Heavy-Chain Isotype Patterns of Human Antibody-Secreting Cells Induced by *Haemophilus influenzae* Type b Conjugate Vaccines in Relation to Age and Preimmunity

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The influence of preexisting immunity on the heavy-chain isotypes of circulating antibody-secreting cells (AbSC) induced by vaccination with *Haemophilus influenzae* type b (Hib) capsular polysaccharide (HibCP) coupled to tetanus toxoid (TT) or diphtheria toxoid (DT) and by vaccination with TT or DT alone in 51 healthy adults and 9 infants was studied. In adults, the isotypes of TT and DT AbSC were dominated by immunoglobulin G1 (IgG1) followed by IgG4 and IgA1. HibCP AbSC were dominated by the isotype IgA1 followed by (in decreasing order) IgG2, IgA2, IgM, and IgG1. The isotype distributions of TT and DT AbSC were independent of whether the toxoids were coupled to HibCP, and the isotypes of HibCP AbSC were not influenced by the nature of the carrier (TT or DT). Furthermore, the isotype distributions were unaffected by recent immunization with components of the conjugates, although this reduced the numbers of AbSC. The heavy-chain gene usage of HibCP AbSC in adults differed clearly from that in infants, which was restricted largely to the genes µ, γ1, and α1, all lying upstream in the heavy-chain constant-region gene locus, while the usage in adults also, to different extents, involved the downstream genes γ2 and α2. The ratio between the numbers of HibCP AbSC using heavy-chain genes from the downstream duplication unit (γ2, γ4, and α2) and those using genes from the upstream duplication unit (γ3, γ1, and α1) correlated with the preimmunization level of natural HibCP antibodies (r = 0.59; P = 0.00002). A possible role of natural exposure for Hib or cross-reactive bacteria on the mucosal surfaces in the shaping of the isotype response to HibCP conjugate vaccines is discussed.

Capsulate bacteria such as *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, and *Neisseria meningitidis* are important causes of serious invasive diseases throughout the world. The incidence is highest in infants because of their low levels of capsule antibodies in plasma. During childhood, natural antibodies against many of these polysaccharides are eventually generated and the incidence of invasive diseases declines. Invasive Hib infections are rare after the age of 5 years.

Conjugate vaccines consisting of Hib capsular polysaccharide (HibCP) covalently coupled to protein carriers have proved effective in inducing protective levels of HibCP antibodies in infants (2, 9, 12) and have recently been included in childhood vaccination programs in many countries. A variety of conjugate vaccines containing polysaccharides from other pathogenic bacteria are currently under development, and more knowledge about the function(s) of the carriers in these vaccines is therefore needed.

Studies with experimental animals suggest that one major role of the carrier protein is to supply T-cell epitopes to the otherwise T-cell-independent polysaccharides (35). This would allow carrier-specific helper T cells to drive the proliferation and differentiation of polysaccharide-specific B cells. This hypothesis can explain the increased immunogenicity in infants of conjugate vaccines compared with pure polysaccharides (13). Furthermore, it explains our recent finding that carrier

priming enhances the response to HibCP conjugated to tetanus toxoid (TT) in infants (3). Diphtheria toxoid (DT) and a nontoxic mutant diphtheria toxin are also used as carriers in some of the registered Hib vaccines, and the induction of carrier-specific helper T lymphocytes by the TT and DT included in the routine vaccination programs is likely to enhance the response to other Hib conjugates in infants (15). However, the quantitative aspects of the response to Hib conjugates are not only regulated by carrier-specific T lymphocytes. We have recently found that maternally acquired TT antibodies inhibit the response of infants to HibCP-TT (3). Furthermore, in adults the effect of recent immunization with TT or DT on the response to HibCP-TT and -DT conjugates is inhibition rather than augmentation, which may be related to the high levels of TT and DT antibodies induced by these booster immunizations (6).

However, T cells not only influence the magnitude of the B-cell responses but also are important regulators of isotype switching. It is therefore possible that differences in preimmunity to the carrier influence the isotype of the antibody produced. Also, the level of naturally acquired immunity to HibCP may influence the isotype usage, since it probably reflects prolonged exposure to environmental antigens with the potential of driving the switch process or selecting certain isotypes. The isotype profile invoked by an Hib conjugate may affect the protective efficacy of the vaccine, since the subclasses differ with respect to the ability to mediate complement-dependent opsonization and killing of capsulate bacteria in vitro (1, 27, 37).

In this report, we examine the possible effects of age and preimmunity to vaccine components on heavy-chain gene

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TABLE 1. Vaccination and blood sample schedule for adults tested in ELISPOT assays[a]

Group	No. of individuals vaccinated	First vaccination (day 0) with:	No. of individuals tested (day 7)	Second vaccination (day 28) with:	No. of individuals tested (day 35)
I	8	HibCP-DT	8	HibCP-TT	8
II	9	TT	5	HibCP-DT	7
III	9	DT	5	HibCP-DT	8
IV	8	HibCP-TT	8	HibCP-DT	7
V	8	DT	8	HibCP-TT	8
VI	9	TT	5	HibCP-TT	8

[a] Blood samples were not available for testing from all vaccinated individuals on both occasions.

usage in the response to two HibCP conjugates: HibCP-TT and HibCP-DT. Infants were found to use primarily heavy-chain genes located upstream in the locus (μ, $\gamma 1$, and $\alpha 1$), whereas two distinct isotype patterns of HibCP AbSC were found in adults: one "infant type" and one involving the downstream genes $\gamma 2$ and $\alpha 2$. The level of natural antibodies to HibCP was found to be more important than age in controlling the relative expression of the two isotype patterns in adults. This indicates that exposure to Hib or cross-reactive bacteria on the mucosal surfaces is shaping the isotype response to systemic administration of HibCP conjugate vaccines.

