The First Dose of a *Haemophilus influenzae* Type b Conjugate Vaccine Reactivates Memory B Cells: Evidence for Extensive Clonal Selection, Intraclonal Affinity Maturation, and Multiple Isotype Switches to IgA2

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The First Dose of a *Haemophilus influenzae* Type b Conjugate Vaccine Reactivates Memory B Cells: Evidence for Extensive Clonal Selection, Intraclonal Affinity Maturation, and Multiple Isotype Switches to IgA2[1]

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The Ab response of a healthy adult to the first dose of a *Haemophilus influenzae* type b capsular polysaccharide (HibCP) conjugate vaccine was studied at the level of Ig gene usage by circulating Ab-secreting cells. Forty-one IgA and 17 IgG mRNA sequences were obtained. The major part of the response was confined to IgA Ab-secreting cells, and 72% of the IgA sequences were derived from the progeny of a single rearranged B cell. These sequences could be arranged in a genealogical tree showing multiple somatic mutations and at least two intraclonal isotype switches to IgA2. Fourteen somatic mutations were shared by this clonal progeny, indicating that extreme clonal selection had occurred early in the clonal development. Taking into account the frequency of somatic mutations and the clone size, it was evident that the responding cell population must have originated from a mutated, highly selected, and expanded population of cells existing before vaccination, i.e., memory B cells. The dominating heavy and light chains of the response were combined in a Fab that bound HibCP. It was shown that the shared heavy and light chain mutations increased the affinity for HibCP considerably, indicating that the clonal selection had been driven by affinity. Pre-existing memory cells in unvaccinated adults may explain several features of Ab responses to polysaccharide vaccines and may play a role in acquiring the ability to respond to pure polysaccharides during infancy. ***The Journal of Immunology*****, 1999, 162: 224–237.**

Conjugate vaccines consisting of *Haemophilus influenzae* type b (Hib)[3] capsular polysaccharide (HibCP) covalently coupled to a protein carrier constitute the first members of a new generation of polysaccharide vaccines aimed at protection against a variety of bacterial infections. Most capsular polysaccharides (including HibCP) are poorly immunogenic in infants, a fact that appears to be related to the thymus-independent nature (TI-2) of the polysaccharides as demonstrated in rodents (1). Covalent coupling of an immunogenic protein to the polysaccharide overcomes this physiological unresponsiveness (2). This procedure introduces other features of thymus-dependent protein vaccines, including the ability of conjugate HibCP vaccines (3, 4) or of the carrier protein alone (5) to prime for booster responses in infants. Nevertheless, Abs induced by conjugate polysaccharide vaccines express features not usually seen with conventional protein vaccines including 1) low intrinsic affinity (6), 2) marked restriction with respect to the V gene usage (usually one of five highly homologous V_H family III genes is used (7–9); although 13 κ and λ light chain genes have been found among HibCP Abs (8–13), a single gene, Vκ$A2$, predominates the response in most individuals) (14), 3) minimal hypermutation as detected by sequencing of purified light chains and light chain genes (heavy chain sequences indicate some mutations, but have been difficult to interpret due to limited knowledge of the V_H repertoire and the considerable polymorphism at this locus; the ratios of replacement to silent mutations in the complementarity-determining regions (CDRs) are usually quite low, suggesting that affinity maturation through selection of amino acid replacements in the CDR does not occur or only proceeds very inefficiently in the B cells responding to vaccination with HibCP vaccines) (10, 15), 4) domination of Abs produced by the progeny of a few virgin B cells (10), and 5) use of about equal amounts of IgG1 and IgG2 (16) and considerable amounts of IgM, IgA1, and IgA2 (17, 18). Especially isotypes IgG2 and IgA2 are rarely used in systemic Ab responses to protein Ags (18).

Because both affinity and isotype may influence the efficacy of the existing and future conjugate polysaccharide vaccines, further studies are warranted to shed light on the clonal commitment of B cells induced by conjugate vaccines, including the acquirement of somatic mutations, affinity maturation, and isotype switch. In this report we address these questions by cloning heavy chain V and C genes involved in the anti-HibCP response of an adult individual and by combining heavy and light chains into a Fab, allowing studies of Ag binding.

Materials and Methods

Purification of HibCP-specific cells

HibCP-specific Ab-secreting cells (AbSC) were purified from the peripheral blood of a 22-yr-old male healthy volunteer, hereafter referred to as To. The procedure has been described in detail previously (10, 19). In brief,

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[3] Abbreviations used in this paper: Hib, *Haemophilus influenzae* type b; HibCP, *Haemophilus influenzae* type b capsular polysaccharide; CDR, complementarity-determining region; AbSC, antibody-secreting cells; TT, tetanus toxoid; FR, framework region; PFU, plaque-forming unit; RS, replacement to silence.

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To was vaccinated s.c. in the shoulder with HibCP-TT consisting of 10 μg of HibCP covalently coupled to 24 μg of tetanus toxoid (TT) (ActHib; Pasteur Mérieux Serum et Vaccines, Lyon, France). He had previously received several routine immunizations with TT but had not received any HibCP-containing vaccine and had no history of invasive Hib infection. Heparinized blood was drawn 9 days after vaccination, and PBMC were isolated using density gradient centrifugation. Serum samples were obtained before and 1 mo after immunization. Total HibCP Ab levels were measured by ELISA as described previously (5). Freshly isolated PBMC (50×10^6) were rotated end-over-end for 30 min at 4°C together with 200×10^6 paramagnetic polystyrene beads (Dynal, Oslo, Norway) coated with HibCP covalently coupled to poly-L-lysine. Beads and bead-binding cells were concentrated on the side of the tube with a Dynal MPC-6 magnet (Dynal) and were washed twice to eliminate nonbinders. The remaining cells were resuspended in 5 ml of medium. Some of these cells were immediately tested in enzyme-linked immunosorbent spot-forming cell assays (20), while aliquots of 0.5 ml were frozen in liquid nitrogen after addition of an equal volume of freeze medium.

Cloning of the heavy chain mRNA sequences

HibCP bead-purified cells from 5×10^6 PBMC obtained on postvaccination day 9 were thawed, mixed with 1×10^6 cells from a T cell line (HUT78) as a source of carrier RNA, and the cells were washed and pelleted. RNA was extracted by the guanidinium thiocyanate method (21), dissolved in sterile water, and stored at −80°C until use. Approximately 1/10th of the RNA was used for heavy chain cDNA synthesis with the Gene Amp RNA-PCR kit (Perkin-Elmer Roche, Foster City, CA). A 20-μl first-strand reaction mix with final concentrations of 1× PEII PCR buffer, 5 mM $MgCl_2$, 1 mM dNTP, 1 U of RNase inhibitor, and 2.5 U of Moloney murine leukemia virus reverse transcriptase was incubated at 42°C for 30 min with 2 pmol of both IgA and IgG CH1 domain primers (IgA, 5′-TGCACGT GAGGTTCGCTTC-3′ (IgA269rc); IgG, 5′-GGGTCCGGGAGATC ATGAG-3′ (IgG264rc)). Four microliters of the first-strand reaction mix was used as template in each of four independent 50-μl PCR reactions, with final concentrations of 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1× PEII PCR reaction buffer, and 1.25 U of AmpliTaq (Perkin-Elmer). The PCRs were hot-started using 1.25 U of *Taq*-Start Ab (Clontech, Palo Alto, CA). Two of the PCR reactions served to amplify IgA cDNA and two IgG cDNA using 5 pmol/reaction of an IgA-specific 3′ primer (5′-GCTGGCTGCTCGTGGTGTA-3′; IgA185rc) and an IgG-specific primer (5′-GCTGCTGAGGGAGTAGAGT-3′; IgG183rc), respectively. In all four reactions 20 pmol of a degenerate V_HIII-specific signal peptide primer, 5′-G(CT)(GT)GCT(AC)T(AT)(AT)TA(AG)(AG) AGGTGTCCA-3′ (V_H3-sig-deg), was used. This primer was designed to amplify all known signal peptide sequences of the V_HIII family available in the EMBL database (per Dec. 1, 1995) and in the directory of Kabat and colleagues (22). All primers were designed by us. After an initial denaturation for 4 min at 94°C, 40 PCR cycles were performed, consisting of 30 s at 94°C, 1 min at 55°C, 1 min at 72°C, and a final 7-min step at 72°C.

The PCR products were cloned into the LigATor vector by use of the LigATor kit (R&D Systems, Minneapolis, MN) in 20-μl reactions containing 100 ng of pTAg vector, 1× ligase buffer, 5 mM DTT, 0.5 mM ATP, 2.5 U of T4 DNA ligase, and 2 μl of PCR product. They were incubated at 16°C overnight. After 30 min of incubation on ice, the ligations were transfected into a 20-μl suspension of competent cells from the LigATor kit by 40 s of heat shock at 42°C followed by 2 min on ice and 1 h of gently shaking at 37°C after addition of 80 μl of SOC medium (20 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 0.5 g/l NaCl, 20 mM glucose) (23). The transformed cells were plated on Luria-Bertoni agar plates (23) with 50 μg of carbenicillin (Sigma, St. Louis, MO) and 10 μg of tetracycline (Sigma) per ml and an indicator system for β-galactosidase activity (23) resulting in blue colonies for wild-type clones and white colonies for clones with insert. White clones were subcloned, and plasmid DNA was purified by an alkaline lysis protocol (24). To analyze insert length, plasmid DNA was digested with *Pst*I (Amersham, Little Calford, U.K.) and *Xho*I (Amersham) in 1× One-Phor-All buffer Plus (Pharmacia, Uppsala, Sweden) and was run on a 2% agarose gel. *Xho*I cuts a single site in the LigATor vector near the insert, and *Pst*I cuts a single site on the other side of the insert. *Pst*I also cuts a single site in the CH1 of IgA1, allowing discrimination between the two subclasses. All IgA2 and IgG clones and 25 IgA1 clones were sequenced corresponding to the entire variable domain (codons 1–113) and a part of the first constant domain (IgA from codons 114–185 and IgG codons 114–183; nomenclature according to Kabat and colleagues (22)).

