens Seruminstitut). A parabola was used as the standard curve, using least-squares curve fitting. Preliminary concentrations of the test samples were determined as the averages of the values obtained from the individual dilutions with ODs paralleling the standard curve. For each serum the inhibitability by antigen was tested in duplicate wells containing the serum in the highest concentration in the presence of free relevant antigen (HibCP, 200 μg/ml; TT, 100 μg/ml). The preliminary concentrations were corrected for unspecific binding by multiplying by the fraction that could be inhibited by free antigen, calculated as $1 - (OD_{inhibited}/OD_{uninhibited})$ (ODs from the highest serum concentration after subtraction of the background). After this correction, the values from the HibCP ELISA correlated closely with values obtained by Helena Käyhty, in the current radioimmunoassay at the National Public Health Institute in Helsinki, over the range of 0.1 to 600 μg/ml (Pearson correlation coefficient for logarithmically transformed data: $r = 0.93$, $n = 20$). The lower detection limit was approximately 0.05 μg/ml, and readings below this were registered as 0.025 μg/ml.

Maternal sera were collected from the majority of the mothers when their infants were 12 to 13 months of age. These sera were analyzed for anti-TT immunoglobulin G (IgG) by using the anti-TT ELISA described above, except that peroxidase-coupled rabbit anti-human IgG (Dako; 1/1,000) was used as secondary antibody. Anti-TT IgG levels were expressed in arbitrary units.

Statistics. The Kruskal-Wallis test was used to evaluate differences in distributions between the three groups. If significant differences were found, individual groups were compared by the Mann-Whitney U test. Fischer's exact test and the chi-square test were used to evaluate nominal data when indicated in the text. Spearman rank correlation analysis was used for all correlations if not otherwise mentioned. $P < 0.05$ was considered statistically significant.

RESULTS

The parents of three infants withdrew after the first immunization and one more infant was withdrawn after the last immunization, leaving postvaccination blood samples from 144 infants (82 male and 62 female) available for testing.

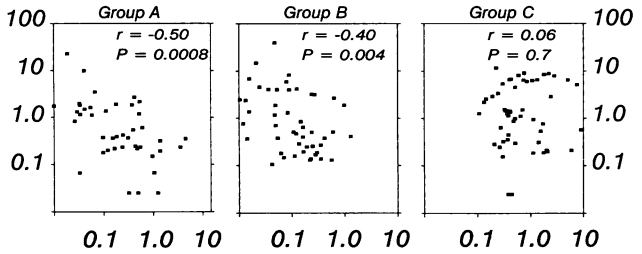

FIG. 1. Correlations between the prevaccination levels of TT antibodies (measured at 5 months) (international units per milliliter, x axis) and the levels of HibCP antibodies 1 month after the first immunization (at 5 months) with HibCP-TT (micrograms per milliliter, y axis). A total of 144 healthy Danish infants were randomized into three groups differing in respect to carrier priming. Group A was not actively immunized with TT. Group B received TT as a part of the DT-IPV vaccine concomitantly with HibCP-TT. Group C was primed at 4 months with DT-IPV and received concomitant DT-IPV immunization at 5 months. Spearman correlation coefficients and corresponding P values are given.

From six infants, blood samples were not collected on all scheduled occasions.

Table 1 shows the experimental design, with three groups receiving HibCP-TT immunizations with different timings of the DT-IPV vaccinations. No significant differences between the groups were found with respect to sex, gestational age, or birth weight (data not shown). The distribution of the two HibCP-TT vaccine lots for the three vaccination groups was, however, slightly skewed, as follows: group A, 19 and 24 infants for S2181 and S2428, respectively; group B, 26 and 22; and group C, 15 and 38 ($\chi^2 = 7.1$; df = 2; $P = 0.03$). The two lots did, however, induce similar levels of HibCP antibodies within the groups as measured at 6 and 7 months (Mann-Whitney U tests; $0.86 > P > 0.12$). Pooled data from both lots are therefore given below.