MATERIALS AND METHODS

Vaccination of adults. Fifty-one adult volunteers were, after informed consent, recruited among hospital staff. The protocol was approved by the regional ethics committee. For inclusion, subjects had to be healthy individuals aged 18 to 45 years. Individuals who had undergone vaccination against tetanus or diphtheria within the last 5 years or previous vaccination against Hib and those who were pregnant were excluded. The median age was 26 years (range, 20 to 44 years; 12 men and 39 women). All had received routine vaccinations against tetanus and diphtheria in childhood, and none recalled experiencing diseases caused by Hib.

The donors were randomized into six groups (Table 1), receiving different combinations of four vaccines: Al(OH)$_3$-adsorbed TT (lot 3801; 5.4 Lf, equivalent to ca. 50 μg), Al(OH)$_3$-adsorbed DT (lot M1202; 12.5 Lf, ca. 100 μg) (both from Statens Seruminstitut, Copenhagen, Denmark), HibCP (10 μg) conjugated to 24 μg of TT (HibCP-TT) (ActHib; lot S2181, Institut Pasteur Mérieux, Lyon, France), or 25 μg of HibCP coupled to 18 μg of DT (HibCP-DT) (Prohibit; lot 5026P26, Connaught Laboratories, Toronto, Canada). Two different vaccines were given subcutaneously in the shoulder on the same side at a 4-week interval.

Heparinized blood was drawn 7 days after each of the two immunizations. Mononuclear cells (MNC) were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway), washed, and resuspended in RPMI 1640 supplemented with penicillin and streptomycin (all three from Gibco, Paisley, United Kingdom) and 10% fetal calf serum (Flow, Irvine, United Kingdom). The cells were used immediately in the enzyme-linked immunosorbent spot-forming cell (ELISPOT) assays. The adults participated in a larger serological study, and a minor portion of the ELISPOT data and the serological data have been published elsewhere (6).

Vaccination of infants. Nine healthy infants were immunized subcutaneously in the thigh with HibCP-TT (lot S2181, S2428, or S2440) at approximately 5, 6, and 12 months of age. Blood MNC were studied 7, 8, or 9 days after the second or the third injection (see Table 5) in HibCP-specific ELISPOT assays. Prior to the HibCP-TT immunization in question, all infants

had received two doses of routine tetanus-diphtheria-inactivated poliovirus vaccine (Statens Seruminstitut). Informed consent was obtained from the parents, and the protocol was approved by the regional ethics committee.

Antibody determinations. Serum samples were collected from the adults immediately before the vaccinations, and total antibodies to HibCP, TT, and DT, as well as immunoglobulin G1 (IgG1) and IgG2 antibodies to HibCP, were measured by using enzyme-linked immunosorbent assays (ELISA) as described elsewhere (6).

Detection of TT and DT AbSC. Antibody-secreting cells (AbSC) were determined in ELISPOT assays by using the principles originally described by Czerkinsky et al. (11) and Sedgwick and Holt (38). All reagents were added in 100-μl aliquots, and unless otherwise stated, incubations were performed at room temperature. Nunc-Immunoplate Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 10 μg of purified TT or DT (both from Statens Seruminstitut) per ml diluted in sodium bicarbonate buffer (pH 9.6). The plates were washed three times with 0.5 M sodium chloride containing 0.044% (vol/vol) Tween 20 (Merck, Darmstadt, Germany) (washing buffer) and once with phosphate-buffered saline (pH 7.4) (to remove detergent). MNC were added in supplemented RPMI 1640, and the plates were incubated for 3.5 h at 37°C in a humified atmosphere containing 5% CO$_2$ and washed three times with washing buffer. Secondary antibodies were diluted in phosphate-buffered saline containing 1% bovine serum albumin (dilution buffer; Sigma, St. Louis, Mo.). The following antibodies were used for the detection of heavy-chain isotypes: horseradish peroxidase-coupled rabbit anti-human IgM, IgG, or IgA antibodies (diluted 1:1,000; Dako, Glostrup, Denmark); biotinylated murine monoclonal antibodies specific for human IgG1 (1:500; clone HP6069B), IgG2 (1:1,000; clone HP6002B), IgG3 (1:1,000; clone HP6047B), or IgG4 (1:2,000; clone HP6023B) (all from Hybridoma Reagent Laboratory, Baltimore, Md.); and murine monoclonal antibodies specific for human IgA1 (1:1,000; clone NIF2; Oxoid, Bedford, United Kingdom) or IgA2 (1:2,000; clone NI512; Nordic, Tilburg, The Netherlands).

