

Variable Region Diversity in Human Circulating Antibodies Specific for the Capsular Polysaccharide of *Haemophilus influenzae* Type b

Preferential Usage of Two Types of VH3 Heavy Chains

Gregg J. Silverman* and Alexander H. Lucas†

*Sam and Rose Stein Institute for Research on Aging and the Department of Medicine, University of California, San Diego, La Jolla, California 92093; and †Children's Hospital Oakland Research Institute, Children's Hospital Medical Center of Northern California, Oakland, California 94609

Abstract

Antibodies against capsular polysaccharides are important in the defense against many pathogenic bacteria. To determine the mechanism for the variability in responses to polysaccharides, a panel of well characterized serologic reagents that identify diagnostic primary amino acid sequences in the framework and hypervariable regions of heavy (H) and light (L) chains were created to characterize the variable region diversity in circulating human antibodies. 10 normal adult volunteers were immunized with the type b capsular polysaccharide of *Haemophilus influenzae* (Hib PS). By immunoblot analyses each individual was found to use at least three different variable L (VL) families, but all had preferential usage of VH3-derived H chains. Four individuals had lesser populations of VH1-derived H chains and three had populations of VH4-derived H chains, but anti-Hib PS antibodies derived from the VH2, VH5, and VH6 families were not detected. The anti-Hib PS antibodies from all subjects were also identified by serologic markers for two specific types of VH3 H chains. These H chains are structurally related to the 20P1 and 30P1 VH genes that are preferentially rearranged in the early human repertoire. These findings document the VH restriction of physiologic responses to Hib PS immunization, and demonstrate a technique to directly assess the structural and genetic diversity of specific serum antibodies. (*J. Clin. Invest.* 1991. 88:911-920.) Key words: cross-reactive idiotype • vaccine • variable region • antibody • polysaccharide

Introduction

Capsular polysaccharides are known virulence factors for many pathogenic bacteria. Antibodies against these immunodominant structures protect against invasive disease, and polysaccharide (PS)¹ vaccines provide broad clinical efficacy (1).

Address correspondence to Gregg J. Silverman, M.D., Department of Medicine, 0945, University of California, San Diego, La Jolla, CA 92093-0945.

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1. Abbreviations used in this paper: BBS, borate buffered saline; CRI, cross-reactive idiotype; FR, framework; H, heavy; Hib, *Haemophilus influenzae* type b; HV, hypervariable; IEF, isoelectric focusing; L, light; PM, powdered milk; PS, polysaccharide; RF, rheumatoid factors.

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However, many individuals are unable to mount adequate responses to PS antigens and are at increased risk for these infections. To determine the mechanisms for nonresponsiveness to these antigens, the structural and genetic basis of anti-PS antibody production must be determined.

Haemophilus influenzae type b (Hib) is a leading cause of meningitis in infants, and it is also responsible for other forms of invasive disease (2). In most populations the peak incidence of Hib meningitis and bacteremia is between 6 and 15 months of age. Susceptibility correlates with a period of anergy to Hib PS and low or absent circulating levels of protective antibodies (3). Also, certain ethnic groups, such as Native Americans, are at high risk of infection with Hib, and they appear to be unable to produce adequate levels of protective antibodies (3-5).

The immune response to immunization with the Hib PS has been extensively studied. Isoelectric focusing (IEF) studies have documented the restricted heterogeneity of human antibodies induced by Hib PS, and Insel et al. have reported that most individuals have only one to three distinct antibody spectrotypes that were each presumed to be clonally derived (6, 7). A cross-reactive idiotype (CRI) has been found on anti-Hib PS antibodies, which has suggested that these antibodies share variable (V) region structural features (8, 9). Scott and co-workers recently provided the first sequence data on human antibodies to a bacterial PS. They reported partial protein sequences of V regions from circulating IgG anti-Hib PS antibodies that were purified to monoclonality. The heavy (H) chains were derived from a single, large VH family, while light (L) chains from at least four VL gene families were found in different individuals (10). Subsequently, the anti-Hib PS antibodies from four selected individuals were shown to have L chains with protein sequences that are characteristic of the germline V κ 2 gene, A2 (11).

To survey the genetic and structural V region diversity of specifically induced human circulating antibodies, we have developed a technique of serologic characterization that employs anti-peptide antibodies that are specific for sequence motifs that are characteristic of V region gene families, and subsets of families. In this report we have directly analyzed the circulating anti-Hib PS antibodies from 10 normal adult volunteers. These antibodies were precipitated with Hib PS, and the H and L chains from these precipitates were electrophoretically separated. Immunoblots were then probed with a panel of well characterized serologic reagents that identify diagnostic primary amino acid sequences in the framework (FR) and hypervariable (HV) regions of H and L chains (12-14). By this independent method, we confirmed earlier findings that induced anti-Hib PS antibodies employ L chains from several VL families. In contrast, the H chains of these antibodies were found to have restricted VH region heterogeneity. The anti-Hib PS anti-

bodies were shown to preferentially use VH3 H chains. Reagents specific for four types of VH3 H chains were also used in these analyses, but only two types of VH3 H chains were detected in the anti-Hib PS antibodies. These VH3 H chains are structurally related to VH genes that are preferentially rearranged in the early human repertoire. The implications of the restricted use of these VH regions in antibacterial antibodies is discussed.