Amplification of germline gene sequences

Genomic DNA was isolated from 5×10^6 MNC by a salting out procedure (25). Five hundred nanograms of DNA was used for PCR amplification of the 3-23 and 3–73 germline genes using 0.625 U of *Taq* polymerase (Life Technologies, Paisley, U.K.) mixed with 0.625 U of *Taq*-Start Ab, 0.2 mM dNTP (Pharmacia), and 5 pmol of gene-specific primers placed in framework region 1 (FR1) and FR3, respectively (3-23: 3–23cn1, 5′-TGTGAG GTGCAGCTGT-3′; and 3–23c89rc, 5′-TCTTTCGCACAGTAATAT-3′; 3–73: 3–73c19, 5′-CCTGGGGGGTCCCTGAA-3′; and 3–73c83rc, 5′-GGCCGTGTCCTCGGTTT-3′). PCR was performed in a final volume of 50 μl containing 1× PCR buffer (Perkin-Elmer). After an initial denaturation for 4 min at 94°C, 30 PCR cycles were performed consisting of 30 s at 94°C, 1 min at 55°C, 1 min at 72°C, and a final 7-min step at 72°C. The PCR products were cloned using the LigATor kit (R&D Systems) as described above.

DNA sequencing

DNA sequencing was performed with the Sanger dideoxy method (26) using the Ready Reaction kit (Perkin-Elmer) and an ABI 373 automatic sequencer (Perkin-Elmer) according to the instructions of the manufacturer. The plasmid DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), pH 6.7/8.0 (Amresco, Solon, OH), before use as template.

Candidate germline V genes were assigned to each clone based on maximum homology (codons 1–94) with sequences of the EMBL database. Similarly, candidate germline J_H minigenes were assigned based on maximum homology from codons 101–113 with the published germline sequences (27). D_H segments were assigned to a germline D_H gene if they matched at least seven continuous bases or if they matched more than eight continuous bases except for a single mismatch. D_H gene candidates (22, 28, 29) were considered in all reading frames and in both directions.

P insertions were defined as junctional bases reverse complementary to the coding end of the V_H, D_H, and/or J_H gene beginning at the position corresponding to the first nucleotide of the heptamer. N additions were defined as junctional bases that could not be assigned to any other category.

Estimation of Taq *errors*

Of 10,837 sequenced bp of the constant region genes, seven unique substitutions were found and considered to be *Taq* errors. This led to an overall estimated prevalence of accumulated PCR mutations of 1 of 1,548 bp, corresponding to 0.24 (0.10−0.49, Poisson 95% confidence interval) PCR mutations/variable domain (codons 1–113; 366 bp) after 40 PCR cycles. This is in agreement with the reported frequency of *Taq* errors of about 1×10^{-4} substitutions/base/PCR cycle (30).

Cloning of Fab in a bacteriophage expression vector

The phage surface display vector, pFab73HHui (31), was provided by Dr. M. Dziegiel. This vector permits display of Fab on the surface of the phage fused to the carboxyl-terminal part of the pIII minor coat protein. We modified the vector by removing the *gIII* gene (encoding the pIII protein) by digestion with *Eag*I (New England Biolabs, Beverly, MA) followed by ligation with T4-DNA ligase (Boehringer Mannheim, Mannheim, Germany). Thereby, soluble Fab could be purified directly from the periplasmic space of infected bacteria. The vector encodes the human IgG1 CH1 domain with a His^6 tail appended at the carboxyl terminus. The resulting phagemid vector was electroporated into *Escherichia coli* TOP10/F′Tet^R cells (British Biotechnology, Edinburgh, U.K.) and was grown under carbenicillin and tetracycline selection. The phagemid DNA was purified on a Qiagen-500 column (Qiagen, Hilden, Germany), and 12 μg was digested with *Nhe*I and *Apa*I (New England Biolabs).

Heavy and light chain sequences were obtained from plasmid clones and prepared for cloning into the pFab73HHui vector by amplification of suitable PCR products. These PCRs were either performed with *Taq*/anti-*Taq* polymerase following the protocol used for amplification of germline genes (see above) or with a mixture of PFU polymerase and *Taq*/anti-*Taq* to minimize the risk of misincorporations. In the PFU-*Taq*/anti-*Taq* protocol, 1× PFU buffer, 0.2 mM dNTP, 5 pmol of gene-specific primers, and a mixture of 0.78 U of PFU polymerase (Life Technologies), 0.55 U of *Taq* polymerase, and 0.55 U of *Taq*-start Ab were used in a final volume of 50 μl. Twenty to thirty PCR cycles were performed consisting of 30 s at 94°C, 1 min at 55°C, 1.5 min at 72°C, and a final 10-min step at 72°C.

The predominant heavy chain sequence of the anti-HibCP response of individual To was represented by the phagemid clone ToPA135. The V_H domain was amplified using 100 ng of ToPA135 as template for a 20-cycle PCR amplification with the PFU-*Taq*/anti-*Taq* protocol, and gene-specific primers were placed in FR1 and FR4 of the gene containing an *Nhe*I or an

*Apa*I site (3–73Fab3′, 5′-CTCGCGAATTGGGCCCTTGGTGGAG GCTGGGGAGACGGTGACCAG-3′;3–73Fab5′,5′-GGATTGTTATTGC TAGCAGCACAGCCAGCAATGGCAGAGGTGCAGCTGGTGGAG-3′; the restriction sites are underlined). The PCR product was size purified, digested with *Nhe*I and *Apa*I, and cloned into the phagemid vector. The resulting phagemid vector was electroporated into TOP10/F′Tet^R cells and grown under carbenicillin and tetracycline selection. The phagemid DNA was purified on a Qiagen-500 column. The V_H domain sequence was verified by sequencing, and 2 μg was digested with *Sfi*I and *Asc* I (New England Biolabs).

A DNA sequence encoding the light chain V domain was obtained from a previously published plasmid (ToP027) (10) containing a sequence encoding the predominant κ light chain of HibCP-specific B cells from the same immune response as the heavy chain. The L chain was amplified in three parts that were later assembled by PCR. An *Rsr*II site was introduced in codons 98–100 without changing the amino acid sequence of the L chain. The following primers were used: codons 1–6: A3Fab, 5′-GATC CTCGCGAATTGGCCCAGCCGGCCATGGCGGATATTGTGATGAC TCAG-3′; codons 104–96: Jk3FabV, 5′-TTTGGTCCCCGGTCCGAA AGTGAA-3′; codons 96–104: Jk3FabC, 5′-ACTTTCGGACCGGGG ACCAAAGTG-3′;codons122–117:CK117rc,5′-CATCAGATGGCGGGA AGAT-3′; 117–122: CK117, 5′-ATCTTCCCGCCATCTGATG-3′; and codons 214-209: HCK.FORW, 5′-GTCTCCTTCTCGAGGCGCGCCT CACTAACACTCTCCCCTGTTGAAGCT-3′ (*Sfi*I, *Rsr*II, *Rsr*II, and *Asc*I restriction sites are underlined). Each PCR was performed with PFU-*Taq*/anti-*Taq* for 20 cycles. The resulting full-length κ light chain PCR product was size purified and digested with *Sfi*I and *Asc*I and cloned into the vector already containing a sequence encoding the heavy chain. The resulting phagemid was called pFab3–73/A3 encoding the Fab Fab3–73/A3.

Two other plasmids were constructed encoding an unmutated version of the light and the heavy chain, respectively. The plasmid with the germline-encoded light chain, pFab3–73/A3gl, was made by replacing the light chain gene of the pFab3–73/A3 plasmid by a backmutated L chain made with a two-step assembly PCR under the same conditions as described above. The germline-encoded tyrosine was introduced in position 30 by PCR mutagenesis using the primers A3Tyr30 5′-CATAGTAATGGA**T**ATAACTATT TGG-3′ and A3Tyr30rc 5′-CCAAATAGTTATA**T**CCATTACTATG-3′ (the deviating nucleotides are shown in bold). The plasmid with the germline-encoded heavy chain, pFab3–73gl/A3, was made by replacing the heavy chain gene of pFab3–73/A3 with the predicted germline heavy chain. Codons 1–48 were obtained from the plasmid ToPA132 using 5 pmol of the primers 3–73Fab5′ and 3–73gl42rc 5′-ACCCACTCCAGC CCTTTCC-3′. Codons 19–88 were made from the germline plasmid To7301 using 5 pmol of the primers 3–73c19 and 3–73c83rc. Codons 83–108 were made by primers covering the area using 10 pmol of 3–73gl83 (5′-AAAACCGAGGACACGGCCGTGTATTACTGTACTAG ATATTACTAT-3′) and 3–73glFab3′ (5′-GACCAGTGGCAGAGGAGT CGGAGGTGGTTCCCGGGTTAAGCGCTC-3′) and 1 pmol of 3–73gl92rc (5′-GCCCCAGTATACACCGTAACCACTACTATCATAGT AATATCTAGTACA-3′) and 3–73gl100b (5′-TACGGTGTATACT GGGGCCAGGGAACCCTGGTCACCGTCTCCTCA-3′). The three resulting fragments were size purified and mixed in a new PCR reaction with 5 pmol of the primers 3–73Fab5′ and 3–73glFab3′ under the same conditions as described above. The resulting heavy chain was cloned into Fab3–73/A3 exchanging the existing heavy chain. Both the heavy and light chain inserts of pFab3–73/A3, pFab3–73/A3gl, and Fab3–73gl/A3Asn30 were sequenced to verify the correct sequence of the cloned genes.

Purification of Fab

To prepare soluble Fab, a 5-ml overnight culture of pFab3–73/A3-infected TOP10/F′Tet^R cells was used to inoculate 1 L of Luria-Bertoni medium containing 50 mg of carbenicillin, 10 mg of tetracycline, and 20 mM $MgCl_2$. Cultures were grown for 6–7 h at 37°C with shaking, induced with isopropyl β-D-thiogalactoside (1 mM; Sigma) and 2 mg/l of cAMP (Sigma), and cultured overnight at 30°C with shaking. Bacteria were harvested by centrifugation at 6,520 × *g* for 30 min, and the pellet was solubilized in 20 ml of PBS, pH 7.4. Soluble Fab were extracted from the periplasmic space by three cycles of freezing at −80°C and thawing at 37°C. Bacterial debris was removed by centrifugation (48,000 × *g*, 45 min). The supernatants were filtered through a 0.45-μm pore filter and mixed with an equal volume of column wash buffer (300 mM NaCl, 50 mM sodium phosphate, and 10% glycerol, pH 7.8) containing 50 mM imidazole (Sigma; giving a final imidazole concentration of 25 mM). The resulting solutions were applied on a 1-ml bed volume of Ni-nitrilo-tri-acetic acid(NTA)-superflow-resin (Qiagen, Chatsworth, CA) in a Polyprep column (Bio-Rad, Hercules, CA). After washing with 20 ml of column wash buffer containing 20 mM imidazole and 4 ml containing 50 mM imidazole, the Fab were eluted with 10 ml of washing buffer containing 250 mM imidazole. Samples were concentrated, and buffer was changed to PBS using Centricon-30 centrifugal concentrators (Amicon, Beverly, MA). The Fab preparations were analyzed by unreduced SDS-PAGE followed by silver staining to assure proper m.w. and to estimate the degree of purity. The Fab concentrations were determined by ELISA with a highly purified Fab preparation as a reference. The reference Fab fragment was purified by applying the Fab-containing supernatant on a Ni-NTA column mixed with 1 vol of washing buffer containing 100 mM imidazole (final concentration, 50 mM imidazole). After washing, the Fab was eluted with 30 ml of a washing buffer imidazole gradient ranging from 50–500 mM imidazole. The fractions were analyzed by SDS-PAGE, the purest fractions were pooled, and buffer was changed and the Fab preparation was concentrated. The preparation was >95% pure as judged by silver staining, and the protein concentration was determined by absorption at 280 nm using an extinction coefficient of 1.33 l/g calculated from the amino acid composition by the formula of Gill and von Hippel (32).

