The V-Region Repertoire of *Haemophilus influenzae* Type b Polysaccharide Antibodies Induced by Immunization of Infants

GOOK H. CHUNG,¹ KYUNG H. KIM,¹ ROBERT S. DAUM,⁴ RICHARD A. INSEL,³ GEORGE R. SIBER,² SUNIL SOOD,⁴ RAJESH K. GUPTA,⁵ COLIN MARCHANT,⁶ AND MOON H. NAHM^{1*}

Department of Pathology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110¹;

Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115²; Department of Pediatrics,

University of Rochester Medical Center, Rochester, New York 14642³; Department of Pediatrics,

University of Chicago, Chicago, Illinois 60680⁴; Massachusetts Public Health Biologic Laboratories,

Boston, Massachusetts 02130⁵; and Department of Pediatrics, Tufts University,

School of Medicine, Boston, Massachusetts 02111⁶

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Haemophilus influenzae type b (Hib) is a significant pathogen for young children, and three Hib vaccines (named PRP-OMPC, HbOC, and PRP-T) are currently available for young children. Extensive studies of anti-Hib polysaccharide (PS) antibodies (Abs) have shown that the V regions of Abs against the Hib PS comprise a VH gene in the VH3 gene family and a VL gene from various V_κ and V_λ subgroups. To study immunogenic properties of the three vaccines in young children, we determined the VL subgroups and avidities of anti-Hib-PS Abs induced by the three clinically available conjugate vaccines. Ab avidity was measured by determining the concentration of a Hib-PS oligomer that abrogates half of the binding of immunoglobulin G anti-Hib-PS Abs to microwells. The PRP-OMPC vaccine induced lower-avidity Abs than the precensure HbOC vaccine ($P = 0.05$). When we compared anti-Hib-PS Abs expressing V_κIa, V_κII, and V_λ subgroups, a greater Ab response was induced by the precensure HbOC vaccine than other vaccines ($P < 0.05$). When anti-Hib-PS Abs with the V_κIII subgroup were compared, however, both PRP-T and precensure HbOC vaccines induced a comparable response, which in turn was greater than those induced by the PRP-OMPC or the postlicensure HbOC vaccine ($P < 0.001$). The VL repertoire of Abs induced with the precensure HbOC or PRP-T vaccine in young children is dominated (about 80%) by anti-Hib-PS Abs using subgroup V_κII. However, anti-Hib-PS using V_κII VL accounts for only about 40% of the total anti-Hib-PS Abs induced with the PRP-OMPC vaccine or the postlicensure HbOC. Our data suggest that immunogenic properties of Hib vaccines in young children vary depending on the vaccine preparations as well as the vaccine types.

Haemophilus influenzae type b (Hib) was the main cause of bacterial meningitis in young children (6 to 24 months old) until recently. Currently, all children are vaccinated to induce antibodies (Abs) to Hib capsular polysaccharide (Hib-PS) which can protect children from Hib infection. There are currently three vaccines that have been licensed for use in 2- to 6-month-old children (2, 4). Two vaccines are prepared by conjugating Hib-PS to the *Neisseria meningitidis* outer membrane protein (OMP) or tetanus toxoid (TT). The two vaccines are referred to here as PRP-OMPC and PRP-T vaccines, respectively. The third is prepared by conjugating a short oligosaccharide of Hib-PS to a nontoxic mutant of diphtheria toxin. This vaccine is referred to here as the HbOC vaccine. The three conjugate vaccines differ in immunologic properties. For instance, the PRP-OMPC vaccine displays several characteristics associated with a type I T-cell-independent antigen. The carrier, OMP, itself can induce polyclonal proliferation of lymphocytes in vitro, like other T-cell-independent antigens (13, 23). Single immunization with the PRP-OMPC vaccine induces a good Ab response, and repeat immunizations given during the routine immunization schedule do not further increase Ab levels (7). In contrast, the HbOC and PRP-T vaccines readily induce an anamnestic response characteristic of a T-cell-dependent antigen (7).