Methods

Collection of donor sera. All volunteers were healthy Caucasian adults between 25 and 42 yr old without history of rheumatoid arthritis or other significant medical ailments, recurrent or recent infections, or hematologic abnormalities. Serum samples were obtained immediately before and after subcutaneous immunization with 40 μ g of purified Hib PS vaccine (prepared by Dr. Porter Anderson, University of Rochester, Rochester, NY). Postimmunization specimens were drawn 1–2 mo later. Subjects were selected if anti-Hib PS antibody levels showed twofold or greater increases after vaccination. Samples were stored at -20°C until used, and aliquoted to avoid frequent freeze-thaws.

Synthetic peptides. Peptides were synthesized by R. Houghten using a modified solid phase technique (15). Sequences were selected from immunodominant portions of Ig H and L chains, and were used to generate antisera (16). The peptide sequences for the first FR region that are specific for the V_{κ} (13) and VH gene families (12, 14) have previously been described. In addition, a reagent was made that identifies the first FR sequence of the VH3-related 20P1/9-1 gene, designated VH3-FR1a, that differs by a single amino acid residue from the VH3 prototype sequence, VH3-FR1 (12, 17). Antisera were also generated to portions of the second HV regions of all reported VH genes isolated from a 130-d fetal liver cDNA library. This highly restricted VH repertoire had 14 independent rearrangements that were derived from only 9 distinct VH germline genes (18). In a previous report we described anti-peptide antibodies to the homologous portion of genes from the VH4 family and VH6 family that are expressed in this fetal repertoire (14). For this study we created additional second HV-specific reagents for the two VH1 and four VH3 distinct sequences from this library. The peptides derived from the VH3 genes in this collection were designated VH3-HV2a, VH3-HV2b, VH3-HV2c, and VH3-HV2d. Also, a diagnostic portion of the third FR of one of the VH3 genes, 20P1/9-1, was also used to make an anti-peptide antibody, and was designated anti-VH3-FR3a. The L chains in anti-Hib PS antibodies have been reported to have V region sequences homologous with a $V_{\kappa}2$ gene, A2 (11). Therefore, a peptide with the sequence EVSNRFSGVDPDRGC was also made with a second HV region sequence taken from the A2 gene, and designated VK2-HV2a. Sequences of the VH3 genes and their source are compiled in Table I. The sequences for the H and L chain constant regions, taken from first domain regions include C_{μ} (SASAPTLFPLVSC), C_{α} (SPTSPKVFPVSLC), C_{γ} (STKGPSVFPLAPC), C_{κ} (VFIFPPSDEQLKSGTASVVC), and C_{λ} (VTLFPPSSEELQANKATLVLC).

Generation of anti-peptide antibodies. Synthetic peptides were conjugated at their carboxy-terminal cysteine to the carrier, keyhole limpet hemocyanin, by using m-maleimido-benzoyl-N-hydroxysuccinimide ester (19). For the generation of anti-peptide sera, 4–6 wk-old female New Zealand white rabbits were immunized at 2-wk intervals with the peptide conjugate. The initial injections contained 0.5 mg of peptide equivalent in an emulsion with Freund's complete adjuvant, and the subsequent injections used 0.2 mg of peptide equivalent in incomplete adjuvant. Rabbits were bled 1 wk after the third immunization, and assayed for reactivity with peptides, as previously described (13). Sera were stored at -20°C until used.

Immunoblot reactivity of anti-peptide antibodies. To demonstrate the binding specificity of the peptide-induced reagents, Western immunoblot analyses were performed with Ig proteins of known and unknown primary amino acid sequence. Briefly, the H and L chains of Igs

were electrophoretically separated in 10% polyacrylamide gels containing 0.1% SDS, after boiling for 2 min in reducing buffer with 4% 2-mercaptoethanol. Before electrotransfer, immobilon P membrane (Millipore Corp., Bedford, MA) was wet in methanol, then equilibrated with transfer buffer. After transfer, nonspecific binding sites were quenched by incubation with borate buffered saline, pH 8.2 (BBS) containing 1% powdered milk (PM). The blotted membranes were then reacted for exactly 1 h with an anti-peptide serum diluted 1:200 with 1% PM-BBS, on a rocking platform. After washing with Tween 20 0.05%-BBS (vol/vol), membranes were incubated with ^{125}I -protein A in 1% PM-BBS (sp act 0.1 $\mu\text{Ci/ml}$) (ICN Radiochemicals Inc., Irvine, CA). After washing, the membranes were dried and exposed to Kodak XAR-5 film at -70°C for 4 and 16 h. To document that replicate blots had equivalent Ig, after use they were reprobbed by first rewetting with methanol, then equilibrated in 0.2 M glycine-HCl, pH 2.5. Stripping was aided by two cycles of 45-min incubation in an Autoblot apparatus (Bellco Biotechnology, Vineland, NJ) at 70°C . Blots were then rinsed with distilled water, and neutralized with 1% PM-BBS. To document stripping blots were next incubated with ^{125}I -protein A, and 24-h autoradiograms exposed. After final probing, blots were retested with antisera to constant region sequences to document that membranes had retained Ig protein. Immunoblot reactivity of anti-peptides directed at κ , λ , μ , α , γ immunoglobulin chain constant regions, and V region first FR region directed, VH and V_{κ} family specific reagents have previously been described (12, 13, 20, 21).