Determination of Fab concentrations

ELISA plates (Costar, Cambridge, MA) were coated overnight at 4°C with 100 μl/well of a 10 μg/ml dilution of goat Abs to $F(ab')_2$ of human IgG (Pierce, Rockford, IL). After four washings in PBS containing 0.05% Tween-20, the plates were blocked for 1 h at 37°C with BSA (Life Technologies; 3% in PBS). Then, 50 μl of purified Fab (20 ng/ml and twofold dilutions) in PBS with 1% BSA were incubated in triplicate at 37°C for 1 h. The concentration standard used was the highly purified Fab preparation (20 ng/ml and twofold dilutions). After four washings, 50 μl/well of goat anti-human κ light chain Abs conjugated with alkaline phosphatase (Sigma) were added, diluted 1/500 in PBS with 1% BSA. After 1 h at 37°C, the wells were washed, and 50 μl/well of *p*-nitrophenyl phosphate (Sigma) in AP substrate buffer (2.03 g/l $MgCl_2$, 8.4 g/l Na_2CO_3, and 1.0 g/l sodium azide, pH 9.8) was added. OD was measured at 410 nm after about 60 min at room temperature.

Evaluation of HibCP binding and specificity

ELISA plates (Immunolon 2, Dynatec, Chantilly, VA) was coated overnight at 22°C with 100 μl/well of a 2 μg/ml dilution of HibCP oligomer coupled to human serum albumin (HbO-HA lot.15 D, Lederle-Praxis Biochemicals, Pearl River, NY). After four washings in PBS containing 0.05% Tween-20, the plates were blocked with BSA (3% in PBS) for 1 h at 37°C. Then, 50 μl of purified Fab (20 μg/ml in PBS with 1% BSA and twofold dilutions of that) were incubated at 37°C for 2 h (all in duplicate). The remaining ELISA procedure was performed as described above.

Detection of binding to native HibCP in solution

An inhibition assay was performed using the anti-HibCP ELISA preceded by an initial 1-h incubation of 10 μg/ml of Fab with different concentrations of soluble high m.w. HibCP (Connaught, North York, Canada) or high m.w. *E. coli* K100 capsular polysaccharide (*E. coli* K100CP; supplied by Uffe Skov Sørensen, Statens Seruminstitut, Copenhagen, Denmark) at 37°C to demonstrate specificity.

Detection of cross-reactivity with other polysaccharides

ELISA plates (Nunc 269620, Nunc, Roskilde, Denmark) were coated with 100 μl/well of a 4 μg/ml dilution of phenylated pneumococcal capsular polysaccharides (PP1, PP4, PP6B, PP7F, PP14, and PP18C, supplied by Dr. Uffe Skov Sørensen) in PBS overnight at room temperature. After washing and blocking, 50 μl of purified Fab (5 μg/ml) were incubated at 37°C for 2 h. Then the ELISA plates were developed as described above. A serum pool (HSP1) made from 10 donors vaccinated with pneumococcal capsular polysaccharides or HibCP-conjugated with TT or diphtheria toxoid, diluted 1/100 and 1/1000 in PBS, was used as a positive control.

Detection of cross-reactivity with TT

ELISA plates (Maxisorp, Nunc) were coated with 100 μl/well of a 1 μg/ml dilution of TT diluted in PBS overnight at 4°C. After washing and blocking, 50 μl of purified Fab (10 μg/ml and 2-fold dilutions of that) were incubated at 37°C for 1 h. After washings, 50 μl/well of a 1/500 dilution of biotinylated mouse anti-human κ light chain Abs (Zymed, San Francisco, CA) was added in PBS with 1% BSA and incubated for 1 h at 37°C. After new washings, 50 μl/well of a 1/500 dilution of streptavidin conjugated with alkaline phosphatase (Kirkegaard & Perry, Gaithersburg, MD) was added in PBS with 1% BSA. After 1 h at 37°C, the wells were washed, and 50 μl/well of *p*-nitrophenyl phosphate in alkaline phosphatase substrate buffer was added. OD was measured at 410 nm after about 60 min at room temperature. The HSP1 serum pool was used as a positive control.

Table I. *Pre- and postvaccination status of a healthy adult male (To) vaccinated with a single dose of HibCP coupled to TT*[a]

		Ig- and Ab-Secreting Cells (SC)/10^6 PBMC															
					Day 9 after vaccination												
Plasma HibCP Ab (μg/ml)[b]		Before vaccination			Before purification						After purification[c]						
Before vaccination	1 mo after	Total IgSC	HibCP AbSC[d]	TT AbSC[d]	Total IgSC	HibCP AbSC	IgM (%)	IgG (%)	IgA (%)	TT AbSC	HibCP AbSC	Recovery (%)	TT AbSC	IgM (%)	IgG (%)	IgA (%)	κ L chain (%)
2.6	177	836	0	0	19,684	16,316	3	6	91	509	6,350	39	4	3	1	96	90

[a] The plasma HibCP Ab levels and the numbers of circulating Ig-secreting cells (IgSC) and HibCP and TT Ab-secreting cells (AbSC) are given as well as the recovery of AbSC after purification with HibCP-coupled paramagnetic beads. Data with permission from Ref. 10 (copyright 1996, The American Association of Immunologysts).
[b] Lower detection limit of the ELISA was 0.05 μg/ml.
[c] AbSC in the HibCP-binding fraction expressed per 1×10^6 PBMC subjected to purification.
[d] A total of 0.6×10^6 PBMC were tested for each isotype.

Measurement of relative K_d *values*

ELISA plates (Immunolon 2) were coated overnight at room temperature with 100 μl/well of 2 μg/ml HibCP-HSA. After washing and blocking, 100 μl of purified Fab (20 μg/ml in PBS with 1% BSA and twofold dilutions of that) were incubated in duplicate at 37°C for 25.5 h to assure equilibrium. Six additional wells were incubated with 100 μl of Fab (11 μg/ml of Fab3–73/A3gl and 10 μg/ml of Fab 3–73/A3), but after 22 h of incubation, the concentration of free Fab in three of the six wells were determined by moving the 100 μl to a new well. At the same time, a new dilution series was made starting with 20 μg/ml Fab to serve as reference. After the remaining 3.5 h of incubation, the plate was washed four times and incubated for 1 h at 37°C with 100 μl of goat anti-human κ light chain Abs conjugated with alkaline phosphatase diluted 1/200 in PBS with 1% BSA. After the wells were washed, 100 μl/well of *p*-nitrophenyl phosphate in alkaline phosphatase substrate buffer was added.

The dissociation constant K_d is defined from the Law of Mass Action (Equation 1). [Ag] and [Ab] are the concentrations of unbound (free) Ag and Ab (in this situation Fab) at equilibrium, respectively. [AgAb] is the concentration of Ag-Ab complexes. Because care was taken to assure that neither the amount of secondary Ab nor that of the substrate was a limiting factor in the ELISA, [AgAb] was proportional with OD_{410} (Equation 1). The constant, *k*, was unknown, but was equal for all Fab binding to the same epitope on the HibCP oligosaccharide.

$$K_D = \frac{[Ag][Ab]}{[AgAb]} = \frac{[Ag][Ab]}{k \times OD_{410}}. \quad (1)$$

The ratio between the K_d values of two Fab, Fab3–73/A3gl (K_{d1}) and Fab3–73/A3 (K_{d2}), could then be determined by Equation 2.

$$\frac{K_{D1}}{K_{D2}} = \frac{[Ag]_1[Ab]_1 \times OD_2}{[Ag]_2[Ab]_2 \times OD_1}. \quad (2)$$

Results and Discussion

This report deals with the B lymphocyte response of an adult male (To) immunized s.c. with a single dose of HibCP-TT. Vaccine-induced HibCP-specific circulating B cells were purified from peripheral blood. Table I shows the numbers of Ab producers before and after purification. After vaccination the vast majority of the cells secreted Ab of the IgA isotype, and 90% were of the κ light chain type. We recovered 39% of the HibCP-specific cells after purification, and the purity of the recovered cells was evaluated using the accompanying AbSC specific for the carrier protein, TT, as indicators for cells of other specificities than HibCP (Table I). In an earlier study by us (10) it was estimated that 99.6% of the Ig-secreting cells of the purified cell fraction were specific for HibCP (10, 19). In that study, sequencing of cloned cDNAs revealed that 90% of the κ AbSC (i.e., ~81% of the total number of HibCP AbSC) used a slightly mutated version of the VκII gene A3/A19 rearranged to Jκ3 and represented the progeny of a single virgin B cell. Based on the huge number of clonal HibCP-specific cells in the circulation ($\sim 1 \times 10^8$) and on an analysis of the somatic mutations, we suggested (10) that the responding cells must have arisen from a selected and expanded population of mutated memory B cells already existing before immunization.

In this report we extend the analysis of the HibCP-specific Ab response of the same individual (To) to comprise the heavy chain V gene usage to further characterize the response including somatic heavy chain hypermutation, isotype switch, and affinity maturation within the dominating clone.

Identification of the predominant heavy chain

IgA SC dominated the purified AbSC constituting about 96% of all HibCP AbSC (Table I). It was therefore expected to find a considerable population of clonally related V gene transcripts in this population representing the heavy chain of the cells donating the predominant light chain gene transcripts (A3/A19) (10). A total of 52 IgA clones from two different PCR amplifications (ToPA101–184 and ToPA203–280, respectively) were analyzed by restriction fragment length polymorphism; 36 (69.2%) were IgA1, and 16 (30.8%) were IgA2. The whole V_H and a part of the CH1 domain of 25 of the IgA1 clones and all of the IgA2 clones were sequenced. Twenty (80%) of the 25 IgA1 clones and 10 (63%) of 16 IgA2 clones were highly homologous. Their V gene sequences all had maximum homology (92.0–95.3%, codons 1–94) with the published germline gene 3–73 (33). All 30 sequences showed identical rearrangements using a J_H gene with maximum homology with the published JH4b1 gene (27) and a D element with maximum homology to D3–22 (IMGT nomenclature, former called DXP3 (34); Fig. 1, rearrangement 1). A seven-nucleotide N addition was inserted between the D and the J_H element and was shared by all clones except for two clones with a single base substitution in this region (Fig. 2). Combined, these observations strongly suggested that these 30 clones represented mRNA from the progeny of a single virgin B cell.