Influence of carrier priming. Since TT is a component of the DT-IPV vaccine as well as the carrier molecule in the HibCP-TT conjugate, the experimental design allowed us to examine the impact on the response to HibCP-TT of priming with TT. One month after the first injection of HibCP-TT (i.e., at 6 months), different median anti-HibCP levels were obtained for the three groups (Table 1) (Kruskal-Wallis test; $P = 0.02$). A threefold-higher median antibody level was obtained for group C, receiving both prior and concomitant carrier immunization, than for the unprimed group (A) ($P = 0.01$). In group C, the anti-HibCP levels also exceeded those in group B (only concomitant carrier immunization; $P = 0.08$). The anti-HibCP levels in the latter group (B) did not differ significantly from those in the unprimed group (A) ($P = 0.23$).

After the second immunization with HibCP-TT, all groups had received at least one DT-IPV injection, and no significant differences between the anti-HibCP levels were found (Kruskal-Wallis test; $P = 0.25$).

Influence of maternally acquired TT antibodies. Figure 1 shows the negative correlations in groups A and B between the anti-HibCP levels recorded after the first vaccination with HibCP-TT and the prevaccination levels of TT antibodies ($r = -0.50$, $P = 0.0008$, and $r = -0.40$, P = 0.004, respectively). Similar trends were seen after the second dose of HibCP-TT, but these were not statistically significant (data not shown). The infants of these groups had not been actively immunized with TT before the blood sample was drawn at 5 months, and the TT antibodies were therefore expected to be of maternal origin. This assumption was confirmed by the demonstration of a close correlation between these antibody levels and the levels of anti-TT IgG in the maternal sera (group A, $r = 0.94$, $P < 0.000001$, $n = 32$; group B, $r = 0.87$, $P < 0.000001$, $n = 31$). In fact, in group A a negative correlation between the anti-HibCP levels at 6 months and the maternal anti-TT IgG levels was found ($r = -0.58$, $P = 0.0007$).

In group C, the anti-TT levels at 5 months were fourfold higher than in groups A and B (Table 1) because of active immunization with DT-IPV 1 month earlier. Under these circumstances, a negative influence of preexisting TT antibodies on the formation of antibody to HibCP was not observed (Fig. 1; $r = 0.06$, $P = 0.7$). Furthermore, no correlation between the maternal anti-TT IgG levels and the anti-HibCP levels obtained in this group at 6 months was found ($r = -0.08$, $P = 0.6$, $n = 39$).

Table 2 shows the numbers of infants from groups A and B (pooled) that obtained defined protective levels of HibCP after the first and second doses of HibCP-TT. The infants were stratified by the median prevaccination anti-TT level into two groups with high and low levels of maternally acquired TT antibodies. After both the first and the second HibCP-TT doses, significantly more infants with high prevaccination levels of TT antibodies failed to reach the defined anti-HibCP level of long-term protection (1 μg/ml) (Table 2). In group C, 22 (of 53 [42%]) infants had less than 1 μg of HibCP antibody per ml after the first dose, and 1 (of 50 [2%]) infants had a level below this limit after the second dose.

Adverse effects. No serious adverse effects were observed in infants vaccinated with HibCP-TT and/or DT-IPV. The adverse effects will be discussed in detail elsewhere.

TABLE 2. Numbers of infants with postvaccination anti-HibCP levels below or above defined limits of short-term (0.15 μg/ml) and long-term (1.0 μg/ml) protection after one or two doses of HibCP-TT[a]

Prevaccination anti-TT level (IU/ml) at 5 mo	No. (%) of infants with the indicated postvaccination anti-HibCP level (μg/ml) after dose:					
	1				2	
	<0.15	≥0.15	<1.0	≥1.0	<1.0	≥1.0
<0.12	3 (7)	41 (93)	17 (39)	27 (61)	1 (2)	43 (98)
≥0.12	9[b] (20)	37 (80)	36[c] (78)	10 (22)	8[c] (18)	36 (82)

[a] The two doses were given at 5 and 6 months, respectively. Individuals from groups A and B were divided into two groups on the basis of a low or high prevaccination level of maternally acquired TT antibodies.