Immunoprecipitation of anti-Hib PS antibodies. A modification of the technique first described by Heidelberger was used to purify precipitating antibodies against the Hib PS (22). Serum samples from normal donors were first filtered through glass wool, placed in 1.5-ml tubes, and then spun for 40 min at 16K relative centrifugal force (RCF) at 4°C . Samples were then placed in clean tubes, and centrifugation repeated. Next, 1 ml of each plasma sample was placed in a clean tube and 2 μ g of purified Hib PS was added, with incubation on a rotator at 37°C for 1 h, then overnight at 4°C . Samples were then centrifuged for 10 min at 4°C at 5K RCF. Supernatants were removed, then pellets were washed once in 0.2% BSA in PBS, pH 7.2 with 0.05% Tween 20, and respun. This procedure was repeated four additional times with PBS-Tween 20 0.05%. Afterwards pellets were resuspended in 2% SDS in 8 M urea. Laemmli reducing buffer was then added, before heating and electrophoretic separation of Ig chains in SDS-polyacrylamide gels. Antibodies from 1 ml of clarified serum were used for two replicate immunoblots. All studies were performed at least three times to ensure reproducibility.

Anti-Hib polysaccharide assay. Total antibody to Hib PS was measured using a radioactive ^{125}I -labeled antigen, as previously described (3). Antibody concentrations were determined from a standard reference curve using dilutions of the U. S. Office of Biologics serum reference pool. Before and after precipitation of antibodies for immunoblot analyses, serum samples were assayed for anti-Hib PS antibody content.

Results

Characterization of anti-peptide antibodies. After three immunizations with peptide conjugates, all rabbits produced high titer antisera ($> 1:10,000$) when tested by direct binding ELISA (data not shown). Immunoblot reactivity of antisera was next tested against Ig of known and unknown sequence.

We have previously demonstrated that the anti-VH3-FR1 reagent identifies only VH3-derived proteins. VH3 H chains are not identified by the reagents derived from other V gene families (12, 14). In Fig. 1, the reactivity of the anti-VH3-FR1 reagent is compared to anti-peptide antibodies against different VH3 sequences. Included in the replicate immunoblots are polyclonal IgM and IgG, five monoclonal VH3 IgM proteins of known sequence (18/2, KIM4.6, 4B4, RIV and LAY) (23–27), three monoclonal IgM proteins of unknown sequence (HER,

Table I. VH3-Related Protein Sequences

First framework region				
Peptide name	Sequence		Immunoblot reactivity	
VH3-FR1	GGLVQPGGSLRLSC		Anti-VH3-FR1	Anti-VH3-FR1a
VH3-FR1a	----K-----		+	+
H Chains			VH3-FR1a peptide	VH3-FR1 peptide
4B4	GGLVKPGGSLRLSC		-	+
18/2	----Q-----		+	-
KIM 4.6	--V-Q--R-----		-	-
RIV	--V-Q--S-----		-	-
LAY	----Q-----		+	-
Second hypervariable region				
Peptide name	Sequence		Immunoblot reactivity	
VH3-HV2a	SKTDGGTTDYAAPVKGC	Encoding genes	Anti-VH3-HV2a	Anti-VH3-HV2b
VH3-HV2b	S--S-Y--DS----	20P1/9-1*	+	+
VH3-HV2c	--SNKY--DS-----	30P1/60P2/63P1/VH26*	VH3-HV2b peptide	VH3-HV2c peptide
VH3-HV2d	TA-D-Y-PGS----	56P1/2P1/13P1/Humhv3005*/1.9III*	+	-
		38P1	-	+
H Chains			VH3-HV2b peptide	VH3-HV2c peptide
4B4	SKTDGGTTDYAAPVKG		+	-
18/2	ISGS--S-Y--DS---		-	+
KIM4.6	ISY--SNKY--DS---		-	-
RIV	MSY--DNKY-VDSK--		-	-
LAY	KYEN-NDKH--DS-N-		-	-
Third framework region				
Peptide name	Sequence		Immunoblot reactivity	
VH3-FR3a	QMNSLKTEDTAVYYCTTC	Encoding genes	Anti-VH3-FR3a	
H Chains		20P1/9-1*	+	
4B4	QMNSLKTEDTAVYYCTT		-	
18/2	----RA-----AK		-	
KIM4.6	----RA-----AK		-	
RIV	----RA-----		-	
LAY	----QA--S-I--AR		-	