Anti-Hib-PS Abs have been extensively studied, and the molecular basis for the V-region diversity of anti-Hib-PS Abs is now well understood in adults. An adult usually produces three to five clones of anti-Hib-PS Ab (11) which use a VH gene belonging to the VH3 gene family and a VL gene selected from many V_κ and V_λ subgroups (20). The product of a V_κII gene (named A2) is used for the majority of anti-Hib-PS Ab clones (19). Anti-Hib-PS Abs using the A2 gene and a gene in the V_λ7 subgroup have been correlated, respectively, with the two idiotypes named HibId-1 (15) and HibId-2 (8). In addition, there are only one or two anti-Hib-PS clones in each VL subgroup, and the VL subgroup can be identified serologically. Thus, the VL subgroup expression of anti-Hib-PS Abs is a convenient marker of an individual Ab clone in many cases.

Abs expressing different VL subgroups can have different immunoregulatory behaviors in mice (24, 26) as well as in human adults (3). The results of a recent study indicated that the PRP-OMPC vaccine elicits anti-Hib-PS Abs with lower avidity than those elicited by the HbOC vaccine in young children and has suggested that Ab V regions induced by the two vaccines may differ (17). A2 Abs (i.e., anti-Hib-PS Abs using the A2 V_κII gene) have higher avidities (16a) and are more specific for Hib-PS (21) than non-A2 Abs. Also, A2 Abs are more commonly of the immunoglobulin G (IgG) isotype than non-A2 Abs, which are mostly of the IgM and IgA isotypes (unpublished observation). Also, the V regions used in young children differ from those in adults (14), and young children may produce Abs with lower avidities than adults. To better understand the differences in the immunogenic proper-

* Corresponding author. Mailing address: Box 8118, Department of Pathology and Medicine, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-3186. Fax: (314) 362-1461.

ties of the vaccines in young children, we have studied the V regions of anti-Hib-PS Abs induced in young children with the three conjugate vaccines.

MATERIALS AND METHODS

MAbs. The monoclonal Abs (MAbs) HG11, HP6016, HK2, HL1, and δ ADA, specific for human IgG1, IgG2, C κ , C λ , and IgM, respectively, have been described (22, 25). MAb OAK-1 from G. Silverman and T. Kipps (San Diego, Calif.) is specific for a subfamily of V κ I L chains (1). It binds the product of the L11 V κ I gene but not of the O8 V κ I gene (3). The V κ I subfamily recognized by OAK-1 is named V κ Ia in this report. The MAbs KB13 and B12 are specific for human V κ II (3) and V κ III L chains, respectively (16).

Antisera from children. Thirty children received the PRP-OMPC (Merck Sharp & Dohme, West Point, Pa.) vaccine prepared before its licensure at 2 and 4 months of age, and blood samples were obtained at 5 months of age. The vaccination study, which was fully described previously (5), was performed in Louisiana with PRP-OMPC vaccine lots 1069 and 1080. Thirty-six children received prelicensure PRP-T vaccine (Institut Merieux, Lyon, France), 27 children received the postlicensure preparation of HbOC vaccine (Lederle-Praxis Biologics, Rochester, N.Y.), and 30 children received a prelicensure preparation of HbOC vaccine (Lederle-Praxis Biologics). The PRP-T vaccine was studied in Massachusetts from 1989 to 1990 using vaccine lots S1982, S1983, and S1984. Postlicensure HbOC was studied also in Massachusetts from 1992 to 1994 using various vaccine lots, including M190HE, M600JA, and M135KA. Prelicensure HbOC vaccine was studied in Pennsylvania using vaccine lot E-12-3. All children were recruited from private practices of physicians in suburban areas, and most (>90%) of them were Caucasian. The children were vaccinated at 2, 4, and 6 months of age, and blood samples were obtained at 7 months of age and stored frozen. Because of the amount of sera available for the study, avidity was measured with 6 children who received PRP-T, 5 who received PRP-OMPC, 15 who received postlicensure HbOC, and 10 who received prelicensure HbOC, and the V-region repertoires were determined with 20 children vaccinated with a prelicensure preparation of HbOC.

Farr-type immunoassay. The amounts of total anti-Hib-PS Ab in postvaccine sera and of purified clonal anti-Hib-PS Abs were measured in a radiolabeled antigen binding assay (Farr assay) using ¹²⁵I-Hib-PS (27). The Food and Drug Administration reference serum containing 70 μ g of anti-Hib-PS Ab per ml was used as the primary assay standard.

