Restricted Immunoglobulin VH Usage and VDJ Combinations in the Human Response to *Haemophilus influenzae* Type b Capsular Polysaccharide

Nucleotide Sequences of Monospecific Anti-*Haemophilus* Antibodies and Polyspecific Antibodies Cross-reacting with Self Antigens

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Abstract

To examine the human antibody repertoire generated against a biologically significant antigen we have obtained sequences of heavy chain variable region genes (IgVH) from 15 monoclonal antibodies specific for the capsular polysaccharide of *Haemophilus influenzae* type b (Hib PS). All VH segments are members of the VH3 family and 9 of 15 are members of the smaller VH3b subfamily. Restriction is evident by the shared use of certain VDJ joints in independent hybridomas from different subjects. Two hybridomas generated from the same subject demonstrate identical heavy chain variable region gene sequences but differ in isotype and rearrange alternative light chain variable region genes (IgVL), suggesting that in a normal immune response, a single pre-B cell clone may use different light chain rearrangements and give rise to progeny capable of reacting with antigen. Using a polymerase chain reaction assay optimized to detect base pair differences among VH genes we demonstrate that at least a portion of expressed anti-Hib PS VH genes have undergone somatic mutation. Anti-Hib PS heavy chain genes are homologous to VH segments encoding autoantibodies and two hybridomas secrete anti-Hib PS antibody that cross-reacts with self antigens (double-stranded DNA and single-stranded DNA). Comparison of VH regions of self-reactive and monospecific anti-Hib PS Ab demonstrates no consistent structural feature correlating with fine antigen specificity. These data demonstrate significant restriction in VH usage and VDJ recombination in the anti-Hib PS response and confirm that autoantibodies may be elicited during normal immune responses. (*J. Clin. Invest.* 1993. 91:2734–2743.) Key words: antibody repertoire • autoimmunity • immunoglobulin heavy chain • somatic mutation • polysaccharide

Introduction

Antigen-specific immunity depends upon the production of a vast number ($\leq 10^{10}$–10^{11}) of individual Ig species. This diversity is generated by molecular mechanisms unique to lymphocytes, including the recombination of one of a number of V, J, and, in the case of IgVH genes, D germline elements, imprecise joining of these segments, and the combinatorial assortment of heavy and light chains (1–8). Further diversity may be generated by somatic mutation of rearranged genes (9). Although these processes may theoretically yield an almost unlimited number of individual antibody specificities, evidence from murine and human systems suggests that these events may not occur randomly (10–13). For example, in early life, the expression of certain V region segments and the utilization of other diversifiers is limited. This programmed utilization of specific V region genes may result in a changing immunoglobulin repertoire during development (14–16). Understanding the molecular mechanisms relevant to the generation of specific antibody in humans will provide important information on the generation of the antibody repertoire in normal health and development, in immunodeficiency states and in autoimmune disorders.

We have used the immune response to the bacterial pathogen *Haemophilus influenzae* type b (Hib)[1] as a model to study the development of the human B cell immune repertoire (17–20). Antibody directed against the capsular polysaccharide of Hib (Hib PS) confers immunity against this important bacterial pathogen. Naturally occuring antibody directed against this and other bacterial polysaccharides, however, develops relatively late (in the second year of life) (21, 22). Another notable feature of this immune response is its pauciclonality; most individuals produce only one to four antibody clonotypes, as detected by isoelectric focusing (23). We and others have shown that although a variety of IgVL genes may encode anti-Hib PS Ab, the IgVH response appears to be restricted to a small group of VH gene segments, in combination with a more diverse group of D and JH elements (17–19, 24, 25). To more clearly understand the nature of the restriction and the regulation of this antibody response in the outbred human population, we have produced additional heterohybridomas secreting human anti-Hib PS antibody (Ab) from immunized subjects. We confirm that most human anti-Hib PS Ab appears to be encoded by a small group of VH3 genes (perhaps three or four) and that additional restraint is evident in CDR-3 regions. Furthermore, both mutated and unmutated Ig genes participate in this immune response. VH segments of these antibodies are highly homologous to segments encoding autoantibodies and

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1. *Abbreviations used in this paper:* Ab, antibody; Hib, *Haemophilus influenzae* type b; Hib PS, capsular polysaccharide of Hib.

the Ab secreted by two of these hybridomas cross-react with self antigens.

Methods

Hybridoma cell lines. Heterohybridomas secreting human monoclonal anti-Hib PS Ab were obtained as previously described (17). Volunteers were immunized with either plain Hib PS vaccine (Praxis Biologicals, Rochester, NY), Hib PS diphtheria toxoid conjugate vaccine (Hib PS-D; Connaught Laboratories, Swiftwater, PA), or Hib-oligosaccharide-CRM^{197} mutant *Corynebacterium diphtheriae* protein conjugate vaccine (Hb OC; Lederle Laboratories, Wayne, NJ). Peripheral blood lymphocytes were harvested 7 d after immunization and fused to the nonsecreting mouse myeloma cell line SP2/OAg14. Anti-Hib PS Ab was detected by binding to Hib PS in an ELISA (26) and by antibody binding to ^{125}I-labeled antigen (27). Specificity was determined by inhibition of binding by 1.25 μg/ml of soluble Hib PS in this ELISA (26). The generation of hybridoma line 16M3C8 has been described previously (28).

Cloning and sequencing of VH genes. Preparation of total RNA and subsequent cloning of rearranged IgVH genes amplified by PCR were performed as previously described (17). PCR products were isolated from low melting point agarose gel (FMC Bio Products, Rockland, ME). Amplification products were either directly cloned into pCR1000 phagemid vectors (Invitrogen, San Diego, CA), or using artificial restriction enzyme sites incorporated in PCR products, directionally cloned into Bluescript II KS+ phagemid (Stratagene, La Jolla, CA) or M13 phage vectors (29).

Single or double-stranded sequencing of cloned inserts was done by the dideoxy technique (30). Two to seven independent clones were sequenced for all VH genes with the exception of SB1/D8 and SD15, for which single clones were obtained.