A common clonal origin was further substantiated by the high number of shared mutations. Of the 657 mutations seen in the V_H domains of the 30 clones, 645 were found in at least two sequences. Thus, in 14 positions substitutions were shared by all sequences, and in an additional 22 positions mutations were shared by at least two sequences (Fig. 2). Because all these mutations were found in clones derived from independent PCR amplifications, these substitutions were not PCR artifacts. However, in principle it was possible that some of the mutations shared by all clones were germline encoded in the vaccinated individual due to unrecognized polymorphisms of the Ig-genes involved. Although unlikely, it could be the case for the two substitutions in the J_H-encoded part (codons 102 and 113), the

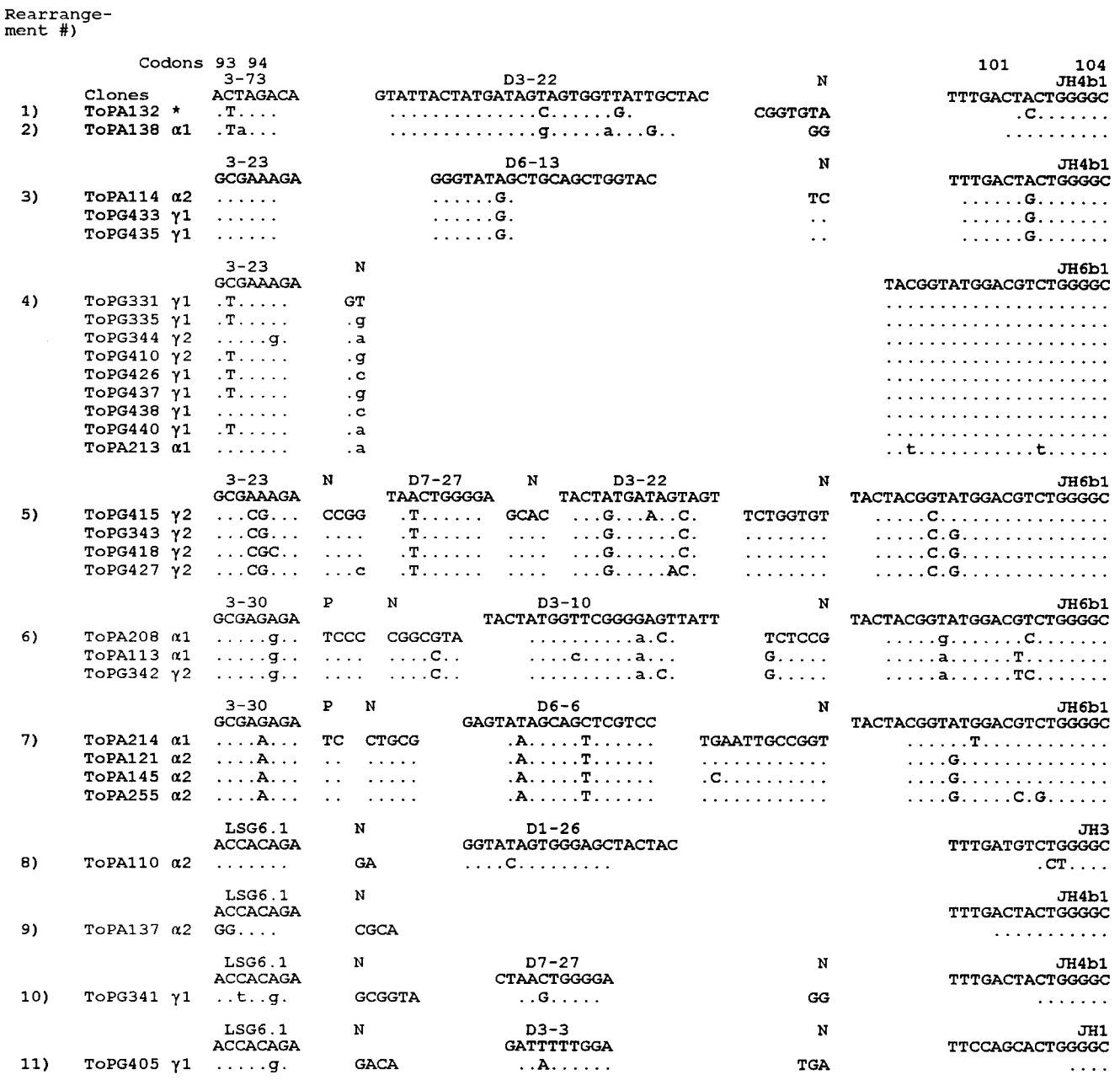

FIGURE 1. V-D-J recombinations (codons 93–104) of PCR-amplified cDNA from HibCP-specific, affinity-purified, circulating B cells/plasmablasts expressing V_H family III mRNA. The cells were obtained 9 days after immunization of a healthy adult male with HibCP coupled to a carrier protein. The complete variable domains were sequenced (codons 1–113) for 41 IgA clones (ToPA101–ToPA280) and 17 IgG clones (ToPG331–ToPG440). Heavy chain subclasses are given for each clone. Dots mean identity with the sequence given above. Capital and lowercase letters indicate amino acid replacing and silent substitutions, respectively. All clones were assigned to the closest V, D, and J germline gene registered in the EMBL database. P indicates nucleotides probably added by P insertion, and N indicates nucleotides probably added by N addition. Different rearrangements are numbered 1–11. All sequences are available from the EMBL/GenBank/DDBJ under accession numbers Z98683-Z98740. ∗, Rearrangement of the dominating genealogical tree.

two substitutions in the D gene-encoded part of the VDJ gene (codons 99 and 100b), and three of the mutations in the V_H gene-encoded part of the VDJ gene (codons 7, 89, and 93) shared by >85% of the clones. It could, however, not be the case for the 11 remaining substitutions in the V_H gene shared by >96% of the clones, because we did not find evidence for such an allele in the germline of To. This was studied by PCR amplification of V_H3–73-like germline genes from MNC employing V_H3–73-specific primers recognizing areas of FR1 and FR3 shared by the published germline gene and by 83% of the 30 related IgA clones. This would give any putative 3–73-like allele the same probability of amplification as the conventional gene. Of 13 cloned germline sequences, from codon 19 through the first base of codon 83, none expressed any of the 11 substitutions carried by most of the rearranged To clones. In fact, all sequences were identical with the published V_H3–73 sequence. If the individual To carried an unknown V_H3–73-like gene expressing the shared substitutions of the rearranged To clones, the lack of this sequences among the 13 clones would be a highly unlikely event even if the novel gene resided as a single copy at an unknown locus (i.e., one of four amplifiable genes; $p = 0.023$, binomial distribution). Thus, at least the eight mutations shared by all 30 clones found in the sequenced part of the germline gene and all 22 mutations shared by some of the