Sandwich-type ELISAs. The amount of anti-Hib-PS Ab expressing a specific isotype, V κ subgroup, or V λ subgroup was determined by sandwich-type enzyme-linked immunosorbent assays (ELISAs) as previously described in detail (3). Briefly, the wells of Immulon II plates (Dynatech, Chantilly, Va.) were coated with tyramine-conjugated Hib-PS (1 μ g/ml), washed, and blocked with phosphate-buffered saline (PBS) containing 1% nonfat milk (Carnation, Los Angeles, Calif.). Either a characterized human serum pool standard (called CRM-pool B) (3) or sera from immunized children were then added, titrated, and incubated for 4 to 6 h. The serum pool was initially calibrated using clonally pure Abs (3). Plates were subsequently washed, and then one of the above-mentioned anti-human Ig MAbs diluted in 1% skim milk in PBS was added to every well. After overnight incubation, goat anti-mouse Ig Ab labeled with alkaline phosphatase was added. The amount of the enzyme immobilized on the well was determined with *para*-nitrophenyl phosphate substrate (Sigma). Optical density at 405 nm was read with a microplate reader (Cambridge Technology, Watertown, Mass.). The amount of Ab in the sample was determined by comparing the optical density of the samples to that of the standard. One unit is approximately 1 μ g of Ab.

Determination of avidity. To measure Ab avidity, we performed a modified ELISA as described by Hetherington (10). Briefly, the wells of Immulon II plates (Dynatech) were coated with tyramine-conjugated Hib-PS (0.1 μ g/ml) by overnight incubation, washed, and blocked with PBS containing 1% nonfat milk powder (Carnation). A serum sample from a child that contained 200 ng of Ab per ml was added to wells in the presence of an oligosaccharide inhibitor at various concentrations (ranging from 0.06 to 960 nM) and then incubated for 2 h at 4°C. The inhibitor is a fragment of Hib-PS with an average of 20 repeating units (range 15 to 35 U) obtained from Lederle-Praxis Biologics. Plates were subsequently washed, and alkaline phosphatase-conjugated goat anti-human IgG Abs (product 62-8422, lot 21212172; Zymed Laboratories, San Francisco, Calif.) diluted in 1% nonfat milk in PBS were added to every well. After several hours of incubation, the wells were washed and the amount of the enzyme immobilized on the well was determined with *para*-nitrophenyl phosphate substrate (Sigma). Optical density at 405 nm was read with a microplate reader (Cambridge Technology). The avidity was defined as the inverse of the concentration of the oligosaccharide that inhibited the binding of the Ab in the sample by 50%.

RESULTS

Measurement of avidities of IgG anti-Hib-PS Abs. To study the V-region differences of anti-Hib-PS Abs, we estimated avidities of Abs in 36 samples by determining the concentra-

TABLE 1. Avidity of anti-Hib-PS Abs of children

Vaccine	<i>n</i>	Avidity [10 ⁶ M ⁻¹ (mean $\pm$ SE)] ^a
PRP-T	6	94 $\pm$ 30
PRP-OMPC	5	73 $\pm$ 27
Prelicensure HbOC	10	160 $\pm$ 32
Postlicensure HbOC	15	190 $\pm$ 69

^a Units are 10⁶ M⁻¹ of the oligosaccharide with 20 repeating units. The differences between mean avidities were not statistically significant except for the prelicensure HbOC vaccinees versus the PRP-OMPC vaccinees.

tion of a Hib-PS oligomer (with 20 repeating units) that can inhibit anti-Hib-PS Ab binding to immobilized Hib-PS (Table 1). Previously, Hetherington (10) showed that the same method can measure the affinities of anti-Hib-PS Abs when a very short-Hib-PS oligomer (with three repeating units) is used as the inhibitor. The avidities measured by this method were independent of the total Ab concentrations (correlation coefficient, 0.16) and of the ratio of V κ II Abs to total Abs (correlation coefficient, -0.25). The average avidity of Abs induced with the PRP-OMPC vaccine was lowest (73 $\times$ 10⁶ M⁻¹), and those induced with HbOC vaccinees were higher (160 $\times$ 10⁶ to 190 $\times$ 10⁶ M⁻¹) (Table 1). The avidity differences between all the groups were not statistically significant except for the difference between the prelicensure HbOC vaccinees and the PRP-OMPC vaccinees (P = 0.05 by two-tailed t test). The postlicensure HbOC vaccine-induced Abs had the highest average avidity, but their avidity results were not significantly different from those of Abs induced by the PRP-OMPC vaccine because of a wide variation in the results.