After overnight incubation, the plates were washed three times. Horseradish peroxidase-coupled streptavidin (1:1,000; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to wells previously incubated with biotinylated monoclonal antibodies, and horseradish peroxidase-conjugated rabbit anti-mouse Ig was added to wells used to detect IgA subclasses (1:1,000; P260, Dako). Wells used to detect IgM, IgG, and IgA AbSC were flooded with dilution buffer. After 2 h, the plates were washed five times and dioctyl sulfosuccinate-tetramethylbenzidine substrate was added in melted agarose as described elsewhere (7). Spots formed within 20 min. The plates were sealed with an adhesive plastic film and kept at 4°C until the spots were enumerated by using a dark-field stereomicroscope at low magnification.

Detection of HibCP AbSC. HibCP AbSC were detected as TT and DT AbSC with the following exceptions. Immulon 2 microtiter plates (Dynatech, Chantilly, Va.) were used as the solid phase and incubated overnight with HibCP coupled to poly-L-lysine (molecular weight of 55,000 as determined by the manufacturer [Sigma]) by a modification of the method of Gray (17). The HibCP–poly-L-lysine conjugate (a gift from Connaught Laboratories, Inc.) was used at a final concentration of 10 μg of HibCP coupled to 2.5 μg of poly-L-lysine per ml.

The detection of IgG (and IgG subclass) HibCP AbSC was facilitated by the addition of $F(ab')_2$ fragments of rabbit anti-kappa and anti-lambda light-chain antibodies (both in a final dilution of 1:500; Dako) to the cell suspension at the initiation of culture. This procedure increases the numbers of IgG HibCP AbSC detected by 40% in this assay, probably by cross-linking the secreted IgG and thereby increasing the avidity for antigen (7). After 3.5 h of incubation, the wells were treated exactly as mentioned above for the ELISPOT assays used for detecting TT and DT AbSC of these isotypes.

Validation of the ELISPOT assays. The specificities of the monoclonal IgG subclass antibodies are well documented in other assay systems (18, 28) and have recently been evaluated in an HibCP subclass ELISA with the same solid phase as in this study (24). The specificity of the IgA subclass assays was confirmed by inhibition experiments with IgA myeloma proteins of both subclasses and by digestion of antibody bound to the solid phase by an Hib IgA1 protease before addition of the monoclonal antibodies.

The sensitivity of the ELISPOT assays was optimized by log 2 titrations of the secondary antibodies in all combinations of isotype and antigen specificities for which AbSC were available in sufficient amounts. For the detection of HibCP AbSC of the IgG4 subclass, the concentration of secondary antibody optimal in the TT- and DT-specific assays was used. For the detection of TT and DT AbSC of the IgM, IgG2, and IgA2 isotypes, the concentrations of secondary antibodies found to be optimal in the corresponding HibCP-specific assays were used. For the IgG3-specific monoclonal antibody, a concentration optimal in our anti-HibCP ELISA was used (24).

Statistics. The absolute numbers of spots as well as relative isotype percentages and ratios obtained in the different vaccine groups were evaluated by using the Kruskal-Wallis test. Individual groups were compared (by using the Mann-Whitney U test) only if the Kruskal-Wallis test indicated a significant difference between the groups. Relative isotype percentages and ratios were not evaluated for individuals with less than a total of 10 spots detected in the isotype-specific assays. Correlation analyses were performed by using the Spearman rank correlation test. A significance level of 0.01 was used to reduce the risk of type 1 errors due to multiple comparisons.

RESULTS

Table 1 shows the vaccination and blood sampling schedule for the six groups of adults. The numbers of TT, DT, or HibCP AbSC present in the circulation 7 days after the first or second immunization are given in Tables 2 to 4, respectively, along with the isotype distributions. The numbers of total IgG and total IgA AbSC are not given but were similar to the sums of the AbSC of the respective subclasses, constituting 118% (median), 124%, and 122% (IgG) and 102%, 121%, and 105% (IgA) of the sums for TT, DT, and HibCP AbSC, respectively.

The total numbers of HibCP- or carrier-specific AbSC were not influenced by immunization 4 weeks earlier with the heterologous carrier (Tables 2 to 4, 0.92 > P > 0.60 [Mann-

TABLE 2. Numbers of TT AbSC per 10^6 MNC and relative heavy-chain isotype distribution 7 days after vaccination of adults with TT or HibCP-TT

Group[a]	Immunization 4 wk earlier	Vaccine	n[b]	Sum of AbSC of all isotypes (median and range)	Mean % distribution (range) for:							n[c]
					IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	
IV, 1st	None	HibCP-TT[d]	8	924 (60–4,800)	0.9 (0–5.6)	82.4 (63–91)	0.6 (0–1.7)	0.1 (0–0.4)	7.3 (0.2–22)	8.1 (2.8–14)	0.6 (0–3.3)	8
V, 2nd	DT	HibCP-TT[d]	8	610 (282–4,149)	0.1 (0–0.7)	86.0 (45–97)	0.5 (0–1.2)	0.1 (0–0.2)	8.8 (0.4–52)	4.6 (1.6–10)	0.0 (0–0)	8
VI, 2nd	TT	HibCP-TT	8	55[e] (8–190)	1.1 (0–4.0)	77.0 (65–86)	0.8 (0–3.5)	0.1 (0–0.9)	11.9 (0–25)	9.1 (3.5–16)	0.0 (0–0)	6
I, 2nd	HibCP-DT	HibCP-TT	8	51[e] (0–232)	1.1 (0–7.7)	82.1 (70–94)	0.8 (0–5.0)	0.0 (0–0)	5.0 (0–15)	11.0 (0–30)	0.0 (0–0)	7
II, 1st + VI, 1st	None	TT	10	584 (78–7,387)	1.0 (0–10)	85.1 (66–100)	0.9 (0–2.6)	0.4 (0–1.4)	5.6 (0–18)	7.0 (0–20)	0.1 (0–0.3)	10
Total					0.8 (0–10)	82.9 (45–100)	0.7 (0–5.0)	0.1 (0–1.4)	7.5 (0–52)	7.7 (0–30)	0.1 (0–3.3)	39