Amino acid sequences were taken from reported VH3 proteins or deduced from cDNA sequences. Dashes represent an identical amino acid residue. The VH3-FR1 sequence is encoded by most VH3 genes, while the VH3-FR1a has only been described in the 9-1 and M26 clones. Germline encoding sequences are *, others are from fetal repertoires. The VH3-HV2a, and VH3-FR3a sequences were deduced from the 20P1, M26, and 9-1 gene. The VH3-HV2b and VH3-HV2c are derived from the VH26 and Humhv3005 germline genes, respectively. The 4B4 H chain is encoded by the germline 9-1 sequence. The KIM4.6 H chain is encoded by the germline Humhv3005 sequence. The 18/2 H chain is encoded by the germline VH26 gene. RIV and LAY are IgM rheumatoid factors with unknown germline origin. To improve binding fine specificity, immunoblot reactivity of immobilized H chains was tested after preincubation of a peptide at 1 µg/ml with certain antisera. Blots are displayed in Fig. 1.

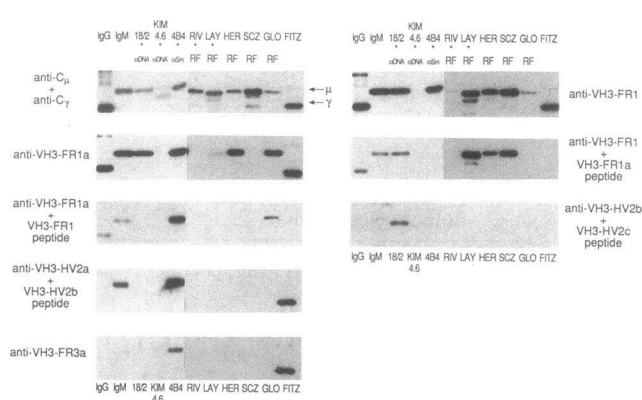


Figure 1. Binding specificity of VH3-related anti-peptide antibodies. Replicate immunoblots were performed of purified human Ig under reducing conditions, and the H chain bands are displayed. IgM and IgG are pooled polyclonal samples. All other samples are monoclonal IgG, except FITZ, which is a monoclonal IgG of unknown binding specificity. Immunoblots were reacted with rabbit anti-peptide sera that identify H chain determinants. For the anti-C μ and C γ reagents, anti-peptide antibodies were created that bind to sequences taken from portions of the first domain of the H chain constant regions. The VH3-FR1, VH3-FR1a, and VH3-FR3a peptides were taken from VH3-specific framework 1 and 3 sequences. The VH3-HV2a, VH3-HV2b, and VH3-HV2c represent second hypervariable region sequences from known germline genes. At times, to demonstrate fine binding specificity with a primary amino acid sequence, anti-peptide serum was preincubated with a peptide with a sequence similar but nonidentical to the immunizing peptide. The FITZ, IgG, is identified by the two of the three 20P1/9-1-derived anti-peptide reagents. IgM proteins marked * have known VH sequences, which are compiled along with peptide sequences and immunoblot reactivity results in Table I.

SCZ and GLO), and one monoclonal IgG of unknown sequence (FITZ). To demonstrate loading, in the top left panel the H chains were reacted with antisera to sequences taken from the first domains of the μ and γ constant regions. At top right, the anti-VH3-FR1 reagent identifies a subset of the polyclonal IgM and IgG, and the VH3-derived H chains of the monoclonal Ig proteins of known sequence and the VH3-derived H chains of the monoclonal Ig of known sequence (18/2, 4B4, LAY), and the H chains of unknown sequence (HER, SCZ, GLO, and FITZ). The H chains of RIV and KIM4.6 are not identified, and sequence comparison confirms that these proteins express variant VH3 first FR sequences associated with two amino acid differences in each (Table I).

The fine binding specificity of an anti-peptide serum can be restricted by preincubation with a peptide with minor variation from the immunizing peptide (21). By this method a preincubated antiserum may identify only proteins that express the exact sequence in the immunizing peptide. In Fig. 1, at right, the second panel demonstrates the restricted fine binding specificity of the anti-VH3-FR1 reagent after preincubation with the VH3-FR1a peptide. Only the proteins with the exact VH3-FR1 sequence (18/2 and LAY), and subsets of the polyclonal IgM and IgG and the HER and SCZ H chains of unknown sequence are identified. At left, the second panel reveals that the anti-VH3-FR1a reagent detects most of these H chains. At left, the third panel reveals that after preincubation with the VH3-FR1 peptide, the anti-VH3-FR1a reagent identifies the H chain of

the 4B4 IgM that expresses the exact VH3-FR1a sequence. In addition, the anti-VH3-FR1a reagent reacts with a subset of polyclonal IgM and IgG and the GLO H chain of unknown sequence.