Measurement of the V-region repertoire of anti-Hib-PS Abs.

To study the Ab V-region repertoire expressed in young children, we measured the anti-Hib-PS Abs expressing various VL subgroups in sandwich ELISAs we have previously described (3). As shown in Fig. 1, the sum of kappa and lambda Ab levels for each individual person correlated with the total Ab levels very well (correlation coefficient, 0.81 for all vaccinees). The sum of V κ Ia, V κ II, and V κ III Abs also correlated with total kappa Ab levels very well (Fig. 1) (correlation coefficient, 0.86 for all vaccinees). These findings indicated that our assays were appropriately calibrated (i.e., 1 U is approximately 1 μ g of Ab [3]), and most anti-Hib-PS Abs were detected by our assays. Nevertheless, for the groups immunized with PRP-OMPC and PRP-T, the sum of geometric means of kappa and lambda levels was less than the mean of the total Ab levels. While this finding may suggest that some antibodies are not detected by ELISA, this is most likely due to a mathematical property of geometric means of values which are distributed over a very large range (see Table 2 for an illustration). In about 10% of cases, the sum of V κ Ia, V κ II, and V κ III subgroup Abs was less than half of that of the Abs expressing the C κ light chain. Some anti-Hib-PS Abs use O8 or O18 V κ I subgroup genes; both code for a protein product with the same amino acid sequence. Since O8 (or O18) V κ I Abs are not detected by our V κ Ia Ab assay, it is likely that about 10% of individuals produce a significant amount of Abs using the O8 or O18 V κ I genes. Consistent with this explanation, we previously found (by amino acid sequencing) O8 (or O18) V κ I Ab clones in 3 of 15 adult vaccinees (18).

Antibody responses elicited by these vaccines are compared in Tables 3 and 4. For the overall Ab responses, the prelicensure HbOC vaccine induced much higher Ab levels than the other three vaccines, which elicited comparable Ab responses. This general pattern was also observed when the Ab responses

FIG. 1. (A and C) Comparison of sums of anti-Hib-PS Abs expressing κ and λ light chains (y axis) with total Ab levels measured by Farr assay (x axis). Solid squares indicate data for sera from children immunized with PRP-T, solid circles indicate prelicensure HbOC vaccinees, open circles indicate postlicensure HbOC vaccinees, and open triangles indicate PRP-OMPC vaccinees. The best-fit line for all the vaccinees determined by the least-square method is shown and is $y = 0.83x + 0.0089$. (B and D) Comparison of anti-Hib Abs expressing κ light chain (y axis) with sums of Abs expressing V_{κ} Ia, V_{κ} II, or V_{κ} III subgroups (x axis). The symbols are the same as for panels A and C. The best-fit line for all the vaccinees obtained by the least-square method is $y = 0.7x + 0.15$.

were compared in C_{λ} , V_{κ} Ia, and V_{κ} II subgroups individually, although the conclusion is not strong for V_{κ} Ia because many samples in these groups had undetectable levels (below 0.5 U/ml) of Abs. Interestingly, the Abs in the V_{κ} III subgroup did not follow the general pattern. The PRP-T vaccine elicited V_{κ} III Ab responses comparable to the prelicensure HbOC vaccine, and in turn, the V_{κ} III Ab responses induced by both vaccines were much greater than that induced by the PRP-OMPC or postlicensure HbOC vaccine ($P < 0.001$).

It was reported that the PRP-OMPC vaccine elicits a low level of Hib Id1⁺ Abs compared with other VL subgroups (8). The Hib-Id1 idiotype is expressed on V_{κ} II anti-Hib-PS Abs (15), and we compared the averages of the ratios of V_{κ} II Abs to the sum of C_{κ} and C_{λ} Abs for all the vaccination groups (Tables 3 and 4). PRP-OMPC vaccinees had a significantly lower ratio [V_{κ} II/($C_{\kappa} + C_{\lambda}$)] than the PRP-T or prelicensure HbOC vaccinees ($P < 0.05$ by the *t* test) (Tables 3 and 4). However, unexpectedly, the ratio [V_{κ} II/($C_{\kappa} + C_{\lambda}$)] for the postlicensure HbOC vaccine was as low as that for PRP-OMPC.