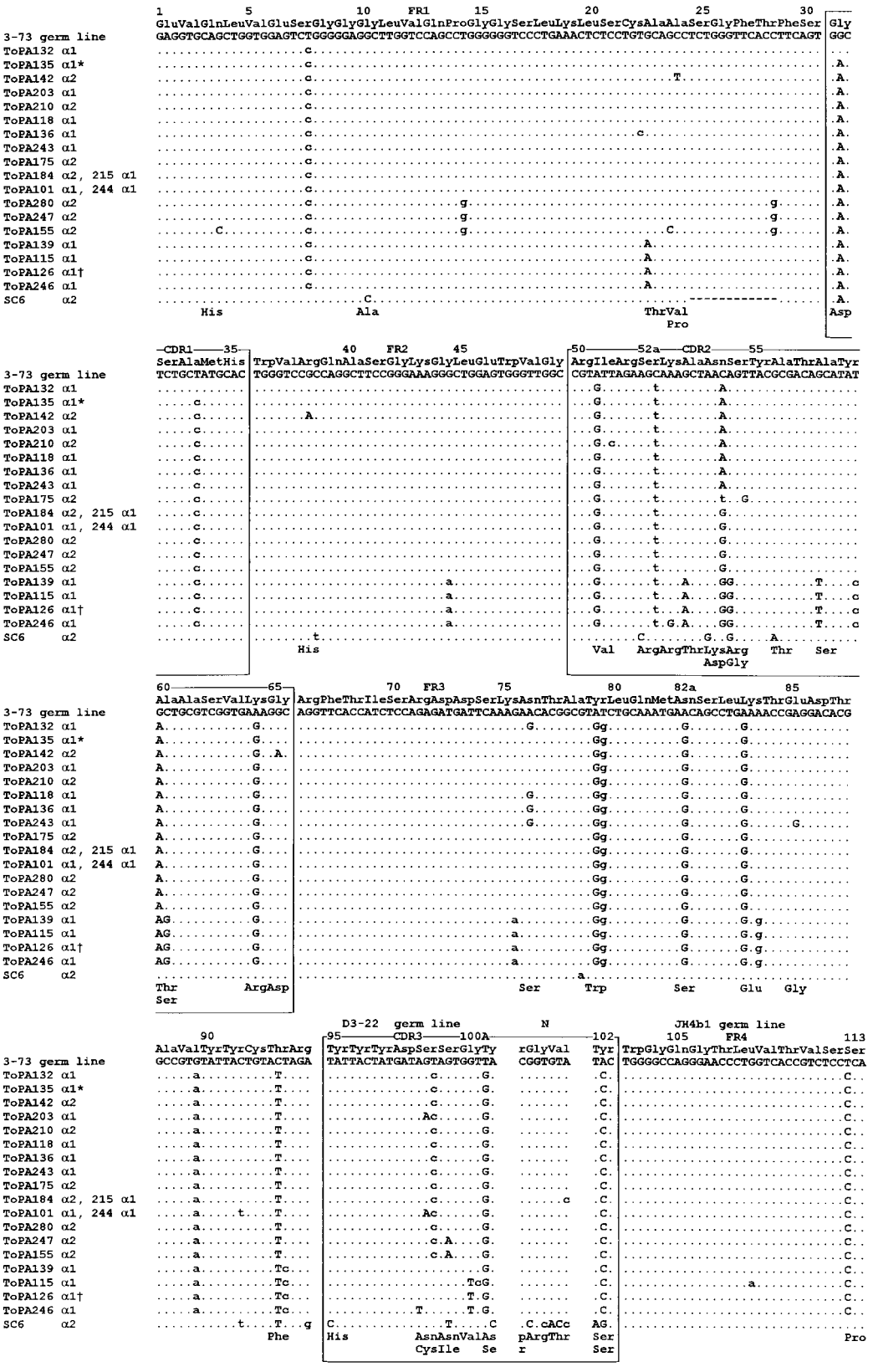

FIGURE 2. Entire variable domains (codons 1–113) of 30 clonally related IgA cDNA sequences using the same rearrangement (rearrangement 1, Fig. 1). The IgA subclasses are given. Dots mean identity with the germline sequence given above, and dashes mean deletions in the sequence. Capital and lowercase letters indicate amino acid replacing and silent substitutions, respectively. Mutant amino acids are given at the *bottom*. When there are two changes in the same position, both are given. N indicates nucleotides probably added by N additions. A high number of shared mutations and a paucity of singular mutations are noted and indicate a common clonal origin of the cells donating the mRNA. Also presented is the sequence of SC6, an HibCP-specific hybridoma cell line from the literature employing the same rearrangement except for the sequence of the seven-base N addition (8). ∗, Identical sequences for ToPA134α1, ToPA156α2, ToPA225α2, ToPA226α1, and ToPA231α2. †, Identical sequences for ToPA127α1, ToPA133α1, ToPA218α1, ToPA230α1, and ToPA232α1.

clones must have arisen by somatic mutation. Twelve of them were amino acid nonreplacing and therefore unselectable. They were thus very likely to be clonal markers.

Besides the uniqueness of the VDJ rearrangement and the high number of shared mutations, probably the best argument for clonal relatedness of the 30 sequences was the extreme paucity of singular mutations. Thus, only 12 of 657 (1.8%) substitutions were not shared by two or more clones, and at least some of them are likely to be *Taq* errors. This would have been an extremely unlikely event if all the 657 mutations had occurred independently. It is therefore concluded that the 30 sequences represented the clonal progeny of a single virgin B cell.

Correspondence with the predominant light chain

It appeared reasonable to assume that the predominant heavy chain sequences were derived from the same clonal progeny that used the predominant light chain previously reported (10). However, because we used a family-specific heavy chain primer, the possibility that some of the HibCP AbSC used V_H genes from other families has to be considered. Serologic studies of the V_H genes used in HibCP-specific Ab have shown, however, that they are totally dominated by the V_HIII family (35), and all HibCP-specific Abs sequenced to date have used a V_H gene from that family (7–9, 12, 36). Because of this, we find it highly unlikely that the predominant clones should not use a V_HIII V gene. Another potential problem that has to be addressed is the possible bias for certain V_HIII genes that the degenerate V_HIII primer might have. Such a bias could skew the amplified repertoire, leading to an over-representation of clones using certain heavy chain genes. To minimize this problem, we used a relatively low annealing temperature during the PCR to allow efficient amplification of all V_HIII genes present in the cDNA preparation despite minor differences in the regions recognized by the degenerate primer. The central question in this respect was whether it was possible to arrive at a reliable estimate of the size of the predominant B cell clone using the frequency of the amplified V genes. Based on the assumption that all V genes used by the anti-HibCP IgA SC were amplified evenly, and after compensating for the deliberate over-representation of IgA2 clones in the material, the 30 IgA clones could be estimated to represent 75% of the IgA-SC or 72% of the total number of purified HibCP-specific cells. With such a high prevalence, the B cell clone donating the 3–73/D3–22/JH4b1 sequences had to be the same as that donating the predominant A3/A19-Jκ3 light chain gene found in our previous study (10). In that study the predominant A3/A19-using clone was estimated to represent 90% of the κ light chain SC and 81% of the total number of HibCP AbSC. Because the latter data were obtained using a V gene unbiased technique (single-sided specific PCR), the close correspondence between the two estimates strongly validated the V_H data and pointed to a clone size of close to three-quarters of all HibCP-specific AbSC recovered by the beads. The only thing needed to finalize this validating argument was an independent demonstration that these heavy and light chain sequences indeed derived from the same cell clone.

In agreement with this, the only described HibCP-specific hybridoma using the A3/A19 light chain (SC6) (8) used the same set of V_H, D, and J_H gene segments and even rearranged in exactly the same positions and D gene reading frame as the 30 IgA clones (Fig. 2). Only the seven-base N addition differed, but it was of the same size and position as in the To-derived clones.

Analysis of a Fab representing the predominant clone

Perhaps the strongest evidence for the derivation of the 3–73/D3–22/JH4b1 heavy chain sequences and the A3/A19-Jκ3 light chain

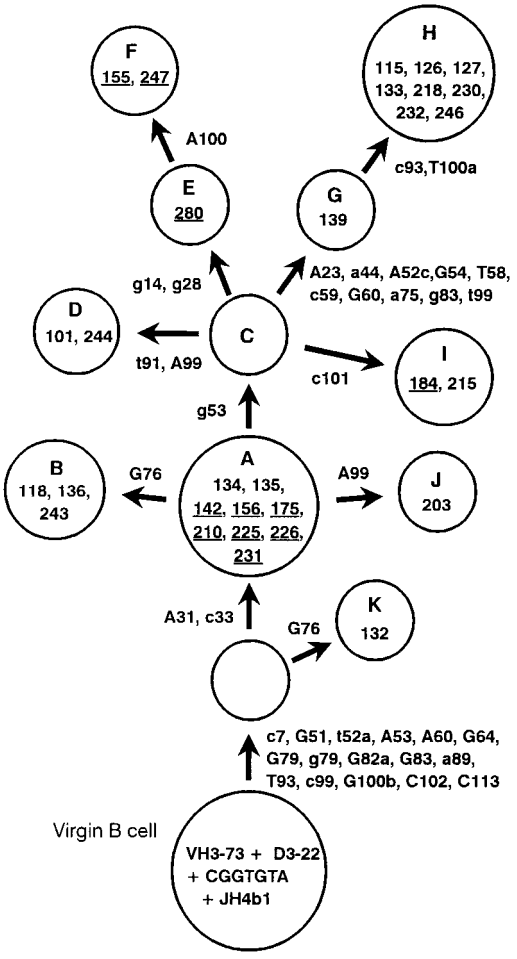

FIGURE 3. Genealogical tree demonstrating a possible clonal relationship between the vaccine-induced HibCP-specific B cells from the individual To using the 3–73 germline gene rearranged to the D3–22 diversity gene segment and the J_H4b1 minigene. Numbers in circles refer to the 30 clones listed in Fig. 2; IgA2-using clones are underlined. Letters identify groups referred to in the text. Only mutations found in at least two clones are indicated. The tree was constructed according to the principle of minimizing the number of mutations that occur twice in the tree. Replacement and silent mutations are indicated by capital and lowercase letters, respectively, followed by codon number. A putative unmutated but rearranged virgin B cell is indicated at the *bottom*. A high number of shared mutations at the basis of the tree indicates that an extreme clonal selection has occurred initially. At least three intraclonal isotype switches to IgA2 are evident.

sequences from the same clonal progeny would be the demonstration that combination of these genes encodes an Ab with specificity for HibCP. To increase the likelihood of combining chains that actually derived from the same cell, we exploited the relatively low frequencies of replacement mutations in the genealogical trees. In fact, 12 of the 30 IgA clones expressed identical V_H region amino acid sequences (Fig. 3, groups A, E, I, and J), and in the corresponding genealogical tree for the light chain, 24 of 42 clones expressed the same amino acid sequence (10). The V(D)J genes from two clones (ToPA135 (Fig. 2) and ToP027 (Fig. 4)) encoding predominant heavy and light chains, respectively, were coexpressed (Fab3–73/A3) using an IgG1 CH1 domain with an appended oligo-histidine tag at the C-terminus facilitating purification.

As shown in Fig. 5, the Fab indeed bound to HibCP oligomers in ELISA, and the binding could be inhibited by the addition of

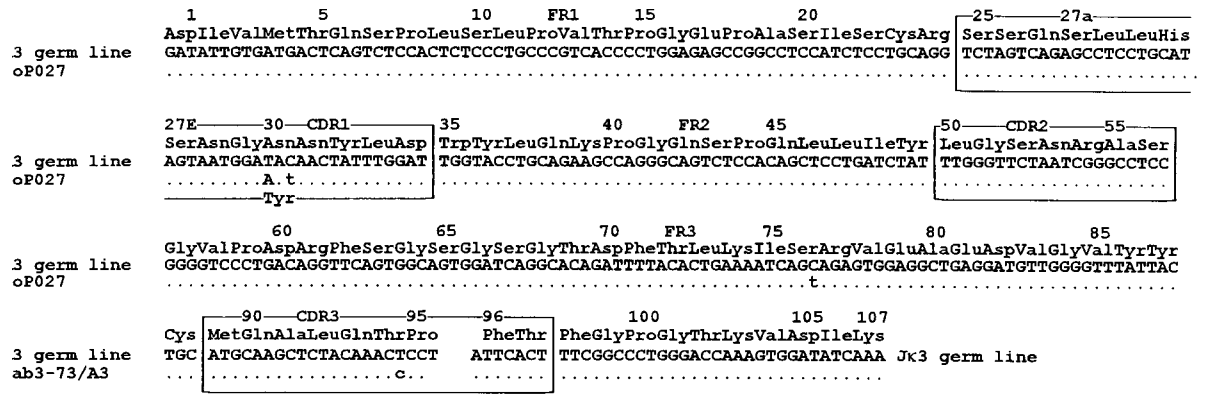

FIGURE 4. Entire variable domain (codons 1–107) of the κ light chain VκA3 (or A19) germline gene (used in Fab3–73/A3gl) and the cDNA sequence of ToP027 used as the light chain of Fab3–73/A3.

soluble HibCP (Fig. 6*A*), demonstrating that the Fab had recognized native high m.w. HibCP in solution. The Fab were not polyreactive, as they did not bind to a panel of pneumococcal capsular polysaccharides (PPS 1, 4, 6B, 7F, 14, and 18C) or to TT in ELISA (data not shown). As expected, the Fab cross-reacted with *E. coli* K100 capsular polysaccharide (Fig. 6*A*), but 60-fold more *E. coli* K100CP than HibCP was needed to inhibit the binding of the Fab to 50% of the initial level. The *E. coli* K100 polysaccharide only deviates from HibCP by a (1→2) glycoside linkage rather than (1→1), and cross-reactivity with this Ag has been observed for most HibCP Abs not using the A2-encoded light chain (11–13). Interestingly, this is the first demonstration of cross-reactivity with an HibCP Ab using a VκII light chain with such high homology (85.9% amino acid homology, codons 1–94) to the (noncross-reactive) A2-derived light chain. It is concluded that the 30 heavy chain clones in the genealogical tree derived from the same clonal progeny as the κ clones previously described (10) and that this clone constituted approximately three-fourths of all HibCP AbSC recovered from the individual To.