[a] Tested after the first or the second immunization (see Table 1).
[b] Number of individuals tested.
[c] Only individuals with at least a total of 10 spots (equal to 16.7 AbSC per 10^6 MNC) detected in the heavy-chain isotype-specific assays were included in the calculation of the average percent distribution and subjected to statistical evaluation.
[d] The results of these two groups did not differ significantly, and the data were pooled (reference population) in the statistical comparisons between groups.
[e] $P < 0.0003$ when compared with the reference population.

TABLE 3. Numbers of DT AbSC per 10[6] MNC and relative heavy-chain isotype distribution 7 days after vaccination of adults with DT or HibCP-DT

Group[a]	Immunization 4 wk earlier	Vaccine	n[b]	Sum of AbSC of all isotypes (median and range)	Mean % distribution (range) for:							n[c]
					IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	
I, 1st	None	HibCP-DT[d]	8	118 (0–13,914)	6.1 (0–43)	44.2 (0–75)	1.4 (0–4.9)	1.7 (0–7.1)	36.5 (3.8–89)	8.3 (0–36)	1.8 (0–7.1)	7
II, 2nd	TT	HibCP-DT[d]	7	178 (0–2,534)	0.0 (0–0.2)	59.0 (25–88)	0.3 (0–1.1)	0.0 (0–0)	36.3 (7.5–72)	4.1 (0.9–8.4)	0.2 (0–1.4)	6
III, 2nd	DT	HibCP-DT	8	54 (13–524)	0.8 (0–5.3)	65.1 (25–100)	1.9 (0–8.3)	2.1 (0–11)	22.6 (0–58)	7.5 (0–22)	0.0 (0–0)	7
IV, 2nd	HibCP-TT	HibCP-DT	7	58 (10–330)	0.6 (0–2.9)	53.5 (22–97)	0.5 (0–2.9)	0.5 (0–2.9)	30.9 (0–78)	14.0 (0–56)	0.0 (0–0)	6
III, 1st + V, 1st	None	DT	13	285 (3–9,247)	0.5 (0–3.2)	69.9 (3.0–93)	0.6 (0–1.6)	0.2 (0–2.5)	22.0 (0.2–96)	6.8 (0.8–25)	0.0 (0–0)	11
Total					1.6 (0–43)	59.7 (0–100)	0.9 (0–8.3)	0.9 (0–11)	28.6 (0–96)	7.9 (0–56)	0.4 (0–7.11)	37

[a] See Table 2, footnote a.
[b] See Table 2, footnote b.
[c] See Table 2, footnote c.
[d] See Table 2, footnote d.

TABLE 4. Numbers of HibCP AbSC per 10[6] MNC and relative heavy-chain isotype distribution 7 days after vaccination of adults with HibCP-DT or HibCP-TT

Group[a]	Immunization 4 wk earlier	Vaccine	n[b]	Sum of AbSC of all isotypes (median and range)	Mean % distribution (range)							n[c]
					IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	
I, 1st	None	HibCP-DT[d]	8	3,798 (546–43,336)	10.1 (1.4–29)	10.4 (0.9–32)	20.2 (1.5–66)	0.4 (0–1.6)	0.0 (0–0.1)	34.2 (8.1–48)	24.7 (5.2–40)	8
II, 2nd	TT	HibCP-DT[d]	7	4,255 (53–16,792)	7.8 (1.1–21)	10.3 (0–22)	33.6 (3.7–86)	0.3 (0–0.7)	0.0 (0–0)	27.5 (2.2–64)	20.5 (6.3–41)	7
III, 2nd	DT	HibCP-DT	8	1,038 (92–27,216)	11.4 (2.5–34)	10.3 (0–24)	24.9 (8.4–60)	1.5 (0–5.7)	0.5 (0–2.8)	28.6 (11–44)	22.8 (7.1–53)	8
IV, 2nd	HibCP-TT	HibCP-DT	7	15[c] (5–148)	4.3 (0–10)	1.5 (0–4.5)	41.3 (17–77)	0.0 (0–0)	0.0 (0–0)	44.9 (14–70)	7.9 (0–18)	3
Total					9.2 (0–34)	9.3 (0–32)	27.7 (1.5–86)	0.7 (0–5.7)	0.2 (0–2.8)	31.9 (2.2–70)	21.0 (0–53)	26
IV, 1st	None	HibCP-TT[f]	8	2,841 (23–13,406)	25.6 (0–72)	7.2 (0–16)	21.3 (2.4–75)	0.1 (0–0.7)	0.0 (0–0)	25.3 (4.6–45)	20.5 (5.6–54)	8
V, 2nd	DT	HibCP-TT[f]	8	2,769 (187–16,406)	16.8 (2.9–38)	6.7 (1.1–11)	18.4 (1.3–45)	0.2 (0–0.5)	0.0 (0–0.3)	30.3 (9.8–72)	27.6 (10–55)	8
VI, 2nd	TT	HibCP-TT	8	498 (40–8,072)	20.8 (2.8–47)	5.5 (0–23)	23.0 (0–62)	0.7 (0–5.0)	0.1 (0–0.7)	29.6 (6.1–60)	20.3 (5.3–59)	8
I, 2nd	HibCP-DT	HibCP-TT	8	36[e] (11–253)	4.4 (0–7.9)	10.7 (0–18)	23.4 (0–79)	0.5 (0–3.3)	0.0 (0–0)	51.4 (17–72)	9.5 (0–27)	6
Total					17.7 (0–72)	7.3 (0–23)	21.4 (0–79)	0.4 (0–5.0)	0.0 (0–0.7)	33.0 (4.6–72)	20.1 (0–59)	30