The 16M3C8 VH gene was obtained by PCR amplification of total RNA reverse transcribed with antisense Cγ primer (5′GATGGGCCCTTGGTGGA 3′) and amplified with the sense VH primer 5′ ATAAGCTTCAGGTGCAGCTGCAGGAGTCTG 3′ and antisense JH primer 5′ ATAGGATCCATGAGGAGACGGTGACCAGGGT 3′. The PCR product was cloned as described above.

Most closely homologous VH and D segments were identified by comparison of these sequences with those contained in the Genbank data base. The sequences of LSF2, RAY4, SB5/D6, SB1/D8, and ED8.4 have been previously reported (17).

Amplification of germline and rearranged VH3b gene segments. To test whether nucleotide differences between the candidate VH3b germline genes LSG1.1 and LSG6.1 and rearranged VH3b segments occurred as a result of somatic mutation or if rearranged genes are encoded by as yet unidentified germline VH3b genes, a PCR-based assay was developed based on the method described by van Es et al. (31). Anti-Hib PS Ab VH segments from two unrelated subjects (LS and RC) contain novel base substitutions in FR3 compared to other rearranged genes and identified germline VH3 genes. To determine if these substitutions occur as a result of somatic mutation, PCR was used to amplify genomic DNA and rearranged anti-Hib PS Ab VH3b gene segments obtained from the two subjects. Two alternative antisense primers were used, corresponding to codons 75–81 of the previously cloned germline VH3b gene LSG6.1 FR3 sequence (5′TTGCAGATACAGCGTGTTTTT 3′)[2] or the same region of the rearranged LSF2 and RC3 VH segments (5′ TTGCAGATACACCGTGCTCTG 3′). These antisense primers, therefore, differ at 4 of 21 nucleotide positions. For both genomic DNA and rearranged genes a sense primer corresponding to codons −4 to 3 of FR-1 (5′ GTGTCCAGTGTGAGGTGCAGC 3′) was used. 200 ng of genomic DNA or plasmid containing the rearranged VH3b segments LSF2 and RC3 was amplified in a 100-μl reaction containing 70 pmol of the FR-1 sense primer and 70 pmol of antisense "germline" FR-3 primer or "rearranged" FR-3 primer. Reaction mixtures contained 50 mM KCl, 10 mM Tris HCl pH8.3, 1.5 mM $MgCl_2$, and 0.2 mM deoxynucleotides. Cycles consisted of 1 min denaturation at 92°C, 1.5 min annealing at 65°C and 2 min extension at 72° for 30 cycles.

PCR products were identified by ethidium bromide staining after electrophoresis in a 0.8% agarose gel, then transferred to a nylon membrane (Duralon UV; Stratagene, Inc.). Amplification products were hybridized with a 250-bp EcoR1 segment from clone ED8.4, which contains a VH3 segment and lacks the associated D and JH segments. Probe was labeled to high specificity using T7 DNA polymerase (32). Membrane was prehybridized and hybridized at 42°C in 2× Pipes buffer, 50% formamide, 1% SDS, and 150 μg/ml sheared and denatured salmon sperm DNA. After hybridization, the blot was washed with 2× SSC/0.1% SDS for 15 min once at room temperature and with 0.2 × SSC/0.1% SDS for 15 min twice at 60°C and exposed briefly to Hyperfilm-MP (Amersham Corp., Arlington Heights, IL).

Immunoassays. Binding of monoclonal Abs to Hib PS and self antigens was measured by ELISA. A panel of self antigens consisted of single-stranded DNA and double-stranded DNA (Calbiochem-Behring Corp., La Jolla, CA), myosin, keratin, actin, tropomyosin, vimentin, laminin, elastin, and hyaluronic acid (Sigma Immunochemicals, St. Louis, MO), and aggregated IgG (supplied by Dr. A. Shikman, University of Oklahoma Health Sciences Center). Hib PS–poly-L-lysine, 10 μg/ml in PBS pH 7.4, or other test antigens, 10 μg/ml in carbonate-bicarbonate buffer, pH 9.6, were coated directly on to microtiter plates (Immunlon 4; Dynatech, Chantilly, VA). Plates were blocked with dilution buffer (1% BSA, 0.05% Tween 20 in PBS) by incubation for 1 h at 37°C. Between steps, plates were washed three times with 0.05% Tween 20/0.9 M NaCl. Monoclonal anti-Hib PS Ab (2–5 μg/ml) was added and incubated at 4° overnight. Bound Ab was detected with alkaline phosphatase–conjugated goat anti-human Ig (Sigma Immunochemicals). For competitive inhibition assays, anti-Hib PS Ab was preincubated overnight at 4°C with 10 μg/ml of soluble Hib PS or 100 μg/ml of dsDNA (Sigma Immunochemicals) or ssDNA (Sigma Immunochemicals) and assayed by ELISA as described above.

Results

Anti-Hib PS hybridoma cell lines. 15 anti-Hib PS secreting hybridomas obtained from 10 individuals were analyzed (Table I). The heavy chain isotypic distribution of studied hybridomas was similar to the total group of hybridomas and to the serum immune response at 7 d after immunization (39, 40, and Shackelford, P. G., manuscript in preparation). Eight hybridomas secreted kappa light chains and seven secreted lambda light chains.