The binding specificities of reagents generated with peptide sequences taken from the second HV region of certain VH3 genes were also evaluated. The IgM autoantibodies, 18/2, 4B4, and KIM4.6 were used as positive controls, because they express the exact coding regions of the 30P1, 20P1, and 56P1 genes from the fetal repertoire, respectively (23–25). To improve fine binding specificity, the same peptide preincubation technique was also used in immunoblot studies of the serologic reagents directed against second HV region sequences from VH3 genes. The VH3-HV2b sequence was derived from the VH3 gene, 30P1, that has an identical encoding region with the VH26 germline gene. In Fig. 1, at bottom right, after the anti-VH3-HV2b was preincubated with a related peptide (VH3-HV2c), only the 18/2 H chain with the exact sequence of VH3-HV2b is recognized. Similarly, at left in the fourth panel, after pretreatment of the antiserum to the second HV sequence derived from the 20P1 gene (anti-VH3-HV2a) the 4B4 H chain is recognized, and it expresses this exact second HV region sequence. A subset of polyclonal IgM is also identified. After similar pretreatment the antiserum from the homologous portion of the 56P1, anti-VH3-HV2c, only identified the cognate sequence in the H chain of KIM4.6 (not shown). The anti-VH3-HV2d reagent, derived from the 38P1 gene, did not identify any of these H chains, but an H chain with the VH3-HV2d determinant was unavailable for comparison. At bottom left, the antiserum to a portion of the third FR region derived from the 20P1 gene (anti-VH3-FR3a) also identifies the cognate sequence in the 4B4 H chain. This reagent displays no cross-reactivity with H chains that express variations of this sequence, so pretreatment was not used. In all immunoblot studies with H chains, reactivity of anti-peptide antibodies was completely depleted by preincubation with immunizing peptide at 0.01 μ g/ml, but not with unrelated peptide at a 1,000-fold higher concentration (not shown). VH region sequences, peptide sequences, and immunoblot reactivities are compiled in Table I.

Comparable studies with anti-peptide antibodies directed against the products of the other members of the early fetal library were also performed. In all cases, antibodies reacted with H chains that express the cognate sequence within the second HV region. Reactivity has been demonstrated with certain VH4 and VH6 H chains (14), and H chains from the VH1 gene, 51P1 (not shown), but H chains encoded by the germline sequences of the VH3 gene, 38P1, and the VH1 gene, 20P3, were not available for analysis.

Analysis of V region diversity in monoclonal Ig. A panel of 55 purified monoclonal human Ig of unknown sequence from patients with Waldenstrom's macroglobulinemia, myeloma, and other lymphoproliferative syndromes was also tested for reactivity with the VH-directed reagents. The panel consisted of 48 IgM, 3 IgA, and 4 IgG, which included 10 IgM rheumatoid factors (RF), but there were no Ig with anti-nuclear, anti-red blood cell, anti-PS, or other known binding activities. Of these, 20 IgM (including 6 RF), 2 IgA, and 2 IgG were identified as VH3-derived due to reactivity with the anti-VH3-FR1 and/or the anti-VH3-FR1a reagent (in Fig. 1, HER, SCZ, GLO, and FITZ are examples). Only the second HV-linked reagents derived from the 20P1 gene identified certain of these proteins. The anti-VH3-HV2a reagent identified 3 IgM and 1

IgG proteins, and all were VH3-derived based on reactivity with the first framework specific reagents. The other 20P1-derived reagent, anti-VH3-FR3a, identified 1 IgM and 1 IgG (FITZ) proteins, but these were a subset of the Ig that were reactive with the anti-VH3-HV2a reagent. To illustrate, in Fig. 1 the monoclonal IgG of unknown sequence, FITZ, is shown to be VH3 derived, and also to coexpress two linear determinants from the 20P1 gene, VH3-HV2a and VH3-FR3a. This suggests that the H chain of this myeloma protein has a VH region that is structurally related to the 4B4 H chain. The antibodies against the other VH3 second HV region sequences (VH3-HV2b, VH3-HV2c, and VH3-HV2d) did not identify any of the monoclonal Ig in this panel. The absence of reactivity of the H chain of FITZ with the reagent directed against the exact first FR sequence from the 20P1 gene may be the result of this H chain being encoded by an allelic, genic, or somatic variant. This may also explain the identical reactivity pattern of the anti-Hib PS antibodies of the subject, DT (see below).