[a] See Table 2, footnote a.
[b] See Table 2, footnote b.
[c] See Table 2, footnote c.
[d] See Table 2, footnote d.
[e] $P < 0.0005$ when compared with the reference population (i.e., pooled data from groups I + II and IV + V for the response to HibCP-DT and HibCP-TT, respectively).
[f] Reference population for the response to HibCP-TT.

TABLE 5. Numbers of HibCP AbSC per 10^6 MNC and relative heavy-chain isotype distribution 7 days[a] after the most recent vaccination of infants with HibCP-TT

Infant	No. of HibCP-TT doses	Age (mo)	Sum of AbSC of all isotypes	% Distribution for:						
				IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
II98	2	6	66.9	0.0	47.9	4.2	0.0	0.0	40.6	7.3
II158	2	7	34.1	56.3	25.0	6.3	0.0	0.0	12.5	0.0
II96[a]	3	9	11.7	—[b]	—	—	—	—	—	—
III84[a]	3	11	47.5	9.4	46.9	0.0	12.5	0.0	31.3	0.0
III92	3	12	1,920.8	0.0	43.9	14.3	0.5	0.0	39.4	1.8
III49	3	12	46.6	27.6	41.4	10.3	10.3	0.0	10.3	0.0
II73	3	12	169.4	0.0	79.8	8.7	0.0	0.0	11.5	0.0
III52	3	12	617.3	1.4	71.8	8.3	0.3	0.0	15.5	2.8
III94	3	12	0.0	—	—	—	—	—	—	—
Mean				13.5	51.0	7.4	3.4	0.0	23.0	2.4

[a] Infants II96 and III84 were tested on postvaccination days 8 and 9, respectively.

[b] Because of limited amounts of blood available, only (0.2 to 1.5) × 10^6 MNC were tested for each isotype. The percent distribution was not calculated in cases with less than a total of 10 spots detected in the isotype-specific assays.

Whitney U test]). This was also true for the isotype distributions of the AbSC, and data from these groups were therefore pooled and used as a reference in the statistical comparisons described below. Smaller numbers of HibCP- and carrier-specific AbSC were, however, seen when vaccination with HibCP-TT or HibCP-DT was preceded by immunization with the homologous carrier or with an HibCP conjugate (Tables 2 to 4), but the reductions were statistically significant in only some of the combinations of vaccines ($0.16 > P > 0.0003$ when compared with the respective reference groups).

Heavy-chain isotype distributions in adults. (i) TT AbSC. Very similar heavy-chain isotype distributions were seen after vaccination with TT and HibCP-TT, and the isotype distribution after vaccination with HibCP-TT was not influenced significantly by prior immunization with components of the vaccine (Table 2, $0.90 > P > 0.07$ for all isotypes [Kruskal-Wallis test of all groups]). On average, 83% IgG1, 8% IgA1, and 8% IgG4 TT AbSC were detected, leaving a few percent to IgM and IgG2. Virtually no IgG3 or IgA2 AbSC were detected.

(ii) DT AbSC. Likewise, the heavy-chain isotype responses to DT and HibCP-DT were similar and the isotype distribution after vaccination with HibCP-DT was not influenced by prior immunization with the carrier or with HibCP-TT (Table 3, 0.99 $> P > 0.03$). Furthermore, the isotype pattern of DT AbSC induced by HibCP-DT or DT was quite similar to that of TT AbSC induced by HibCP-TT or TT except for a higher relative contribution of IgG4 among DT AbSC ($P = 0.00004$).

(iii) HibCP AbSC. HibCP AbSC induced by HibCP-DT and by HibCP-TT did not differ significantly (Table 4, groups I, first immunization, and II, second immunization, versus groups IV, first immunization, and V, second immunization) with respect to numbers of AbSC ($P = 0.38$) or to the isotype distributions ($0.97 > P > 0.03$). In contrast to the results for the protein antigens (TT and DT), the responses to the capsular polysaccharide HibCP were dominated by IgA AbSC (IgA1, 32 and 33%; IgA2, 21 and 20% [for HibCP-DT and HibCP-TT, respectively]), and they showed a quite different IgG subclass distribution dominated by IgG2 (28 and 21%) and IgG1 (9 and 7%), whereas there was only a small percentage of IgG3 and IgG4 AbSC. Furthermore, a significant contribution of IgM HibCP AbSC was found (9 and 18%, respectively). No significant differences were seen between the different immunization regimens with respect to the distributions of subclasses (1.00 $>$ $P > 0.03$).