IgVH nucleic acid sequences. Overall, the nucleic acid sequences of the VH segments used by the 15 hybridomas are 71.0–100.0% homologous to one another (Fig. 1). We have previously found, under similar amplification conditions, an error rate for misincorporation of nucleotides by Taq polymerase of < 1/4,000 bp sequenced (41). Thus, the majority of nucleotide differences between these anti-Hib PS IgVH segments are authentic. All hybridomas use members of the VH3 family (42), and 9 of 15 (SB5/D6, RAY4, CB20, JB32, JB21, RC3, LSF2, SB1/D8 and ED6.1) are members of the smaller VH3b subfamily (Table II) (43). The VH3b-encoded IgVH genes are highly homologous to the previously described germline gene 9.1 (5) and to two germline genes, LSG6.1 and LSG12.1, obtained from subject LS[2]. SB5/D6, RAY4, CB20, JB32, JB21, RC3, and LSF2 are 93.5–98.9% homologous to the LSG6.1 germline gene. SB1/D8 and ED6.1 are more closely homologous (93.2–93.8%) to LSG12.1. ED8.4, SD15, Ann2, Ann6, CB6, and 16M3C8 VH sequences are 85.3–97.8% homologous to the germline gene VH26 (38). The similarities between the anti-Hib PS Ab VH segments and the candidate germline genes suggest that the Ab VH segments may

2. Adderson, E. E., F. Azmi, P. Wilson, P. Shackelford, and W. Carroll. The human VH3b gene subfamily is highly polymorphic. Manuscript submitted for publication.

*Table I. Hybridoma Cell Lines**

Hybridoma	Isotype	Age	Vaccine form	Total anti-Hib PS Ab		VH	VL
				Before	After		
				μg/ml			
SB5/D6	IgA_1/λ	adult	PS-D	3.0	688	VH3 LSG6.1[2]	VλVII 4A (33)
RAY4	IgA_2/λ	adult	PS-D	1.9	28	VH3 LSG6.1	VλVII 4A
CB20	IgA_2/λ	adult	Hb OC	21.0	75	VH3 LSG6.1	VλIII III.1 (34)
JB32	IgA_1/λ	11 yr	PS	4.1	200	VH3 LSG6.1	VλVII 4A
JB21	$1gG_2/\lambda$	11 yr	PS	4.1	200	VH3 LSG6.1	VλII 2.1 (35)
RC3	IgA/κ	adult	PS-D	1.1	300	VH3 LSG6.1	VκII A2 (36)
LSF2	IgA_1/λ	adult	PS	ND‡	138	VH3 LSG6.1	VλVII 4A
SB1/D8	IgG_1/κ	adult	PS-D	3.0	688	VH3 LSG12.1[2]	NK§
ED6.1	IgG_2/κ	4 yr	PS-D	0.8	22	VH3 LSG12.1	Vκ1 clone KC-1 (37)
CB6	IgA/κ	adult	Hb OC	21.0	75	VH3 VH26 (38)	NK
ED8.4	IgM/κ	4 yr	PS-D	0.8	22	VH3 VH26	NK
SD15	IgA_2/κ	11 yr	PS-D	0.4	550	VH3 VH26	NK
Ann2	IgA_1/κ	adult	Hb OC	1.7	110	VH3 VH26	VκII A2
Ann6	IgA/κ	adult	Hb OC	1.7	110	VH3 VH26	NK
16M3C8	IgG_2/λ	adult‖	PS	ND	ND	VH3 VH26	VλII 2.1

* Columns show name of hybridoma cell line, isotype of anti-Hib PS Ab secreted, age of subject at immunization, type of vaccine (PS, plain polysaccharide; PS-D, Hib PS conjugated to diphtheria toxoid; Hb OC, Hib oligosaccharide conjugated to CRM^{197} mutant diphtheria protein), concentration of total serum anti-Hib PS Ab immediately before and 1 mo postimmunization and most closely homologous germ-line VH and Vκ/Vλ segments identified.
‡ Not done.
§ Not known.
‖ Pool of three adult donors.

derive from these genes or a small group of closely related germline elements.

The hybridomas JB21 and JB32 were obtained from the same subject and share identical VH segments and VDJ joinings. The complete homology of VH transcripts in these two hybridomas is unlikely to be a result of cross-contamination of PCR reactions, since each of these VH sequences was repeatedly isolated from these hybridomas and no additional heavy chain transcript was detected. In addition, different Ig heavy chain isotypes and different IgVL segments were used in the two hybridomas (JB21–IgG_2/VλVII, JB32–IgA_1/VλII). Hybridization of HindIII-digested genomic DNA obtained from these cell lines with a human JH probe (44) demonstrated a 7.0-kb band in JB32 DNA (data not shown). No germline or rearranged JH band could be detected in DNA obtained from cell line JB21, reflecting the marked instability of these murine-human heterohybridomas (45).

Four additional pairs of hybridomas were obtained from single subjects. The pairs SB5/D6 and SB1/D8, ED6.1 and ED8.4, and CB20 and CB6 use VH segments that are distinctly different and appear to derive from different germline genes. Ann2 and Ann6, obtained from the same subject use closely related IgVH genes and have identical VDJ joinings (see Table I).

Considerable heterogeneity exists in the CDR-3 region of anti–Hib PS IgVH genes. 10 different D segments are represented (Fig. 2). The length of these segments varies considerably from 1 to 34 bp. In general, these D segments are short and share little homology with known germline or rearranged D segments. The SB5/D6 D segment shares some segmental homology with DHQ52 and JB32/21 with D4C (6, 42, 46). In 16M3C8, "P" nucleotides may be present at the VH-D junction and in RAY4 at the D-J junction. Five different JH segments are used (42). Although a variety of D and JH segments are used, certain D and JH combinations appear to be preferentially combined. Remarkably, in four instances, identical VDJ joints are shared by two or more hybridomas. ED8.4, CB6, and SD15, obtained from unrelated subjects, display identical VDJ joints with different VH segments, as do LSF2 and RC3. Ann2 and Ann6 share identical VDJ joints and use distinct but closely homologous VH gene segments and JB32 and JB21, from the same subject, share identical IgVH segments, as noted above.

Overall, amino acid translation of the nucleic acid sequences of these VH genes shows that ~ 60% of differences in nucleic acid sequence from the putative germline genes result in amino acid replacements (Fig. 3 and Table II). When compared to candidate germline genes LSG6.1, LSG12.1, and VH26 sequences, the replacement/silent ratios of anti–Hib PS hybridoma genes are generally low, but exceed 3:1 in the CDR regions of ED6.1, SB1/D8, Ann2, and 16M3C8 and in FR regions of ED8.4. Although we cannot be confident that these rearranged genes are compared to the appropriate germline sequence, these replacement/silent ratios suggest that some of these VH segments may be mutated. Amino acid homology to candidate germline genes parallels that of nucleic acid homology in most instances. The amino acid homology in CDR regions of SD15 and 16M3C8 to VH26; however, are 73 and 41%, respectively, compared to nucleic acid homology of 85% and 64%. Again, this may reflect selection as a result of somatic mutation or, alternatively, these genes may derive from other germline elements.