The binding specificities of the antibody to the second HV region sequence of the V κ 2 gene, A2, designated VK2-HV2a, was also evaluated by immunoblot analysis. In these studies the L chain of B-G2a was used as a positive control, because it has this exact sequence (11) (kindly provided by M. Scott, Washington University). In contrast, the anti-peptide antibody to VK2-HV2a did not recognize two VK2 L chains of known sequence, Cum and GM607, that have divergent HV region sequences (11). In addition to the panel of 55 Ig paraproteins (which included six V κ 2-derived proteins), we also tested five V κ 2 Bence-Jones L chains of unknown sequence. Only one additional L chain from an IgM of unknown binding activity was identified by the VK2-HV2a reagent (not shown). After preincubation with immunizing peptide at 0.01 μ g/ml, reactivity with these L chains was completely abolished.

Analysis of VH diversity in circulating anti-Hib PS antibodies. In Fig. 2, immunoblots of the H chains of antibodies precipitated with the Hib PS from 10 normal volunteers are displayed. Included are pre (–) and post (+) immunization samples from five individuals (JN, AW, SS, CA, CS), and another five postimmunization samples (DT, NF, PT, HF, DF). At top left, the immunoblot was reacted with antisera to primary sequences from the first domain of μ , α , and γ H chain constant regions. Antibodies of the IgM, IgA, and IgG isotypes are uniformly precipitated in the postimmunization samples, with the greatest band intensities present in the CA (+) sample. Little or no Ig was precipitated from the preimmunization specimens. Consistent with previous studies, detection of an Ig band by this immunoblot technique requires more than 50 ng ([20], and unpublished observations). Incubation with an anionic pneumococcal PS and centrifugation of sera samples did not affect the subsequent recovery of Hib PS-precipitated antibodies (not shown). It is unlikely that there was a significant contamination by coprecipitating anti-IgG autoantibodies. Animal models have demonstrated that PS antigens are poor inducers of RF production (29), and immunization did not increase RF levels in our subjects. Also, depletion of RF by immunoprecipitation was not detected (data not shown). Although the immunoblot method is not quantitative, the relative band intensity of the precipitated anti-Hib PS antibodies was equivalent to the amounts of anti-Hib PS antibodies depleted from the serum samples, as determined by radioimmunoassay (data not shown). By conjecture, residual Hib PS left from the precipitations may have interfered with the radioim-

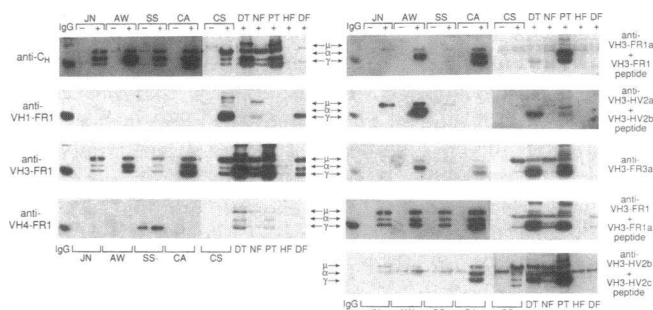


Figure 2. Heavy chain variable region diversity in circulating human antibodies to Hib PS. Replicate blots included controls of polyclonal IgG (shown) and control monoclonal IgMs (not shown). Of five preimmunization samples (–), precipitating anti-Hib PS antibodies were detected in two individuals (SS and CS), but not in the other three. VH3-derived H chains were the major populations in all post-immunization (+) precipitating antibodies. VH4-derived antibodies were detected in four samples, but were smaller subpopulations. Patient SS had VH4-derived antibodies before immunization, but VH3-derived antibodies were later induced. VH1-derived antibodies were detected in three cases, but represented significant populations in only two samples (CS and DF). VH2, VH5, or VH6-derived H chains were not detected (not shown). Many VH3-derived H chains were also identified by the 20P1/9-1-derived antibodies (VH3-FR1a, VH3-HV2a, and VH3-FR3a) and the 30P1/VH26-derived antibody (VH3-HV2b). In addition, anti-peptides to three VH1, two VH2, two VH3, six VH4, one VH5, and one VH6 hypervariable region sequences were also nonreactive with any of the anti-Hib PS antibodies (not shown).

munoassay quantitations, but the overall agreement of the two methods suggests that any imprecision was not significant.

In Fig. 2, at left are displayed the reactivities of replicate blots with reagents specific for the VH families. Corroborating the recent report by Scott and co-workers (10), all 10 donors had major populations of anti-Hib PS antibodies which derived from the VH3 family. The H chains of anti-Hib PS antibodies were not identified by reagents specific for the minor VH families, VH2, VH5, and VH6 (not shown). However, anti-Hib PS antibodies from four individuals (CS, DT, NF, and DF) were also identified by the VH1 family-specific reagent (anti-VH1-FR1). The VH1-derived antibodies induced in CS and DF were predominantly IgG isotype. A subset of the induced anti-Hib PS antibodies from an overlapping set of three individuals (SS, DT, and PT) were also derived from the VH4 family (anti-VH4-FR1). In these panels, subsets of the unfractionated polyclonal IgG were also detected by the VH1, VH3, and VH4 specific reagents, but the VH restriction of the Hib PS precipitated antibodies indicates the absence of detectable contamination with nonspecific Ig. Significantly, only VH4-derived anti-Hib PS antibodies were present in the sample SS before immunization, while immunization induced the new appearance of VH3-derived antibodies.