Analysis of mutations of the dominant clone

The 30 IgA clones using the rearrangement 1 (Fig. 1) on the average carried 17.6 mutations from codons 1–94, which was significantly more than the average of 5.5 mutations found among the corresponding light chain genes (by χ^2 test, $p < 0.0001$). This difference was still evident when only silent mutations were regarded ($p = 0.017$). A similar preponderance of mutations of the heavy chain gene relative to the light chain was seen in 12 of 15

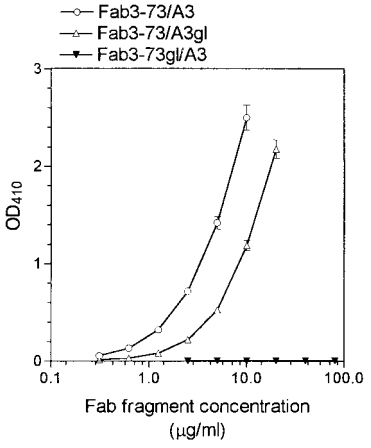

FIGURE 5. Binding of HibCP-specific Fab, Fab3–73/A3 and Fab3–73/A3gl, to HibCP oligosaccharides immobilized on a solid phase (ELISA technique). Each dilution curve represents the mean (error bars indicate 2 × SD) of three independent experiments. The net OD at 410 nm are given. Fab3–73/A3gl is identical with Fab3–73/A3, except for position 30 of the light chain where the former is back-mutated to the germline-encoded tyrosine rather than asparagine. Significantly poorer binding of the back mutant is evident.

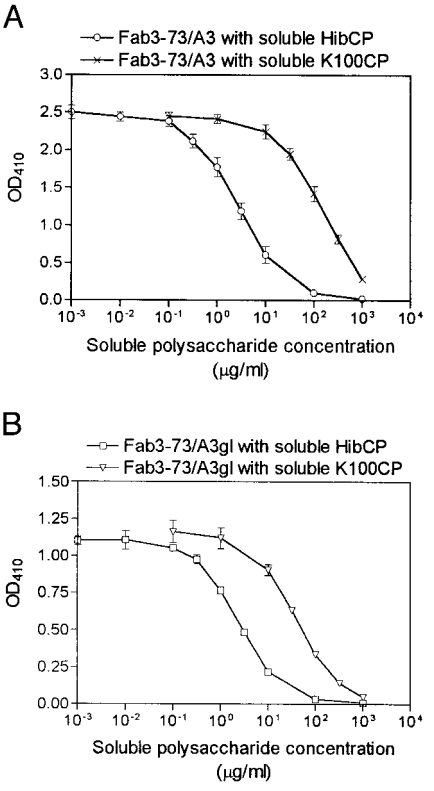

FIGURE 6. Inhibition of binding to solid-phase immobilized HibCP oligosaccharides of Fab3–73/A3 (*A*) and Fab3–73/A3gl (*B*) by various concentrations of soluble high m.w. HibCP and *E. coli* K100CP at 37°C. A fixed concentration of Fab (10 μg/ml) was used. The net OD (410 nm) are indicated. Each curve represents the mean (2 × SD) of three independent measurements. Both fragments are totally inhibitable by the native polysaccharide (HibCP) and cross-react with *E. coli* K100 polysaccharide, but with a lower affinity.

HibCP-specific hybridomas with known heavy and light chain sequences available in the literature (7–9, 12, 13) and has been noted in some murine hybridomas (37, 38), while other have demonstrated a more equal distribution (39). These observations suggest that the process of somatic mutation may proceed more efficiently in the rearranged heavy chain gene than in light chain genes. An alternative possibility is that light chain rearrangement may reoccur in mutated (memory) B cells, a possibility that currently lacks experimental evidence.

The high number of heavy chain mutations shared by all the heavy chain sequences of the predominant clone indicated that a high level of clonal selection must have occurred before a common mutated intermediate precursor subsequently expanded vigorously. According to the genealogy proposed in Fig. 3, a common intermediate precursor B cell should contain 16 substitutions that would demand about 48 cell divisions using a high estimate of the germinal center mutation rate (10^{-3} substitutions/nucleotide/cycle) (40, 41). Even with a low estimate of the doubling time (6 h (42)) this would mean that it would take at least 12 days to generate the common mutated precursor B cell. A further 27 divisions (or 7 days) would be needed to expand this precursor to account for the 0.7×10^8 clonally related HibCP AbSC present in the circulation 9 days after vaccination. This is an absolute minimum because all the cells remaining in the regional lymph nodes or disseminated to tissues elsewhere, such as spleen, bone marrow, and mucosal sites, or those that died from apoptosis have been ignored in the calculation. If one considers the highly mutated group of sequences (group H, Fig. 3) that constituted 27% of the dominant clones (i.e., $\sim 0.2 \times 10^8$ circulating cells), at least 23 days were required to accumulate the 31 shared mutations and another 6 days of expansion were required to yield this number of circulating cells. From these calculations it appears inevitable that the virgin B cell founding the predominant clone of individual To must have proliferated, and its progeny must have been somatically hypermutated and extremely selected (i.e., memory cells) before the time of vaccination. Because the individual To had not previously been immunized with any HibCP-containing vaccine, this means that he responded with a memory cell response to the first systemic vaccination. Indeed, it is likely that cells carrying the 18 shared mutations of group A and cells carrying the 31 shared mutations of group H both existed before vaccination in this individual. Because these cells represent the mutational extremes of the genealogical tree, it is possible that the tree represents the geneology of the memory B cells and that these cells were simply expanded without further mutation in response to the vaccination. However, we cannot exclude the alternative: that some of the mutations were acquired after vaccination due to re-entry of memory B cells into the process of somatic hypermutation.

Intraclonal affinity maturation

Given the extreme selection to which the clone must have been subjected, it was surprising to find quite low ratios of replacement to silent mutations in the CDR regions. Thus, the replacement to silence (RS) ratios of the genealogical tree of the heavy chains were 14/7 (2.0) in the CDR and 8/11 (0.7) in the FR. The corresponding values for the light chains were 5/7 (0.7) for CDR and 4/1 (4.0) for FR (10). These are considerably lower levels than the average for somatically mutated CDR regions (3.2–3.8) in the peripheral repertoire (43, 44), and it does not exceed that found among unfunctional (i.e., unselected) V_H rearrangements in humans (45). A similar low RS ratio were found for the corresponding light chains (10), suggesting that continued selection of better binding CDR mutants has not been the driving force behind the extreme selection of these cells. RS ratios of the CDR <3 have been found in seven of the eight known light chains and in half of the 20 known heavy chains of the published HibCP-specific heterohybridomas (7–9, 12, 13), indicating that it may be a common phenomenon in the response to HibCP conjugates.

Possible explanations for the generally low levels of RS ratios in the CDR are that either affinity maturation does not occur or that a few mutational events increase the affinity to a level yielding a clear selective advantage to the B cell while further selectory power is not conveyed by other replacement mutations. If the latter possibility is true, any of the 11 replacement mutations of the heavy chain shared by all clones are candidates for an affinity-increasing effect, but also the single replacement mutation of the light chain (Tyr→Asn in position 30) that is shared by all clones could represent this gateway signal. Previously, we predicted that the early light chain mutation represented such an event (10). Thus, apart from being the only replacement mutation shared by all light chain clones, it was placed in a position where the first CDR was known often to have contact with Ag (46), and the same mutation was found in the light chain gene of the independently isolated HibCP-specific SC6 hybridoma (8).

To evaluate the influence of the light chain mutation in position 30 in CDR1, we changed the asparagine in that position in Fab3–73/A3 to the germline-encoded tyrosine by PCR mutagenesis. The resulting Fab fragment was called Fab3–73/A3gl and differed only from Fab3–73/A3 at that single position. Fab3–73/A3gl bound to solid phase HibCP (Fig. 5), and the binding could be completely inhibited by native high m.w. HibCP as well as by *E. coli* K100CP (Fig. 6*B*). Fiftyfold more *E. coli* K100CP than HibCP was needed to decrease the amount bound to the solid phase by 50%. No cross-reactivity with pneumococcal capsular polysaccharides (PPS 1, 4, 6B, 7F, 14, and 18C) or with TT was detected. The only difference between Fab3–73/A3 and Fab3–73/A3gl was a twofold lower OD_{410} signal for the latter when measured at the same Fab concentration, which indicated a difference in the affinity of the two Fab for HibCP. Using measurements of free and bound Fab concentrations at equilibrium (at least 22 h of incubation), the law of mass action provided an estimate of the ratio between the K_d values of Fab3–73/A3gl (1) and Fab3–73/A3 (2):

$$\frac{K_{D1}}{K_{D2}} = \frac{[Ag]_1[Ab]_1 \times OD_2}{[Ag]_2[Ab]_2 \times OD_1} = \frac{[Ag]_1 \times 5.4\ \mu g/ml \times 1.461}{[Ag]_2 \times 4.3 \mu g/\mathrm{ml} \times 0.995} = \frac{[Ag]_1}{[Ag]_2} \times 1.84. \quad (3)$$