Analysis of somatic mutation of rearranged VH3b genes. PCR products of the expected size of 245 bp were observed only in reactions in which genomic DNA was amplified using the "germline" FR-3 primer, and in which the rearranged anti–

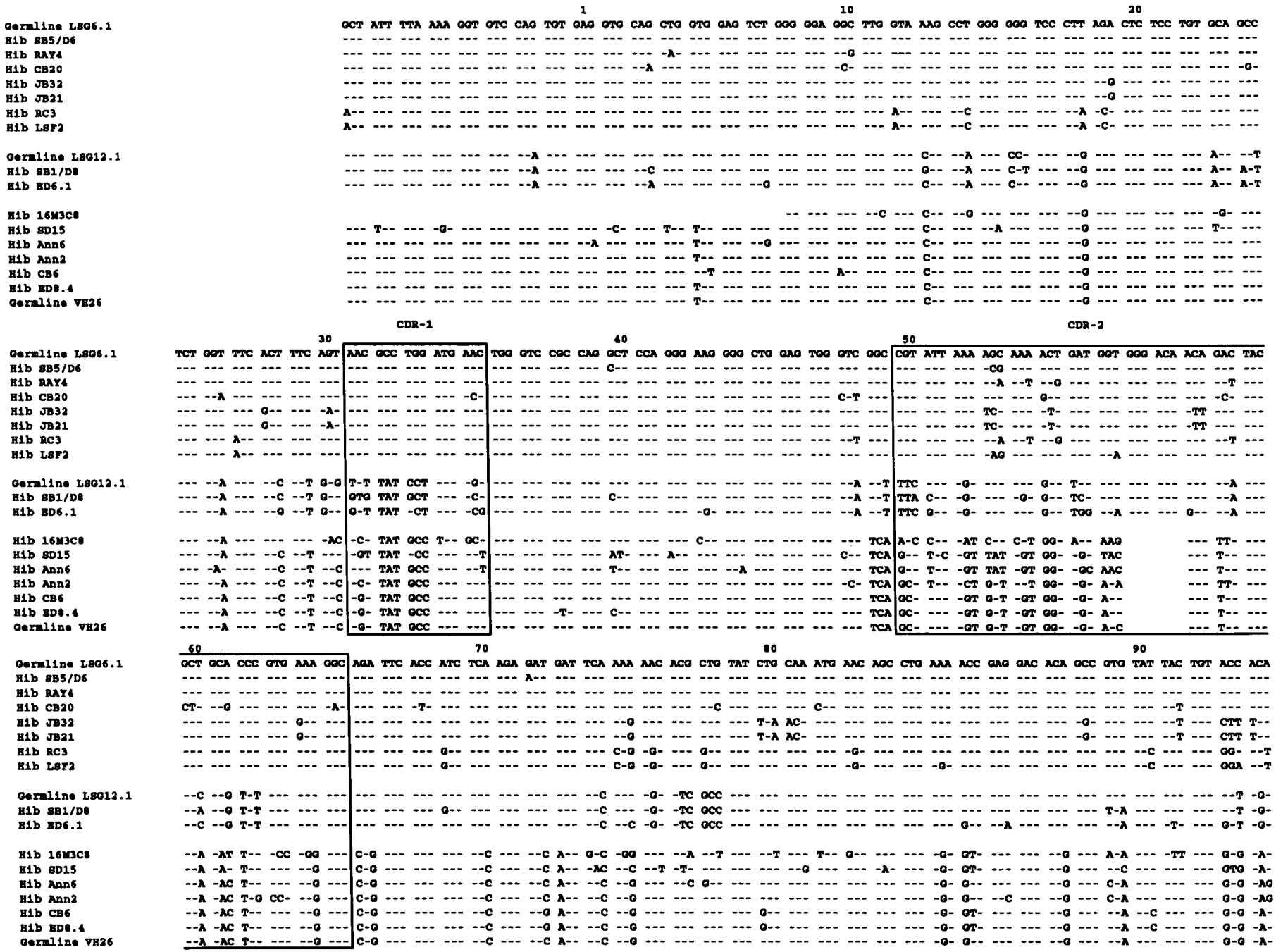

Figure 1. Nucleotide sequences of 15 anti-Hib PS VH segments. Shown for comparison are the nucleotide sequences of the germline genes LSG6.1, LSG12.1, and VH26 (reference 38, and manuscript submitted for publication). These sequence data are available from EMBL/Genbank under accession numbers X56523 (SB5/D6), X56524 (RAY4), L14818 (CB20), M86597 (JB32), M86598 (JB21), M99397 (RC3), X56525 (LSF2), X56527 (SB1/D8), L14819 (ED6.1), M86597 (16M3C8), M86601 (SD15), L14822 (Ann2), L14820 (Ann6), L14821 (CB6), and X56526 (ED8.4).