TABLE 2. Geometric means distributed over large range

Sample	Value for group:		Sum
	A	B	
1	100	1	101
2	1	100	101
Geometric mean	10	10	20

DISCUSSION

To assess immunogenic properties of the three Hib vaccines used for young children, we studied the V regions of Abs by measuring Ab avidity. Schlesinger et al. (17) reported that the PRP-OMPC vaccine elicits Abs with about two- to threefold-lower avidities than the HbOC vaccine in 6-month-old chil-

TABLE 3. Anti-Hib-PS Ab levels in young children

Vaccines (<i>n</i>)	Mean Ab levels (SE) ^a						
	Total Ab	C _κ	C _λ	V _κ Ia ^b	V _κ II	V _κ III	V _κ II/ (C _κ +C _λ)
PRP-OMPC (<i>n</i> = 30)	3.62 (1.18)	1.36 (1.28)	0.33 (1.24)	0.37 (1.1)	0.76 (1.32)	0.12 (1.17)	0.49 (0.057)
PRP-T (<i>n</i> = 36)	3.18 (1.26)	1.74 (1.30)	0.23 (1.29)	0.3 (1.07)	1.35 (1.31)	0.29 (1.15)	0.82 (0.092)
Post-HbOC (<i>n</i> = 27)	4.89 (1.41)	3.40 (1.35)	0.127 (1.28)	0.30 (1.12)	1.52 (1.41)	0.062 (1.20)	0.52 (0.054)
Pre-HbOC (<i>n</i> = 20)	11.47 (1.26)	10.12 (1.28)	1.05 (1.36)	0.61 (1.24)	4.95 (1.54)	0.35 (1.2)	0.79 (0.12)

^a Geometric mean and standard error except for the column V_κII/ (C_κ+C_λ), for which the algebraic mean and standard error is used. Total antibody was determined by Farr assay and is expressed in micrograms per milliliter. All other groups were measured by sandwich-type ELISA, and the results are expressed in units per milliliter; 1 U is approximately 1 μg (3).

^b For samples with V_κIa values below the detection limit (0.5 U/ml), half of the detection level was assigned for calculation. The number of individuals below the detection limits were 29 among 36 PRP-T vaccinees, 17 among 30 PRP-OMPC vaccinees, 3 among 18 prelicensure HbOC vaccinees, and 24 among 27 postlicensure HbOC vaccinees.

dren. Avidity is not a physical constant but is an experimentally defined parameter. Consequently, the avidity results may depend on the measurement methods employed. To confirm whether the PRP-OMPC vaccine elicits Abs with lower avidities than those elicited by the HbOC vaccine, we have used a method of measuring avidity different from Schlesinger's method (17). Methods similar to ours have been used to study avidities of Abs to other antigens (28). Using this method, we have confirmed that the PRP-OMPC vaccine induces about twofold-lower-avidity Abs than the HbOC vaccines, although the difference was statistically significant only with the prelicensure HbOC. The low avidity is not likely due to the fact that PRP-OMPC induces low amounts of Abs expressing Hib-Id1 compared with other vaccines since the level of V_κII anti-Hib-PS Abs was independent of the avidity. Nevertheless, this finding suggests that the immunogenic properties vary among the Hib vaccines. Low avidity may be clinically significant in young children who produce a small amount of Abs to PS antigens, although the biologic significance of low avidity is not yet clear.

Additional differences in immunogenicities among the Hib vaccines were observed when we studied the repertoire of the VL regions of Abs produced by these vaccines. In most subgroups of Abs, the prelicensure HbOC vaccine was found to induce significantly greater responses than the other vaccines. This finding was not surprising since the prelicensure HbOC vaccine has been found to elicit often higher total Ab responses compared with the postlicensure HbOC, PRP-T, or PRP-OMPC vaccine (6, 7). However, when Abs using the V_κIII subgroup were examined, the PRP-T vaccine elicited V_κIII Abs as well as the prelicensure HbOC vaccine and both of these conjugate vaccines elicited much more (*P* < 0.001) V_κIII Abs than the PRP-OMPC vaccine. During our study, it was reported that Hib-Id1, which is expressed on V_κII anti-Hib-PS Abs (15), is expressed only in a minority of anti-Hib-PS Abs induced with the PRP-OMPC vaccine in 6-month-old chil-

dren, whereas the idiotype is expressed in the majority of anti-Hib-PS Abs among HbOC and PRP-T vaccinees (8). Our results were partially consistent with this observation: PRP-OMPC induced the least amount of Ab in the V_κII subgroup compared with PRP-T or the prelicensure HbOC.