HibCP AbSC responses to HibCP-TT in infants. Table 5

shows that infants respond to the second or third HibCP-TT dose by the presence of HibCP AbSC 7 to 9 days after immunization (median, 47.5 HibCP AbSC per 10^6 MNC; range, 0 to 1,921). They were dominated by IgG AbSC, but there were substantial contributions from IgA and IgM AbSC. The utilization of the different heavy-chain constant-region genes was similar between the infants but differed markedly from that seen in adults (compare Tables 4 and 5). The response in infants was largely confined to genes located upstream in the Ig heavy-chain constant-gene locus (μ, γ1, and α1), whereas genes from the downstream duplication unit (γ2, γ4, and α2) constituted only 10% of the total response.

Patterns of heavy-chain constant-gene usage. Prompted by the observation of a marked difference in subclass distribution between infants and adults, we further analyzed the heavy-chain gene utilization among adults responding to one dose of the HibCP conjugates (with or without prior immunization with the heterologous carrier). To analyze if certain isotype patterns were preferentially expressed by some individuals, the correlations between the absolute numbers of HibCP AbSC of different heavy-chain isotypes were studied in pairs (Fig. 1). IgG3 and IgG4 AbSC were not analyzed because too few spots were detected to make the analysis meaningful. Positive correlations were observed for all isotype combinations, but the correlation coefficients varied considerably from 0.40 ($P = 0.12$) to 0.86 ($P = 0.00003$). Similar correlation patterns were obtained for both conjugates. Strong correlations were found between the numbers of AbSC expressing heavy-chain genes located upstream in the locus on chromosome 14 (μ, γ1, and α1), and similar strong correlations were observed between AbSC expressing genes grouped downstream (γ2 and α2). In contrast, weaker correlations between numbers of AbSC expressing isotypes derived from each of the two groups were found, with the notable exception of α1 and α2 AbSC, which correlated strongly.

The preferences for one of the two gene usage patterns were further studied by calculating individual ratios between the number of HibCP AbSC using downstream genes and the number using upstream genes. Individual IgG2/IgG1 and IgA2/IgA1 ratios varied considerably, with median values of 1.8 (quartiles, 0.53 and 8.5) and 0.81 (quartiles, 0.33 and 1.4), respectively. Both ratios correlated with the prevaccination levels of total HibCP antibodies ($r = 0.64$, $P = 0.0001$, and $r = 0.49$, $P = 0.005$, respectively) as well as with the prevaccination levels of anti-HibCP IgG2 ($r = 0.55$, $P = 0.001$, and $r = 0.35$,

UPSTREAM   DOWNSTREAM

M   G1   A1   G2   A2

0.40 ≤ r < 0.50   P > 0.05
0.50 ≤ r < 0.64   P < 0.05
0.64 ≤ r < 0.73   P < 0.01
0.73 ≤ r ≤ 0.86   P < 0.002
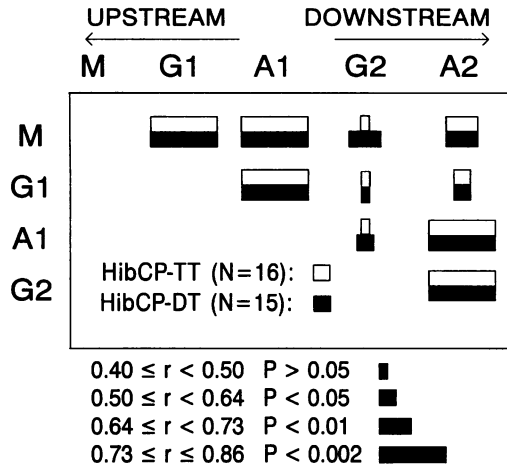
HibCP-TT (N=16): □
HibCP-DT (N=15): ■

FIG. 1. Spearman rank correlation coefficients for the numbers of HibCP AbSC per 10^6 MNC detected for combinations of heavy-chain isotypes. The AbSC were measured 7 days after vaccination of adults with HibCP-TT (open bars, $n = 16$) and HibCP-DT (solid bars, $n = 15$). The isotypes are arranged in the order they appear in the genome. IgG3 and IgG4 AbSC are not included since spot numbers were too small to make correlation analysis meaningful. The indicated P values refer to $n = 16$.