*Table II. Comparative Homology of Anti-Hib PS Antibody VH Segments**

	Nucleic acid homology					Amino acid homology		
	Overall (%)	CDR (%)	(R/S)	FR (%)	(R/S)	Overall (%)	CDR (%)	FR (%)
Consensus LSG6.1								
Hib SB5/D6	98.8	97.2	(2:0)	99.2	(2:0)	97.2	95.8	97.6
Hib RAY498	98.1	94.4	(2:2)	99.2	(1:1)	97.2	91.7	99.0
Hib CB20	94.8	90.3	(6:1)	96.0	(5:5)	90.7	79.2	94.0
Hib JB32	93.8	91.6	(3:3)	94.4	(8:6)	91.7	87.5	92.9
Hib JB21	93.8	91.6	(3:3)	94.4	(8:6)	91.7	87.5	92.9
Hib RC3	93.5	94.4	(2:2)	92.9	(11:7)	88.9	91.7	88.1
Hib LSF2	93.5	95.8	(2:1)	92.9	(12:6)	88.9	95.8	86.9
Consensus LSG12.1								
Hib SB1/D8	93.8	86.1	(8:2)	96.0	(7:3)	87.0	70.8	91.7
Hib ED6.1	93.2	87.5	(7:2)	94.8	(7:5)	88.0	75.0	91.7
Consensus VH26								
Hib ED8.4	97.8	98.5	(0:1)	97.6	(5:1)	95.3	100.0	94.0
Hib CB6	97.8	98.5	(0:1)	97.6	(4:2)	95.3	100.0	94.0
Hib Ann2	95.9	86.4	(8:1)	98.4	(3:1)	90.6	68.2	96.4
Hib Ann6	94.3	87.9	(6:2)	96.0	(4:6)	91.5	77.3	95.2
Hib SD15	90.3	83.3	(8:3)	92.1	(14:6)	83.0	72.7	85.7
Hib 16M3C8	85.3	65.7	(18:4)	91.5	(11:7)	73.3	40.9	83.8

* Columns show names of anti-Hib PS antibody and germline genes these segments are compared to, overall percentage of nucleic acid homology, percent homology within CDR and framework regions and number of nucleic acid differences from the germline gene resulting in nucleic acid replacement (R) or silent (S) changes, and percentage amino acid homology of total VH segment, CDR, and framework regions.

Hib PS VH segments were amplified using the "rearranged" FR-3 primer (Fig. 4). Southern blot analysis using a probe specific for VH3 gene family members detected only those bands visible by ethidium bromide staining. The failure to amplify genomic DNA with the "rearranged" FR-3 antisense primer suggests the germline repertoire of the two subjects studied does not include a gene containing all of the nucleotide differences seen in FR-3 of the rearranged VH segments. At least a portion of these nucleotide differences from the candidate germline genes appear, therefore, to result from somatic mutation.

Immunoassays. Because of the high homology between IgVH segments encoding anti-Hib PS Ab and those encoding autoantibodies, six monoclonal anti-Hib PS Ab were tested for reactivity with a panel of autoantigens. Antibodies Ann2 and CB20 were reactive with both ssDNA and dsDNA (Fig. 5) and synthetic polynucleotides (dATP, dUTP, and dITP; data not shown). The reactivity of Ann2 and CB20 with DNA was low compared to pooled sera from patients with SLE. In a competitive inhibition assay, soluble Hib PS strongly inhibited DNA binding by these Ab. However, binding to Hib PS could not be inhibited by 100 μg/ml of dsDNA, likely reflecting higher avidity for Hib PS. This is in agreement with the lower direct binding of these antibodies to DNA versus native Hib PS (Figs. 5, *A* and *B*). In situ binding to DNA could not be demonstrated by indirect immunofluorescence staining of acetone-fixed rat heart cells (CRL-1446; American Type Culture Collection, Rockville, MD) (47, 48) or *Crithidia,* likely caused by the comparatively lower sensitivity of these assays, compared to the ELISA assay (data not shown).

There was no reactivity of our panel of anti-Hib PS Ab with other autoantigens (myosin, keratin, actin, tropomyosin, vimentin, laminin, elastin, or aggregated IgG).

Discussion

Our laboratory and others have studied the immune response to Hib PS as a model of the development of the human antibody repertoire. Our initial analysis of VH genes in five anti-Hib PS Ab indicated that a small group of VH3 genes appeared to encode this immune response (17). However, in the outbred human population few definitive conclusions can be reached with such a small sample size. We, therefore, have isolated an additional 10 IgVH genes, extending our analysis to 15 total IgVH sequences. The additional genes are also members of the VH3 gene family and appear to be related to three germline genes previously cloned in our laboratory[2] or others (36). Seven of the anti-Hib PS Ab VH segments are highly homologous to the VH3b germline gene LSG6.1, two to the VH3b gene LSG12.1, and six to a VH3a germline gene, VH26 (38). The VH3b anti-Hib PS Ab VH segments are also closely homologous to the previously described germline gene 9.1 (5). Neither IgVH usage nor homology to putative germline genes appears to correlate with subject's age, vaccine formulation, heavy chain isotype, or IgVL usage.

All anti-Hib-PS heavy chains characterized by protein or nucleic acid sequence to date are encoded by members of the VH3 family, although a minority of subjects studied by serologic techniques also appear to express small amounts of antibody encoded by VH1 and VH4 gene family members (17, 24, 49). It is estimated the human VH locus contains 50–300 members (5). The VH3 family is relatively large (25–200 members) and heterogeneous (5, 50, 51). The exclusive usage of only a small number of related VH3 gene elements in the anti-Hib PS hybridomas reported here suggests that the heavy chains encoded by these genes are important for Hib PS binding.