[b] Indicates a tendency for more infants failing to reach the defined short-term protective level among infants with high prevaccination levels of anti-TT ($P = 0.07$, Fischer's exact test).

[c] Significantly more infants failing to reach the defined long-term protective level after one ($P = 0.0001$) and two ($P = 0.01$) doses of HibCP-TT.

DISCUSSION

In 1929, Avery and Goebel (3) showed that coupling of polysaccharides to protein carriers increases the immunogenicity of polysaccharides in experimental animals. Recent studies with humans, first with pure HibCP (24) and later with protein conjugates thereof, have shown that coupling of capsular polysaccharides to protein carriers also greatly improves their immunogenicity in infants (11). HibCP is a good immunogen in adults, but conjugates yield higher antibody responses (15). The role of the protein carrier in the superiority of such conjugates over the pure polysaccharide is, however, still poorly understood. HibCP, like many capsular polysaccharides, has been classified as a T-lymphocyte-independent antigen (type II) in rodents (21). From studies of the response to haptenated proteins in rodents (20, 25), it seems likely that the carrier molecule works by supplying T-cell epitopes to otherwise T-cell-independent polysaccharides. According to our current understanding of the cognate T-cell–B-cell interactions (reviewed in reference 22), presentation of such epitopes for $CD4^+$ T-helper cells by the HibCP-specific B cell leads to activation and clonal expansion of both carrier-specific T cells and polysaccharide-specific B cells. This hypothesis explains the ability of conjugate vaccines to induce higher antibody responses than pure HibCP as well as the ability of conjugates to prime for booster responses (reviewed in reference 30). According to this model, priming with the carrier molecule should enhance the response to conjugate vaccines by increasing the number of carrier-specific T lymphocytes. Enhancement by priming has indeed been found in rodents and rhesus monkeys (1, 28, 29). For humans, however, few studies have addressed this important question, and none has convincingly confirmed the existence of a beneficial effect of carrier priming. However, several observations point to this possibility (16). For example, Anderson and coworkers found that concomitant immunization of infants with HibCP (oligosaccharides) conjugated to DT and diphtheria-tetanus-pertussis vaccine was less efficient in inducing HibCP antibodies than if the injection of the conjugate was delayed for 1 month after the diphtheria-tetanus-pertussis immunization (2). This could be explained by carrier priming, but the result could also be a consequence of the infants in the high-responder group being 1 month older.

From the present study it can be concluded that priming with DT-IPV at 4 months followed by concomitant injection of this vaccine and HibCP-TT at 5 months increases the anti-HibCP response compared with that induced by HibCP-TT administered without DT-IPV. Coadministration of DT-IPV and HibCP-TT at 5 months without priming at 4 months did not result in significant increases in HibCP antibody levels compared with HibCP-TT given alone. Both observations are compatible with the induction of carrier-specific T-cell immunity, since activation, expansion, and dissemination of carrier-specific T cells take time. Consequently, priming 1 month earlier is expected to be more effective than coadministration. However, an enhancing effect of concomitant carrier immunization on the response to HibCP conjugates has been found in experimental animals (29, 31), and our findings do not exclude that coadministration alone could have some effect on the response to HibCP-TT which was overlooked in the present study because of the relatively small numbers of infants examined. It should also be noted that this study has not formally ruled out the possibility that the positive effect of the DT-IPV vaccine was caused by components other than TT. However, we find this possibility unlikely, since we have shown that in adults prior immunization with irrelevant $Al(OH)_3$-adsorbed protein antigens did not interfere with the subsequent response to HibCP conjugates, including HibCP-TT (4).