Replicate immunoblots were also reacted with reagents that identify subsets of VH3 H chains. At top right, after preincubation with the related peptide, the anti-VH3-FR1a antibody directed at the 20P1 derived first FR sequence identifies postimmunization H chains from three samples (AW, CA, and PT). At right, the second panel displays reactivity of the pretreated anti-VH3-HV2a reagent derived from the second HV region sequence of the 20P1 gene, and the α and/or γ H chains of the precipitated anti-Hib PS antibodies from three samples (AW,

DT, and PT) were identified. These same four individuals (AW, CA, DT, and PT) had significant responses that were also identified by the anti-VH3-FR3a antibody, that was taken from the third FR sequence of 20P1. While FR sequences may be shared by different members of the same V gene family, the concurrent expression of HV sequences suggests that H chains structurally related to the 20P1 gene are commonly used in antibodies to Hib PS. Similar to the reactivity of the monoclonal IgG Fitz, the anti-Hib PS antibodies of DT and CA were each identified by two of the three reagents specific for the products of the 20P1 gene, which may suggest that they are either derived from different genic variants of 20P1 or have undergone somatic mutations.

In Fig. 2, at right in the fourth panel, preincubation of the anti-VH3-FR1 reagent did not alter the reactivity of this antibody with the anti-Hib PS antibodies. At bottom right, after preincubation with a peptide related to the immunizing peptide, the antibody specific for the second HV region of the 30P1 gene product, anti-VH3-HV2b, reacted with H chains of IgM, IgA, and IgG isotypes in five individuals (CA, CS, DT, NF, and PT), and also reacted with IgM and IgA antibodies from the five other individuals. Taken together, these reactivities are consistent with identification of the anti-Hib PS responses by certain VH3 family specific reagents. However, the anti-Hib PS antibodies were recognized neither by the serologic reagents against the HV region sequences of other VH3 gene, 56P1 and 38P1, nor to any of the other VH families in the fetal repertoire (data not shown). The reactivities of the antipeptide reagents to VH determinants are compiled in Table II. When compared to monoclonal Ig, the 20P1 and 30P1-associated markers appear to be nonrandomly expressed in anti-Hib PS antibodies (two-tailed Fischer exact test, $P = 0.00042$ and < 0.0001 , respectively). These data suggest that immunization with the Hib PS nonstochastically induces dominant clones of VH3 antibodies

that are structurally related to the products of the 20P1 and 30P1 genes.

To document that VH restriction was due to detection of antigen-specific responses, control precipitation studies were performed. When Sepharose beads were used, the V region distribution of the precipitated Ig was identical to unfractionated serum Ig (data not shown). In contrast, after immunization of 14 normal subjects with pneumococcal vaccine, the precipitates with the pneumococcal PS type 14 immunogen had equivalent subsets of Ig from two VH families in seven samples, while the remainder had antibodies from three VH families (Silverman, G. J., manuscript in preparation).

Analysis of VL diversity in circulating anti-Hib PS antibodies. Previous immunochemical analyses have documented that anti-Hib PS responses are dominated by V_{κ} -derived L chains (30), and over $\frac{1}{3}$ of patients have responses with $> 90\%$ kappa L chain restriction (31). Illustrated in Fig. 3 and Table II, all 10 subjects were found to have a major induction of V_{κ} -derived L chains. Only three individuals (AW, CS, and DF) had significant populations of λ -derived L chains, and five additional subjects had minor λ L chain populations. All 10 individuals also had induced responses that included $V_{\kappa 2}$ -derived L chains, of which 6 (CA, CS, DT, NF, PT, and DF) were also identified by the anti-VK2-HV2a reagent that identifies the products of the A2 gene. Compared to the panel of monoclonal Ig, the occurrence of A2-linked L chains in anti-Hib PS antibodies appears to be nonrandom ($P < 0.0001$).

All individuals had a population of the anti-Hib PS antibodies with $V_{\kappa 3}$ -derived L chains, and seven individuals also had a minor subset of $V_{\kappa 1}$ -derived antibodies. None of the anti-Hib PS responses reacted with the $V_{\kappa 4}$ gene family specific reagent (not shown) (data compiled in Table II). Of note, the patient SS had a significant population of $V_{\kappa 3}$ -derived antibodies before immunization, but immunization predominantly induced $V_{\kappa 1}$