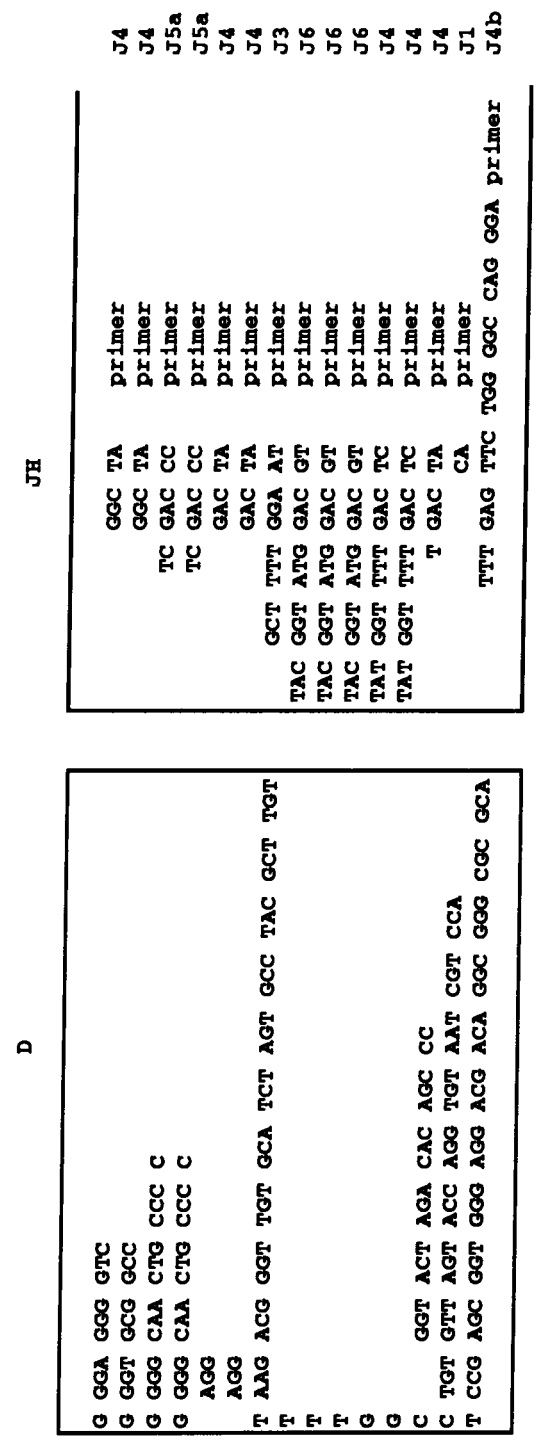

Figure 2. Nucleotide sequences of 15 anti-Hib PS IgVH CDR-3 regions. Also shown are the two base pairs of the VH segments of germline genes LSG6.1 and VH26 that are proximal to the heptamer recombination sequence.

Two pairs of hybridomas were obtained from each of five subjects and analyses of these pairs are particularly interesting, since the antibody response to Hib originates from a few B cell clones (23). Three pairs of hybridomas (SB5/D6 and SB1/D8, ED6.1 and ED8.4, as well as CB6 and CB20) use distinct VH genes and VDJ joints. Thus, each hybridoma originates from a distinct B cell clone. The hybridomas JB32 and JB21 share identical IgVH gene segments but differ in heavy chain isotype and light chain usage. Since the number of VH and D segments potentially available for combinatorial assortment is great, a "shared" VDJ segment would be expected to occur infrequently. This might occur, however, if a single pre-B cell were to give rise to distinct progeny with specific antigen binding potential. The use of shared VDJ segments and distinct IgVL rearrangements has previously been described in a hybridoma specific for antiinfluenza virus hemagglutinin Ab (52). The identification of two progeny from a single B cell clone implies that clonal precursors capable of Hib PS binding may be relatively rare in the human immune repertoire. An alternative explanation is that IgVH usage and VDJ association may be so restricted that expression of identical IgVH genes in different anti-Hib PS B cell clones may be much more common than expected. Our unexpected finding of identical VDJ joints in IgVH genes obtained from unrelated subjects supports this hypothesis. We were unable to definitively determine which of these two possibilities is correct, since Southern blot analysis of IgVH genes of these hybridomas revealed a rearrangement in JB32 only. The lack of a detectable rearrangement in JB21 reflects the instability of these heterohybridomas. Both of these theories are consistent with the previous observation that individuals display only one to three clonotypes of anti-Hib PS Ab by isoelectric focusing and that these Ig patterns may be shared among individuals (23).

The hybridomas Ann2 and Ann6 share identical VDJ joinings and, although closely related, multiple replacement and silent base differences are noted in the VH segments. Both hybridomas secrete kappa light chains, and it is possible that these hybridomas, like JB21 and JB32, may be progeny of a single B cell. If encoded by a single germline gene, the differences in the Ann2 and Ann6 VH segments would likely be the result of somatic mutation. Using a PCR-based assay to detect base pair differences, we demonstrate that some anti-Hib PS IgVH segments (LSF2 and RC3) are mutated. However, somatic mutation appears to be limited in these anti-Hib PS genes and may occur in only a portion of the immune response. The presence of mutation does not appear to correlate with antigen formulation (subject LS received plain polysaccharide vaccine and subject RC, polysaccharide-protein conjugate vaccine). The absence of basepair differences in VH segments of clonal progeny JB21 and JB32 suggests that, in contrast to LSF2 and RC3, some anti-Hib PS IgVH genes are unmutated. Previous analysis of light chain variable region usage also demonstrated that a portion of these antibodies was encoded by unmutated versions of germline genes (18, 24, 36). Both the paucity or absence of somatic mutation and the extreme restriction of anti-Hib PS VH usage may explain the poor response to Hib PS immunization and the increased incidence of invasive Hib disease in certain individuals and ethnic populations (53–55). That is, such individuals and groups may lack one or more important VH segments on the basis of genetic polymorphism and may be unable to be improve upon a less optimal germline gene by antigen-driven somatic mutation.