Because the binding to the solid phase oligosaccharides was not saturable at the available Fab concentrations, the exact values for free Ag, $[Ag]_1$ and $[Ag]_2$, were not determined. However, a rather narrow interval for the ratio $[Ag]_1/[Ag]_2$ could be estimated because doubling the total concentration of Fab3–73/A3 led to an approximate doubling of the OD value, showing that less than half of the antigenic epitopes were occupied at a free Fab concentration of 4.3 μg/ml (data not shown). Therefore, the ratio $[Ag]_1/[Ag]_2$ had to lie between 1 (corresponding to virtually all Ag unbound) and $(1 - 0.5 \times 0.995/1.461)/(1 - 0.5) = 1.32$ (half-saturation at $[Ab]_2$). Accordingly, a relatively precise estimate for the ratio of dissociation constants of the two Fab would be: $1.84 < K_{d1}/K_{d2} \leq 2.43$. This indicates that replacing the germline-encoded tyrosine in position 30 of the light chain by asparagine increased the affinity for HibCP by a factor of approximately 2. This increase in affinity was probably accompanied by a considerable increase in avidity assuming bivalent binding of Ab to the repetitive epitopes of HibCP and may represent an important event in the selection of this clonal progeny. This did not exclude, however, that one or

more of the 11 (three in CDR) replacement mutations of the heavy chain shared by all 30 clones had similar effects on affinity. To evaluate the effect of the 11 shared replacement mutations, we constructed a Fab using the germline sequence in the V_H domain (same as the germline sequence in Fig. 2 throughout codon 113). This Fab (Fab3–73gl/A3) was not able to bind HibCP monovalently in a concentration of 80 μg/ml in the ELISA (Fig. 5), indicating that at least some of the 11 shared heavy chain mutations are important for Ag binding. However, this must not be taken to indicate that the virgin B cell founding the predominant clone carried an Ag receptor without HibCP binding capability. The B cell could benefit from bivalent binding of the individual receptor and from engagement of many receptors resulting in binding with a reasonable avidity to the multivalent polysaccharide Ag. What it did indicate, however, was that the shared heavy chain mutations had increased the affinity considerably. A minimum value for the increase in affinity between Fab3–73gl/A3 and Fab3–73/A3 could be estimated at equilibrium using the background OD plus 3 $\times$ SD as OD_1 in the above formula (representing the maximum OD value for the nonbinder Fab3–73gl/A3. By this method, the increase in affinity was at least a factor of 475. It is therefore concluded that the predominant B cell clone has undergone a considerable affinity maturation, indicating that clonal expansion and selection have been affinity driven. Because the mutated Fab represented the least mutated sequences in the genealogical tree, further affinity maturation may have occurred in the B cells represented by the branches of the tree. In this context it should be mentioned that affinity maturation within the clone opens the possibility that the PCR-amplified repertoire is skewed in the direction of the most mutated sequences, because the B cells are selected based on their ability to bind to Ag-coated beads. While such an effect could influence the shape of the genealogical tree in Fig. 3 somewhat, it does not interfere with any of the major conclusions of this paper. The multipoint attachment nature of the binding between Ag-coated beads and cells probably makes affinity of little importance for cell recovery. The fact that as many as 39% of the circulating HibCP AbSC were actually recovered in this experiment clearly limits any possible affinity bias in this study.

Intraclonal isotype switches

The Ab response of adults to HibCP and HibCP conjugates consists of IgG, IgA, and IgM, mentioned in order of decreasing plasma levels (17). The IgG component consists of IgG1 and IgG2, but little IgG3 and IgG4 (17). At the level of circulating AbSC, about equal amounts of IgG and IgA SC are found, probably reflecting the shorter plasma half-life and secretion across the mucosal barriers of IgA. On the average, about equal numbers of IgA1 and IgA2 SC are found (18), a feature that characterize AbSC directed against polysaccharides (47) and contrasts to the predominance of IgA1 among AbSC of specificity for protein Ags (18). We have shown that IgG2 and IgA2 AbSC dominate in adults with high levels of natural Ab to HibCP, while IgG1 and IgA1 AbSC dominate in adults with low levels of natural Abs and in infants (18). The individual To had relatively low levels of natural Abs (2.6 μg/ml) and correspondingly a predominance of IgA1 (69.2%) over IgA2 (30.8%).

Evaluation of intraclonal isotype switches is complicated by the possible presence of PCR or cloning crossing-over artifacts that could have misplaced a few sequences in the genealogical tree (48). This problem was addressed by sequencing the available part of the α-chain CH1 (codons 114–184, 189 bp) in all 41 IgA clones. In this area, there are 10-bp differences between IgA1 and IgA2, the most 5′ in codon 127 and the most 3′ in codon 176 (22). Thirty-nine (95%) of the sequences were unequivocal, while two clones (5%) carried the IgA2 sequence in nine of these positions, but the IgA1 sequence in the most 3′ position (codon 176). These hybrid clones (ToPA110 and ToPA155) were classified as IgA2 sequences, and one of them (ToPA155) was included in the genealogical tree (Fig. 3). Although *Taq* errors could not be ruled out, the hybrids were most likely the result of cloning or PCR crossing-over, suggesting that this artifact did occur, but at a relatively low rate.

At least three different intraclonal isotype switches were seen in the genealogical tree. Clones of both isotypes were seen in groups A and I (Fig. 3), indicating that at least two independent switches occurred in these groups. A third switch must have occurred in either group C or group E. If the isotype switch had happened in group C, further somatic hypermutations were acquired after isotype switching. Because both IgA1 and IgA2 clones of group A were derived from independent PCR amplifications, and because several IgA2 sequences were found in groups E and F, at least two of the three switch events could not result from PCR or cloning artifacts but must result from in vivo switching.

Intraclonal switching to IgG probably did not occur, because none of the 17 IgG sequences (10 IgG1 (59%) and seven IgG2 (41%)) analyzed showed any signs of clonal relatedness with the predominant IgA clone (Fig. 1). This means that while switching has occurred repeatedly in the expanding clone, it has been very selective and comprised $\mu \rightarrow \alpha_1$ and $\alpha_1 \rightarrow \alpha_2$ or $\mu \rightarrow \alpha_2$ switches only. The cytokine TGF-β could be involved, as it is known selectively to induce these type of switches, probably by inducing germline transcription of α_1 and α_2 genes (49). Our data do not allow us to discriminate between direct switches from μ to α_1 and α_2 or sequential switches ($\mu \rightarrow \alpha_1 \rightarrow \alpha_2$). Sequential switching has been described in other situations (50). Finally, it should be mentioned that it is unclear whether these isotype switches occurred before vaccination during the generation of natural memory B cells or after vaccination during the recruitment of memory B cells.

All seven IgG2 sequences had a nucleotide characteristic of IgG1 in the last base of position 138 (G instead of C). The difference is not amino acid replacing, and it probably represents a hitherto unknown polymorphism of the IgG2 gene, but this must be confirmed by sequencing of the germline of individual To.

Analysis of used canonical V genes

While the dominant clone uses the A3/A19 light chain, most of the remaining (three of four sequences accounting for ~7% of the κ HibCP AbSC) use the VκII gene A2a rearranged to Jκ3 with the introduction of an extra arginine codon in position 95a (10). HibCP Ab using the latter light chain usually dominates postvaccination sera and may be detected in 85% of individuals immunized by the expression of the HibId-1 Id (51). The allelic gene A2c may replace A2a in these Abs (7, 52), and the rearrangement may be to Jκ1, Jκ2, and Jκ3 (14). Following the proposal by Pinchuk and colleagues (8), we suggest that these gene combinations be called canonical with respect to HibCP following the terminology used in murine systems. Twelve other light chain genes have been detected in HibCP antisera and hybridomas (8–13), including the A3/A19 gene, but are much less prevalent than the A2 gene and will accordingly be called noncanonical.

Judged by the sequences of four of the five published HibCP-specific hybridomas using the canonical light chain (8, 12, 13), the corresponding canonical heavy chains consist of either the V_HIII gene VH26 (53) or the highly homologous (99.7%, codons 1–95) gene 3–23 (54) rearranged to D6–13 (34) (formerly DN1) and J_H4 or directly to J_H6 forming a CDR3 region of only six amino acids (codons 95–102) starting with a common motif: Gly-Tyr-Gly (7, 8). Indeed, these VDJ rearrangements have been found in seven of

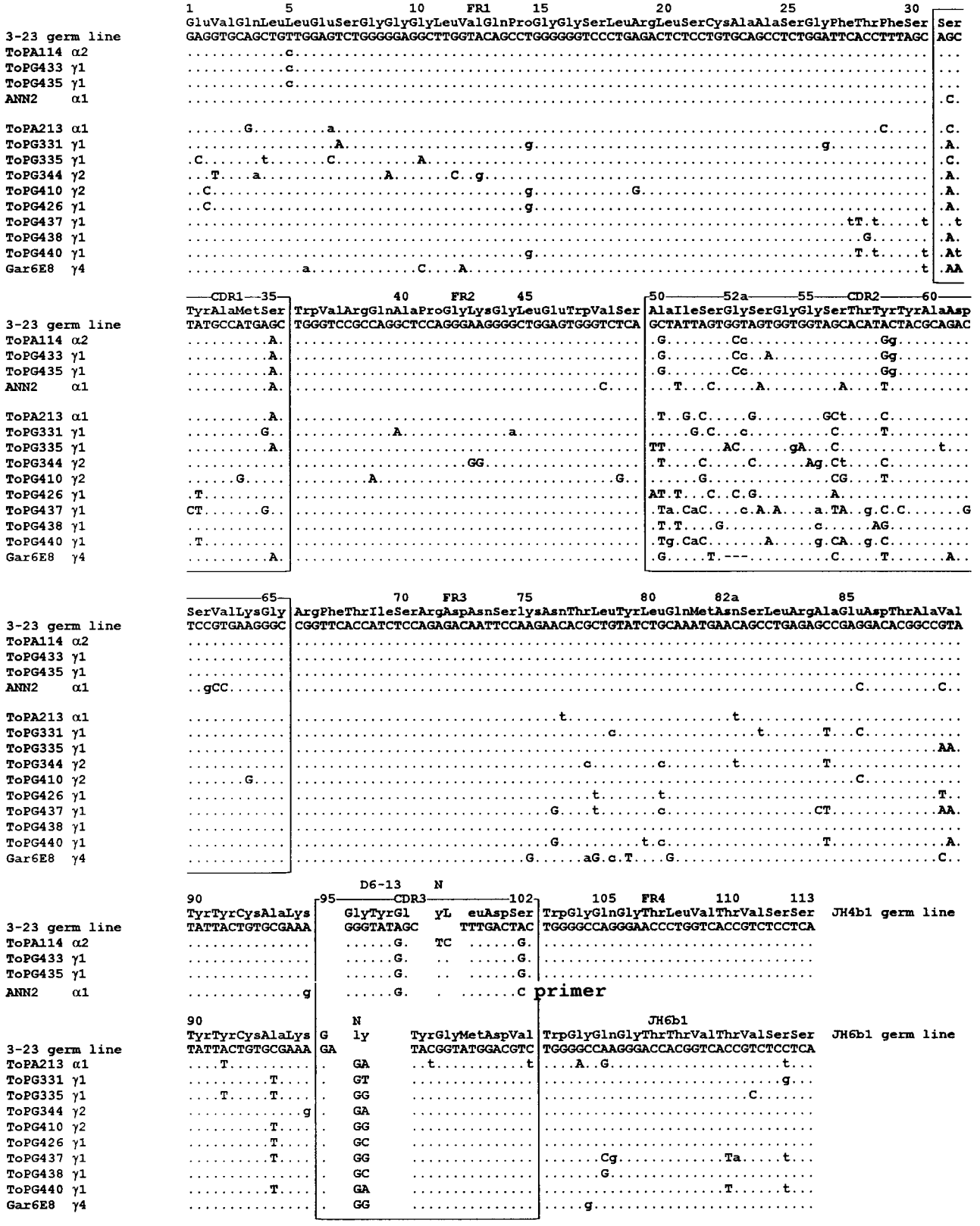

FIGURE 7. Entire variable domains (codons 1–113) of 12 cDNA clones using the two canonical rearrangements (rearrangements 3 and 4, Fig. 1). Heavy chain subclasses are given. Dots means identity with the germline sequences given above. Capital and lowercase letters indicate amino acid replacement and silent substitutions, respectively. Also presented are the sequences of two HibCP-specific heterohybridomas from the literature (ANN2 and Gar6E8) (7, 8), using the same rearrangements.