Ratio

Infants

Adults

r = 0.59
P = 0.00002
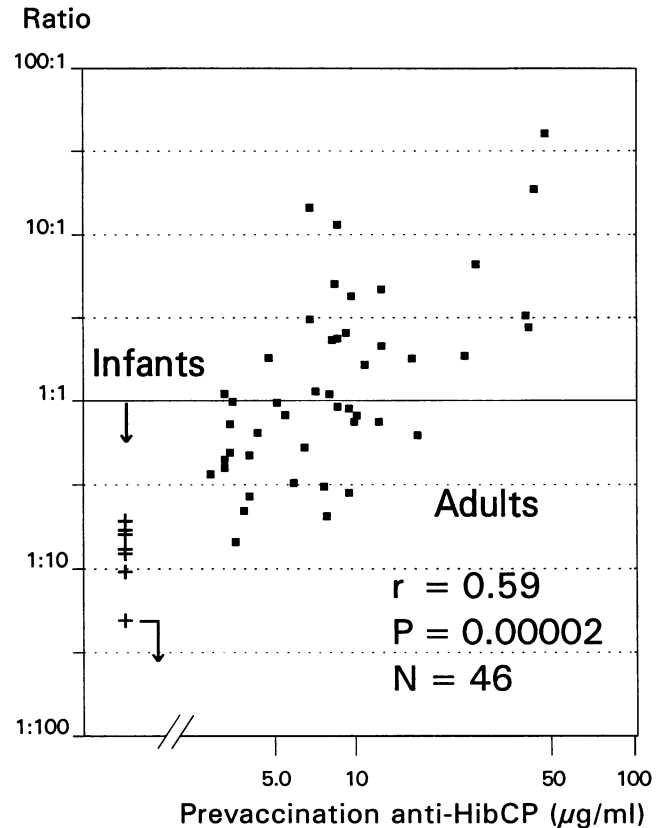N = 46

Prevaccination anti-HibCP (μg/ml)

FIG. 2. Correlation between the prevaccination levels of HibCP antibodies in adults and the ratios between the numbers of HibCP AbSC using heavy-chain genes from the downstream (γ2, γ4, and α2) and the numbers of AbSC using genes from the upstream (γ3, γ1, and α1) duplication unit. AbSC were measured 7 days after immunization with one dose of HibCP-TT or HibCP-DT (solid squares). Ratios from seven infants vaccinated with HibCP-TT are given for comparison (+). One of the infants had no detectable AbSC using genes from the downstream duplication unit, whereas 28 AbSC using genes from the upstream duplication unit were found (angled arrow).

$P = 0.07$, respectively) but not with the IgG1 levels ($r = 0.14$, $P = 0.45$, and $r = 0.07$, $P = 0.71$, respectively). No differences in these ratios between individuals receiving HibCP-DT and those receiving HibCP-TT ($P > 0.93$) or between these vaccinees and the group receiving the same conjugate after immunization with the homologous carrier ($P > 0.35$) were observed.

These observations could indicate that the HibCP AbSC to a variable degree had switched to heavy-chain genes of either the downstream duplication unit (γ2, γ4, and α2) or the upstream duplication unit (γ3, γ1, and α1) depending on the level of natural HibCP antibodies but irrespective of previous immunization with the homologous or heterologous carrier molecule. For all these individuals, the correlation was calculated between the prevaccination anti-HibCP levels and the ratios between the numbers of HibCP AbSC using genes from the downstream duplication unit and those using genes from the upstream duplication unit (Fig. 2). Ratios for the infants are given for comparison. The correlation coefficients for adults were 0.59 ($P = 0.00002$, $n = 46$) for total antibodies, 0.51 ($P = 0.0003$) for IgG2, and 0.04 ($P = 0.8$) for IgG1. A slightly weaker correlation was found between the age of the adults and the gene utilization ratios ($r = 0.47$, $P = 0.0009$). The contributions from the prevaccination antibody levels and from age were largely independent since no correlations were found between these two parameters (total anti-HibCP versus age: $r = -0.01$, $P = 0.94$, $n = 46$; IgG2 anti-HibCP versus age: $r = 0.12$, $P = 0.41$).

No correlations between the age or the prevaccination levels of total HibCP antibodies and the total numbers of HibCP AbSC measured after one dose of HibCP-DT ($P > 0.29$) or of HibCP-TT ($P > 0.49$) (with or without prior immunization with the heterologous carrier) were found. None of the parameters (age, prevaccination levels of antibody to HibCP, or prevaccination levels of anticarrier antibody) correlated with the ratios between downstream and upstream isotypes of DT AbSC induced by HibCP-DT ($0.74 > P > 0.43$) or of TT AbSC induced by HibCP-TT ($0.84 > P > 0.27$).

DISCUSSION

Systemic immunization of humans leads to B-cell activation and differentiation in the lymphoid tissues. Eventually, vaccine-specific AbSC are liberated into the circulation, where they can be detected from postvaccination days 5 to 14 (4, 43). Their numbers correlate with the increases in antibody levels of the corresponding isotypes in plasma (5). The heavy-chain isotype of the secreted antibody is determined by the usage of one of nine functional heavy-chain constant-region genes located on chromosome 14. Virgin B lymphocytes express on their surface the products of the μ and δ genes, which are located upstream in the locus. Approximately 60 kb downstream of these, a cluster containing the γ3, γ1, and α1 genes is located, and 75 kb further downstream a second cluster contains the γ2, γ4, ε, and α2 genes. Evidence suggests that these clusters have arisen by gene duplication (14), and they are referred to as the upstream and downstream duplication unit, respectively.

Upon antigenic stimulation, B lymphocytes may secrete IgM or switch to one of the downstream genes by a mechanism deleting the intervening DNA segment. Sequential switches to genes further downstream may also occur (33). Switch recom-

bination is a regulated phenomenon preceded by increased germ line transcription of the target gene (47). Considerable evidence points to important roles for T cells and cytokines in the regulation of the B-cell isotype switch. Thus, interleukin-4 promotes switching from μ to ε in both mice (31) and humans (41). Transforming growth factor β induces a sequential switch from μ via γ to α in mice (26) and is also involved in IgA switching in humans (46).