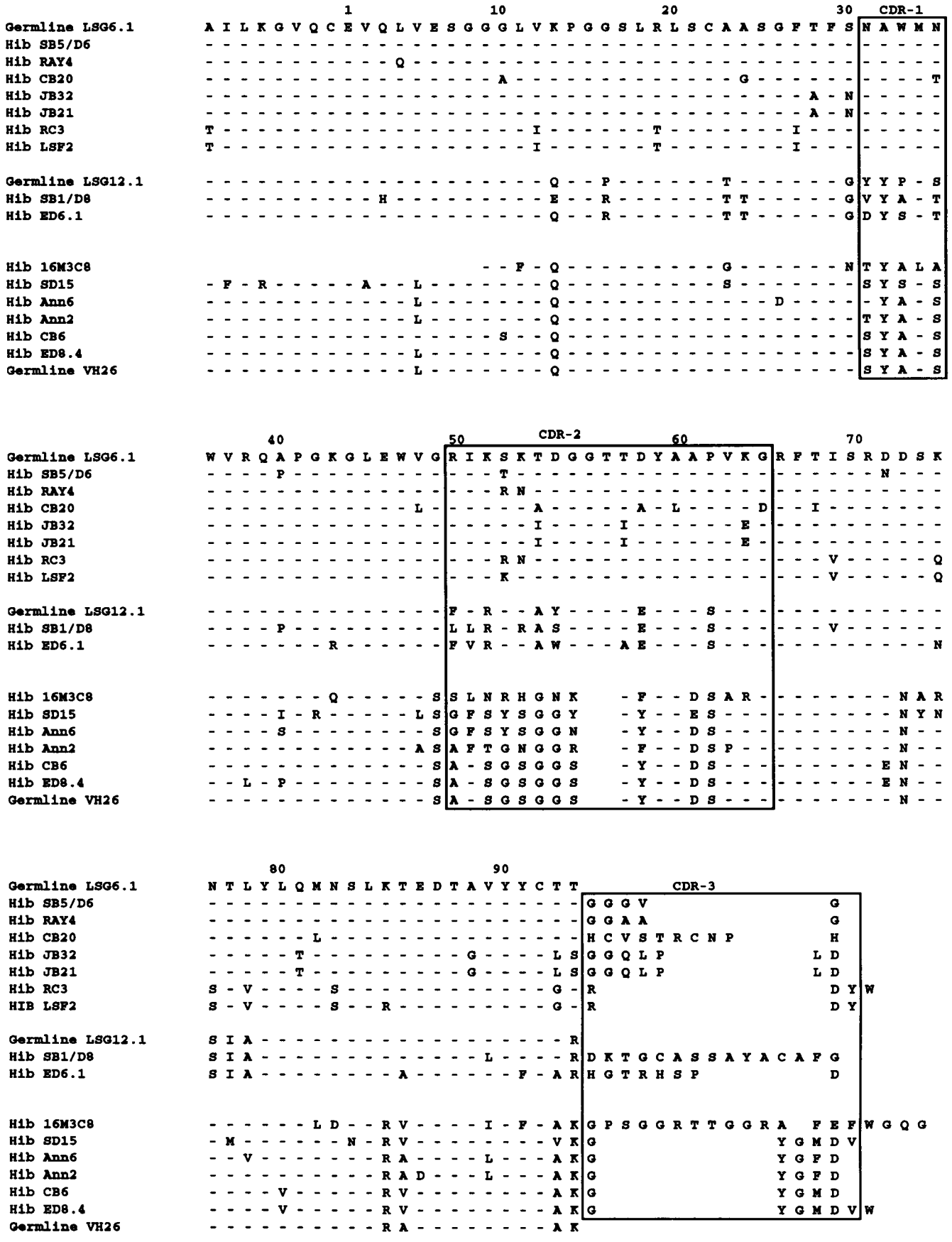

Figure 3. Translated amino acid sequences of 15 anti-Hib PS IgVH segments (see Figs. 1 and 3). Shown for comparison are the translated amino acid sequences of the germline genes LSG6.1, LSG12.1, and VH26.

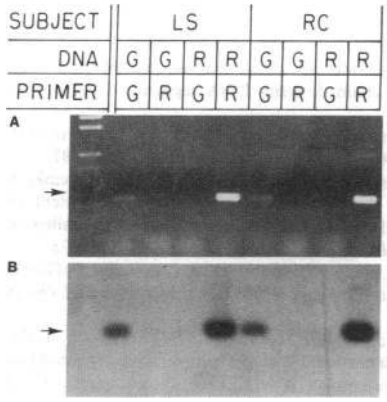

Figure 4. PCR analysis of somatic mutations in the rearranged anti-Hib PS IgVH genes LSF2 and RC3. Lanes show either genomic DNA (*G*) obtained from subjects LS and RC, or plasmid containing the rearranged anti-Hib PS IgVH gene (*R*) obtained from each subject amplified by primers corresponding to candidate germline genes (*G*) or rearranged gene segments (*R*). (*A*) an ethidium bromide stained agarose gel showing amplified products. (*B*) Southern blot of specific amplified products hybridized with a ^{32}P-labelled VH3 probe. Arrows indicate amplification products of expected 245 bp.

A striking feature of the anti-Hib PS IgVH genes is the finding of shared VDJ joints among unrelated subjects. The disparity of length of D segments implies that a variety of structural configurations of CDR-3 may permit antigen binding. However, about half of the D segments are extremely short (one to three bases) and the remainder of moderate length. It is conceivable, therefore, that CDR-3 length may identify Ab recognizing different epitopes ("groove" vs "cavity" type) (56). The shared VDJ rearrangements noted in this study may also occur as a result of selection at the DNA level. That is, junctional diversity might be limited in B cells responsive the Hib PS. A prominent feature of the murine fetal Ab repertoire is the paucity of "N" addition (57), the use of relatively short D segments compared to adult animals and the use of a restricted number of VH and JH segments (58). Acquisition of N addition also procedes more slowly in the murine Ly1$^+$ B cell subset, as compared to conventional B cells (59). Thus, the conserved pattern of VDJ rearrangement seen in anti-Hib PS IgVH genes might reflect rearrangement of precursors early in ontogeny or derivation from a discrete B cell subpopulation. Further characterization of both Hib PS epitopes and B cell subpopulations responding to Hib PS will be required to establish the molecular basis for restricted VDJ usage.