the 14 known HibCP-specific κ-bearing hybridoma cell lines (7–9). We found two compatible sequences among the 41 IgA clones (7%, corrected for over-representation of IgA2 clones); they are given in Fig. 7 compared with two hybridoma sequences from the literature. Despite the low number (1%) of IgG-secreting cells among the affinity-purified HibCP-specific cells, we also examined

the possibility that some of the AbSC using the canonical light chain could be of the IgG isotype. Seventeen IgG clones from two independent PCR amplifications (ToPG331–344 and ToPG405–440, respectively) were sequenced, and 10 (59%) of these used the canonical heavy chain (Fig. 7). It is therefore likely that a minor portion of the HibCP AbSC carried the A2 gene combined with the heavy chain sequences listed in Fig. 7. This would indicate that four isotypes, γ_1, γ_2, α_1, and α_2, and at least two VDJ rearrangements have been involved. While the three clones rearranged with J_H4 carried almost identical somatic mutations, and therefore were very likely to share a common clonal origin, clonality was less evident among the nine clones rearranged with J_H6. However, the possibility that they had a common clonal origin but had diverged considerably by somatic mutation could not be ruled out.

Like the dominant noncanonical clone (Fig. 3), somatic mutations were much more frequent among the canonical heavy chains (average homologies (codons 1–94), 97.2 and 93.6% for the J_H4 and J_H6 clones, respectively) than among the corresponding canonical light chains (average homology, 98.8%) (10). Similar differences are seen in the published canonical HibCP-specific hybridomas (7, 8). The interpretation of this difference has, however, been complicated by lack of knowledge of the germline genes of the individual from whom the hybridoma was obtained. To determine whether To had a V_H gene of higher homology to the nine canonical clones than 3–23, we amplified genomic DNA with 3–23/V_H26-specific primers, cloned the product, and sequenced 11 clones. All clones were identical with the 3–23 germline gene, which would be an unlikely finding had the individual To carried an unrecognized allele of the 3–23 gene ($p = 0.042$ for existence of another allele in a genome with four copies of the germline gene). A condition for this argument was that the unrecognized allele was identical with 3–23/V_H26 in the primer regions. This was very likely because the consensus sequences of the HibCP-specific canonical clones were identical with 3–23 germline sequence in these areas.

All the clones shared the Gly-Tyr-Gly motif in codons 95–97. In the case of the clones employing the J_H4 gene, these amino acids are encoded in the D6–13 germline segment. However, as illustrated in Fig. 1 (rearrangement 4) the codon for the initial glycine is dependent on the outcome of a 2-bp N addition. The finding that all nine clones shared a G in the crucial second position of codon 95, while all four possible nucleotides were represented in the third position, led us to suggest that the Gly in position 95 of the canonical heavy chain will prove to be essential for the function of the Ab. In fact, all seven HibCP-specific hybridomas using a canonical heavy chain have this conserved Gly residue in position 95 (7, 8).

The RS ratios in the CDR regions of the canonical heavy chains covered a very broad range (range, 1.5 to ∞; median, 3.8). A similar broad range was found in the seven hybridomas with canonical heavy chains (7, 8) (range, 0.0–8.0; median, 2.7). The canonical light chains in this material (10) and the four canonical light chains known from published hybridomas (8, 12, 13) are sparsely mutated and have a low combined RS ratio in the CDR regions (8:5 (1.6)). This shows that the presence of low RS ratios is also common in the canonical heavy and light chains.

Minor B cell clones

Of the remaining 16 clones (nine IgA and seven IgG), 11 could be grouped in three small genealogical trees (rearrangements 5, 6, and 7 in Fig. 1), and the last five clones originated from different rearrangements (2 and 8–11, Fig. 1). The entire V region sequences of the 16 remaining clones are available in the EMBL database under accession numbers Z98713 and Z98726–Z98740. The cloned cDNAs could derive from HibCP-specific B cells using λ light chains or from minor κ using B cell clones. Because the purity of the isolated HibCP-specific AbSC was not 100%, it cannot be excluded that some of these sequences derived from contaminating B cells not specific for HibCP. Some of these sequences, however, used VDJ gene combinations previously seen among HibCP-specific hybridomas, strongly suggesting their origin from HibCP AbSC.

Concluding remarks

The recruitment of pre-existing, highly selected, hypermutated HibCP-specific memory B cells after systemic HibCP conjugate immunization of adults may explain several characteristic features of the response to this and other polysaccharide vaccines. 1) HibCP vaccination in healthy adults induces substantial increases in HibCP Ab levels, usually ranging from 15–150 μg/ml after a single dose (17, 55). These levels are much higher than usual for primary responses to thymus-dependent Ags, but are similar to those obtained after good recall responses to proteins such as TT (56). The presence of a highly expanded set of memory cells is a plausible explanation for the prompt and pronounced primary Ab responses to HibCP. The use of presumed thymus-dependent protein conjugates of HibCP yields only two- or threefold higher Ab levels in adults (57), showing that the effect of the carrier protein is limited in adults. 2) HibCP Ab is predominated by switched isotypes such as IgG and IgA after the first vaccine dose, unlike protein Ags, where IgM usually predominates. This is easily explained by the accumulation of switched memory cells of which many had switched to downstream isotypes such as IgG2 and IgA2 characteristic of HibCP Abs (18). 3) HibCP Abs are limited to a low number of so-called spectrotypes consisting of Ab molecules with a narrow spectrum of isoelectric points (58, 59). This is understandable given the demonstrated vast selection of the progeny of a few precursor B cells combined with the limited mutation of the light chain and the generally low RS ratios making amino acid changes (and thereby charge changes) sparse within the individual clones.

An important remaining question is when in life these memory B cells arise. It seems likely that the origin of these cells is related to the acquirement of natural Ab to HibCP that occurs within the first years of life (60). The mucosa-associated lymphoid tissues are candidate sites for engagement of B cells producing this natural Ab, because several bacteria capable of inducing anti-HibCP Abs regularly colonize the mucosal surfaces from infancy, including Hib itself (61) and *E. coli* K100 (62).

The fact that the ability to respond effectively to systemic immunization with pure (TI-2) polysaccharides (such as HibCP) is usually delayed in ontogeny is a major obstacle for the development of effective polysaccharide vaccines against many serious infectious diseases of infancy. This problem is usually ascribed to some ill-defined age-related immaturity of the B cell compartment in infants. Based on our finding in this report of HibCP-specific hypermutated memory B cells in the not previously Hib vaccinated but naturally immune individual, we propose that the generally strong Ab responses to pure HibCP in adults are due to activation of memory B cells that, unlike unexperienced B cells of the newborn, may be activated by the polysaccharide without the need for T cell epitopes. According to this hypothesis, the infant is not waiting for the immune system to mature, but for colonization by an organism capable of inducing HibCP-specific memory B cells in a thymus-dependent way. This fits with the fact that the acquirement of natural HibCP Abs coincides roughly with the time when the ability to respond to pure HibCP appears (63). Furthermore, it

explains the puzzling observation that infants primed with an HibCP conjugate vaccine are able to respond effectively to pure HibCP at an age when infants are usually unresponsive to the pure polysaccharide (64). This observation clearly shows that unresponsiveness of infants to pure HibCP is not an absolutely age-related feature of the B cells themselves, but depends on prior thymus-dependent stimulation of the cells leading to formation of memory cells.

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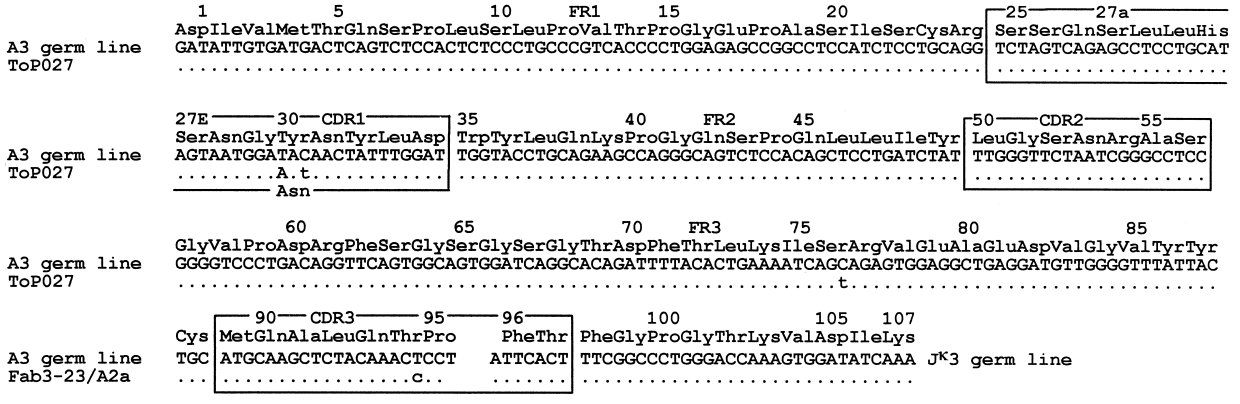
CORRECTION

Lotte Hougs, Lars Juul, Henrik J. Ditzel, Carsten Heilmann, Arne Svejgaard, and Torben Barington. The First Dose of a *Haemophilus influenzae* Type b Conjugate Vaccine Reactivates Memory B Cells: Evidence for Extensive Clonal Selection, Intraclonal Affinity Maturation, and Multiple Isotype Switches to IgA2. *The Journal of Immunology* 1999; 162:224–237.

In Figure 4, the first letter in the germ line gene name and the first letter of the clone names are missing. In addition, the Tyr and Asn amino acid mutation in codon 30 are mixed up. The correct figure is shown below.



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