Studies of isotype patterns of human antibodies and B-cell lines have suggested the existence of certain switch programs. Thus, switches from μ to the genes of the upstream duplication unit ($\gamma 3$, $\gamma 1$, and $\alpha 1$) and possibly also to $\gamma 4$ appear related and may occur sequentially in the course of an immune response (19, 20, 42). Our finding that TT and DT AbSC were dominated by IgG1 followed by IgG4 and IgA1 with very small contributions from other isotypes is compatible with such a program. This pattern was unaffected by coupling of the toxoids to HibCP and by prior immunization with the toxoid. Neither was it influenced by the prevaccination levels of toxoid or HibCP antibodies. Similar isotype profiles have been reported for these antigens in serological studies in both children and adults (IgG subclasses) (30, 39) and for TT in a cellular study (IgA subclasses) (45).

We found a similar isotype pattern among HibCP AbSC induced by HibCP-TT in infants. Some of these cells secreted IgM, but the majority had switched to the $\gamma 1$ and $\alpha 1$ genes of the upstream duplication unit, while only 10% produced downstream isotypes IgG2 and IgA2. This could indicate that similar regulatory mechanisms govern isotype switch in HibCP- and protein-specific B cells in infants when systemically challenged with T-cell-dependent vaccines. A predominance of IgM and IgG1 in the HibCP response in infants and young children has been documented in serologic studies (10, 16), but this is the first report showing that IgA1 is the preferential IgA subclass utilized by infants. This may have clinical importance, since many pathogenic capsulate bacteria including Hib secrete IgA1 proteases, which are likely to be important virulence factors (29).

A more complex isotype pattern was found among HibCP AbSC in adults, with considerable contributions from isotypes IgM, IgG1, IgG2, IgA1, and IgA2. Similar isotype profiles have been found in several studies of recall antibody responses to HibCP and other polysaccharides in adults (22, 34, 39, 40, 45). The isotype distributions induced by the two conjugates were indistinguishable, and, like the isotype profiles of DT and TT AbSC, they were not affected by prior immunization with the carrier. Neither did prior immunization with another HibCP conjugate seem to influence the isotype distribution significantly. However, because of the low responses to the second conjugate, the latter data were difficult to evaluate.

Many investigators have noted that the relative contributions of IgG1 and IgG2 HibCP antibodies may vary considerably among individuals (25, 39). The present study confirms this and shows that it is also the case for the contributions of IgA1 and IgA2. Our correlation analysis indicated that this was due to a variable utilization of two distinct isotype response patterns in adults. The first consisted of IgM and switch variants from the upstream duplication unit (IgG1 and IgA1) and was similar to that used by infants. The second isotype pattern consisted of switch variants from the downstream duplication unit (IgG2 and IgA2) with contributions from IgA1 and possibly from IgM. The relative utilization of switch variants from the downstream duplication unit was found to correlate positively with the prevaccination levels of natural HibCP antibodies and with the age of the individual.

The gradual appearance with age of natural antibodies is a

characteristic of the human immune response against many polysaccharides including HibCP. Substantial evidence indicates that HibCP antibodies are induced by colonization of the mucosal surfaces by Hib or cross-reactive bacteria such as the apathogenic enterobacterium *Escherichia coli* K100 (21, 36). Studies with experimental animals have shown that mucosal colonization with *E. coli* K100 primes for responses to systemic challenge with Hib, involving differentiation of HibCP-specific AbSC in the Peyer's patches, mesenteric lymph nodes, and spleen (21). Priming can exist even in the absence of detectable plasma antibodies. It is therefore possible that memory B cells resulting from mucosal exposure are involved in the systemic response to HibCP in humans. Since the microenvironment in the mucosa-associated lymphoid tissues favors an isotype switch to IgA1 and IgA2 (44), we and others have proposed that the many IgA-committed B cells involved in the systemic response to capsular polysaccharides could have such an origin (23, 32). This hypothesis is supported by the high IgA2/IgA1 ratios among such cells, which are seen among immunoglobulin-secreting cells in the mucosa-associated lymphoid tissues only, especially in the distal parts of the gut including the Peyer's patches and the mesenteric lymph nodes (8).

Mucosal B cells which have undergone a deletional isotype switch to the $\alpha 1$ gene are, at least in principle, still able to switch to genes of the downstream duplication unit (e.g., IgG2 and IgA2). Therefore, we propose that the HibCP AbSC utilizing the downstream isotype pattern are reactivated memory B cells originally primed in the mucosa-associated lymphoid tissues in response to environmental antigens. In contrast, we propose that the HibCP AbSC utilizing the upstream isotype pattern represent a pure systemic response, which with respect to isotype switch mechanisms may be related to that involved in the systemic B-cell responses to protein antigens.

It is tempting to speculate that the different IgG2/IgG1 ratios of HibCP antibodies induced in children by HibCP conjugates and the pure polysaccharide (25) reflect different abilities of these antigens to activate B cells of the two pathways.

ACKNOWLEDGMENTS

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