A most unexpected finding is that the postlicensure HbOC elicits less Abs in the V_κII subgroup than the prelicensure HbOC vaccine or PRP-T vaccine. The observed difference is unlikely due to differences in vaccinees, who were mostly Caucasians recruited in a similar manner in both cases. Only quantitative differences have been reported so far in the Ab response between pre- and postlicensure HbOC vaccines (6, 8) or between PRP-T vaccine preparations (9). We now report a qualitative difference in Abs induced by different HbOC vaccine preparations; the postlicensure HbOC vaccine induces less V_κII Abs than the prelicensure HbOC vaccine. The findings of our retrospective analysis need to be confirmed by a prospective study of a large number of samples. Nevertheless, this difference may arise from altered physical conjugation of oligosaccharide termini to carrier protein, inasmuch as non-V_κII Abs may bind to the termini of Hib-PS (16a). One of several possible speculations is that a larger fraction of the short oligosaccharide chain bound to the carrier protein at only one end instead of at both ends in the postlicensure preparation. While it is not yet clear whether these immunochemical differences lead to significant functional differences, additional studies should be performed to confirm the observed difference.

Differences in the immunogenicities of Hib conjugate vaccines have been generally explained by the possibility that Hib-PS and PRP-OMPC vaccines are T-cell-independent antigens and PRP-T and HbOC vaccines are T-cell-dependent antigens. Although this is consistent with several observations made with Hib conjugate vaccines, conjugate vaccines against *Streptococcus pneumoniae* containing OMP protein display the anamnestic response like a typical T-cell-dependent antigen

TABLE 4. *P* values for all comparison pairs of groups in Table 3

Comparison pair	<i>P</i> value ^a						
	Total Ab	C _κ	C _λ	V _κ Ia	V _κ II	V _κ III	V _κ II/ (C _κ +C _λ)
PRP-T-PRP-OMPC	NS	NS	NS	4.7 × 10 ⁻²	NS	1.2 × 10 ⁻⁴	3.7 × 10 ⁻³
PRP-T-Pre-HbOC	3.1 × 10 ⁻⁴	1.4 × 10 ⁻⁵	7.6 × 10 ⁻⁴	4.7 × 10 ⁻³	1.8 × 10 ⁻²	NS	NS
PRP-T-Post-HbOC	NS	NS	9.9 × 10 ⁻²	NS	NS	2.6 × 10 ⁻⁸	8.8 × 10 ⁻³
PRP-OMPC-Pre-HbOC	3.1 × 10 ⁻⁴	9.8 × 10 ⁻⁷	4.7 × 10 ⁻³	5.3 × 10 ⁻²	1.1 × 10 ⁻³	6.1 × 10 ⁻⁵	2.7 × 10 ⁻²
PRP-OMPC-Post-HbOC	NS	2.4 × 10 ⁻²	7.0 × 10 ⁻³	NS	NS	8.5 × 10 ⁻³	NS
Pre-HbOC-Post-HbOC	4.8 × 10 ⁻²	8.4 × 10 ⁻³	6.9 × 10 ⁻⁶	8.1 × 10 ⁻³	4.4 × 10 ⁻²	3.5 × 10 ⁻⁸	4.8 × 10 ⁻²

^a *P* values were obtained by using the two-tailed *t* test on the logarithm-transformed values for the Ab concentrations. NS, not significant (*P* values greater than 0.05).

(12). In addition, we found that different preparations of HbOC vaccines display striking differences in immunogenic behavior. Our understanding of the immunologic behavior of conjugate vaccines is still insufficient not only to predict the immunogenic behavior of conjugate vaccines but also to even reliably manufacture a conjugate vaccine. These limits should be recognized, and studies of immunogenicity of Hib conjugate vaccines should be generalized with caution.

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