We show here that the anti-Hib PS Abs CB20 and Ann2 bind dsDNA, ssDNA and polynucleotides. It has been hypothesized that the basis for cross-reactive immune recognition of bacterial polysaccharides and DNA is the structurally similar repetitive phosphodiester linkage common to both macromolecules (56). Analysis of IgVH segments of these anti-Hib PS Ab suggests a molecular basis for anti-DNA cross-reactivity, although the precise features distinguishing polyreactive from monospecific Ab are not obvious. The VH segments described here are highly homologous to gene segments encoding autoantibodies. An anti-Sm Ab has a nucleic acid sequence identical to the 9.1 germline sequence and rheumatoid factor and anti-DNA Abs (16/6 Id$^+$) are encoded by gene segments identical to VH26 (38, 42), germline genes closely homologous to CB20 and Ann2, respectively. However, several nonautoreactive anti-Hib PS VH genes are more homologous to the 9.1 and VH26 genes than CB20 and Ann2. Although CDR-3 has been implicated in determining autoreactive potential (60), CB20 and Ann2 VDJ joints are similar or identical to non-cross-reactive anti-Hib PS clones. The presence of Arg in CDR-3, common in murine anti-DNA Ab (61) is inconsistent in these anti-Hib PS Ab, although nearly invarient in IgVL segments (18, 19, 25, 36). Analysis of anti-DNA antibodies from MRL/lpr mice indicates that the heavy chain provides essential determinants for antigen binding but that the various associated light chains modulate binding activity (62). The CB20 IgVL gene is encoded by a VλIII gene family member that appears to be infrequently expressed in this immune response and the Ann2 Vκ segment differs from other described VκII A2-encoded anti-Hib PS Ab in that its nucleic acid sequence differs at a number of positions from that of the germline A2 gene (19). Thus, it is possible that combinatorial assortment or light chain usage may play a role in mediating fine specificity of these antibodies. Finally, antigen-driven somatic mutation has been shown to result in the acquisition of cross-reactivity and self-reactivity (63, 64). The anti-Hib PS Ab described in this study appear to be mutated but no single amino acid replacement distinguishes cross-reactive from monospecific antibody. Further characterization of bacterial and self epitopes, x-ray crystallographic studies of antibody-antigen complexes and the construction of chimeric antibodies combining portions of anti-Hib PS and autoantibodies may clarify which of the subtle differences in these antibodies is responsible for fine specificity.

Considerable data associates the immune response to bacterial polysaccharides with the generation of anti-DNA Ab. The autoimmune disease of NZB/W mice is diminished in a germ-free environment (65) and mice homozygous for the xid mutation demonstrate a marked reduction in both anti-DNA and in antipolysaccharide Ab (66). In humans, autoreactive antibodies are elicited by infection or by LPS-stimulation of normal B cells (67) and in response to infection and thus may be a feature of many normal immune responses. For example, the immune response against both *Streptococcus pneumoniae* and *Klebsiella* species is marked by the expression of anti-DNA associated idiotypes (68, 69). Hib is a ubiquitous pathogen, and most individuals have acquired protective levels of anti-Hib PS Ab by 5 yr of age (22). The vast majority of individuals exposed to Hib PS do not develop autoimmune disease, sug-

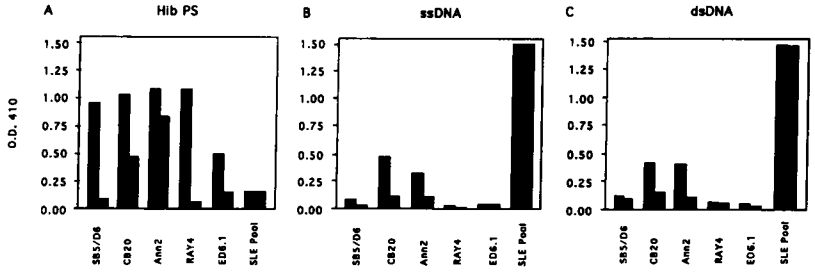

Figure 5. Fine antigen specificity of five monoclonal anti-Hib PS Ab. Graphs show reactivity of anti-Hib PS Ab to (*A*) Hib PS, (*B*) single-stranded DNA, and (*C*) double-stranded DNA in the presence (*stippled bars*) or absence (*solid bars*) of preincubation with 10 μg/ml of soluble Hib PS. Also shown is the reactivity of pooled sera from patients with SLE.

gesting that proliferation and expression of anti-Hib PS Ab reactive with self antigens may be limited. That is, by the process of generating hybridomas, we may have captured a B cell that, although present at 7 d after immunization, is not expanded and may not contribute to the normal serum anti-Hib PS Ab repertoire. However, under certain conditions the antigen-driven expansion of autoantibody-producing B cells may be stimulated or perpetuated by exposure to microorganisms. It has been hypothesized that disordered regulation of variable gene usage by B cells, such as might result from the deletion of pivotal genes in the anti-Hib PS repertoire, may lead to aberrant expression of autoreactive clones in response to Hib PS or other external antigens. Olee and co-workers have shown that, in humans, deletion of the Humhv 3005 VH segment is noted significantly more frequently in patients with systemic lupus erythematosus and rheumatoid arthritis than in normal controls (70). An alternative explanation for the infrequent occurrence of autoimmune disease in individuals exposed to bacterial polysaccharides is that "polyreactive" antibodies with low affinity for DNA may differ from pathologic autoantibodies. The affinity of CB20 and Ann2 for DNA appears to be significantly lower than that for Hib PS. "Naturally occurring" autoantibodies, in general, are of the IgM isotype, are polyreactive and relatively low affinity, whereas autoantibodies implicated in the pathogenesis of autoimmune disorders are often IgG, high affinity, and monospecific (71). Finally, somatic mutation might result in the acquisition of low affinity polyreactivity or pathologic autoreactivity, but occur at a very low rate (64).

We have demonstrated that the IgVH sequences of human anti-Hib PS Ab appear to be encoded by a limited number of VH3 germline elements closely homologous to sequences expressed preferentially in the early fetal repertoire. These VH genes are very similar to those encoding autoantibodies, and two of these Abs cross-react with DNA, suggesting that a role may exist for Ab encoded by these genes in both the maturation of the normal immune repertoire and in the pathogenesis of autoimmune disease. Genetic polymorphisms involving critical IgVH segments may explain the increased susceptibility of individuals and populations to invasive Hib infection. Similarly, such polymorphisms may be associated with the induction or exacerbation of autoimmune disorders. Ongoing studies of the expression and structure of these important gene segments will provide insight into these issues.

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