Another finding of this study is that maternally acquired TT antibodies present at the time of immunization with HibCP-TT suppressed the response to the polysaccharide part of HibCP-TT in a dose-dependent manner (Fig. 1, group A). This effect was not overcome by coadministration of TT (group B) but was overcome by active priming with TT 1 month earlier (group C). Infants (groups A and B) with high prevaccination levels (above the median) failed to reach the defined long-term protective level (1 μg/ml) of HibCP antibody more often than those with low levels, even after the second immunization (Table 2). Claesson and coworkers (7) also found a negative influence of maternally acquired TT antibodies on the response to HibCP-TT given at 3 and 5 months of age, but unlike us they found a negative influence on the response to the TT component only, not on the anti-HibCP response.

The demonstration in this study of opposite effects of passively (antibody-mediated) and actively (carrier-specific T-cell-mediated?) acquired immunity to the carrier on the response to HibCP-TT points to several possible ways to optimize conjugate polysaccharide vaccines for future use in childhood immunization programs. The negative influence of maternal antibodies may explain the superiority of HibCP coupled to meningococcal outer membrane protein as compared with TT or DT conjugates when infants are immunized at 2 months of age (9, 14), since maternal antibodies to this outer membrane protein are not expected to be present in significant amounts. Thus, in populations in which very early protection against Hib is needed, the use of carriers that are not part of the routine vaccination protocols may be the most rational approach. Alternatively, prevaccination with the carrier could be tried as a means to increase the response to the first dose of the HibCP conjugate. It should be noted, however, that with the protocols used in the present study, we found no significant differences in the median anti-HibCP levels after the second dose of HibCP-TT.

Although the present study demonstrates that the inhibitory influence of preexisting TT antibodies on the response to HibCP-TT can be counteracted by active immunization with the carrier, this may not be the case in situations in which the anti-TT levels are very high. In fact, we have recently shown for adults that recent preimmunization with TT inhibits the anti-HibCP response to HibCP-TT (4). In that study, preimmunization induced very high anti-TT levels, and it is likely that the suppression was in some way caused by these antibodies.

Suppression by carrier priming is well documented in studies of the antibody response to haptenated proteins in rodents. Suppression is usually reported in experiments in which very large amounts of carrier are used for priming (often together with an adjuvant) and high anticarrier antibody levels are achieved. Passive transfer of large amounts of anticarrier antibody has been shown to inhibit the response to a hapten-carrier conjugate (18). In some systems, including that described in a recent report on suppression of the response to a polysaccharide conjugate by carrier priming in mice (23), suppression was abrogated or even changed into enhancement if the amount of carrier used for priming was reduced (27) to a level causing a low antibody response to the carrier. A similar effect has been achieved by removing B-cell epitopes from the carrier by dodecanoylation (27)

or by using peptide fragments containing T-cell epitopes but no B-cell epitopes instead of the intact carrier (13).

The success of the HibCP conjugates has greatly stimulated interest in the development of polysaccharide-protein conjugates for the protection of children and adults against a variety of capsulate bacteria, including *Neisseria meningitidis*, *Streptococcus pneumoniae*, group b streptococci, *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhi*, and *Pseudomonas aeruginosa*. With the present tendency to use a limited number of B-cell immunogenic proteins as carriers, there is a risk that anticarrier antibodies induced by one conjugate will suppress the response to another (or the same) conjugate. Such negative interactions between vaccines could take place in one individual or be transferred by maternal antibodies from mother to infant. In the long run, it may therefore be necessary to design protein carriers with reduced ability to invoke synthesis of anticarrier antibody but still containing T-cell epitopes. Denaturation of the carrier protein or the use of oligopeptides containing well-defined T-cell epitopes may be ways to achieve this goal.

ACKNOWLEDGMENTS

We thank Tonni Hansen and Marianne Pedersen for excellent technical assistance, Helena Käyhty for assistance with assay calibration, and Connaught Laboratories for supplying reagents.

This work was supported by the Lundbeck Foundation, the Danish Medical Research Council (grants no. 12-0251-1 and 12-0251-2), the Danish Hospital Foundation for Medical Research (region of Copenhagen, the Faroe Islands, and Greenland), and Pasteur Mérieux A/S.

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