Table II. Diversity of Postimmunization Antibodies to Hib PS

Subject	VL Reactivity							VH Reactivity					
	C λ	C κ	V $\kappa 1$	V $\kappa 2$	A2-associated VK2-HV2a	V $\kappa 3$	V $\kappa 4$	VH1	VH4	VH3	20P1/9-1 associated		30P1/VH26-associated VH3-HV2b*
											VH3-HV2a*	VH3-FR3a	
1. JN	+	++++	-	+	-	+++	-	-	-	+++	++	-	++
2. AW	+++	++++	++	++++	-	++	-	-	-	++++	++++	++	++
3. SS	+	++++	++	++	-	+++	-	-	++	+++	-	-	++
4. CA	+	++++	+	+++	++++	++++	-	-	-	++++	-	++	++++
5. CS	+++	++++	+	+++	+	+	-	+++	-	++++	-	++	++++
6. DT	-	++++	+	++++	++	+	-	+	++	++++	+++	++++	++++
7. NF	-	++++	+	+++	++	+	-	+	-	++++	-	++	++++
8. PT	+	++++	+	+++	+++	+	-	-	+	++++	++	++++	++++
9. HF	+	++	-	+	-	+	-	-	-	++	-	-	++
10. DF	+++	++	-	+++	+	++	-	++	-	++++	-	++	++
	8	10	7	10	6	10	0	4	3	10	4	7	10

Variable region diversity determined by immunoblot reactivity. The V_{κ} and VH families were determined with antipeptide antibodies to first FR determinants. The reactivity of anti-VH3-HV2a and anti-VH3-HV2b were determined after preincubation with a related synthetic peptide, to improve fine binding specificity. Reactivity of antisera to second HV and a third FR region sequences are displayed below the gene from which they derive. In each blot, after adjustment for total Ig detected by constant region specific reagents relative band intensity indicated +, ++, +++, +++++. (-) indicates not detected, and the limits of detection were < 50 ng. H chains were not recognized by VH2, VH5, or VH6 family specific reagents (not shown).

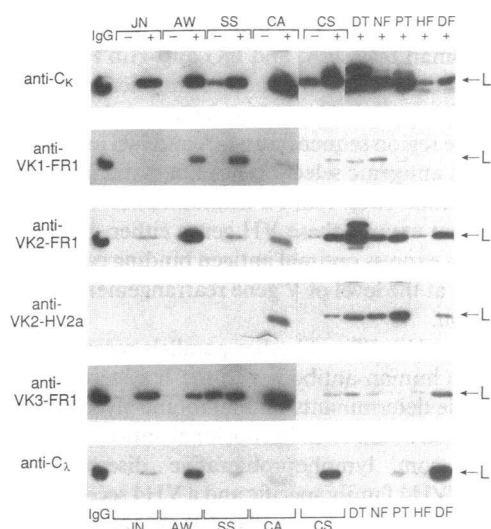


Figure 3. L chain variable region diversity in circulating anti-Hib PS antibodies. Replicate blots are displayed with the L chain bands from the blots used in Fig. 2. The anti-C κ and anti-C λ reagents identify sequences in the constant regions of kappa and lambda L chains, respectively. V κ family specific reagents identify diagnostic sequences in the first framework regions (FR1), and the anti-VK2-HV2a identifies a diagnostic sequence in the second hypervariable region of the V κ 2 gene, A2. As in Fig. 2, only two patients have detectable anti-Hib PS antibodies before immunization (–). For SS, these L chains are predominantly V κ 3-derived, and for CS they are V κ 1-derived. Of the 10 individuals, 6 have L chains identified by the anti-VK2-HV2a reagent that was derived from the second hypervariable region of the V κ 2 gene, A2 gene. This antibody does not react with unfractionated IgG, or IgA and IgM (not shown). Anti-Hib PS antibodies derive from the V κ 1, V κ 2, and V κ 3 families, but not V κ 4 (not shown). Three individuals also have a major population of anti-Hib PS antibodies with λ L chains (AW, CS, and DF).

and V κ 2 antibodies. As previously described, this individual had VH4-derived anti-Hib PS antibodies, but immunization induced the appearance of VH3 antibodies, which may suggest that not all cross-reacting B cell clones are stimulated by Hib PS immunization.

Discussion

Our study documents the restricted VH diversity in circulating antibodies induced by immunization with *Haemophilus influenzae* capsular polysaccharide. Based on studies with anti-peptide antibodies that recognize diagnostic FR and HV region determinants, the anti-Hib PS antibodies from unrelated individuals preferentially express VH3 H chains. Although reagents specific for four types of VH3 H chains were also employed, these antibodies use only two of these types of H chains. Minor populations of VH1 and VH4 antibodies to the Hib PS are expressed by some individuals, but the products of the VH2, VH5, and VH6 gene families are not used. These direct analyses of human antibody repertoires demonstrate that the physiologic responses of individuals in an outbred population can all use similar VH regions. Moreover, the two types of VH3 regions are structurally related to known VH genes. Our findings suggest that Hib PS immunization causes a specific induction of oligoclonal B cell clones that express 20P1 and 30P1-related V regions.

Previous reports have concentrated on VL expression in these antibodies. We confirm and extend the observation that the VL regions of anti-Hib PS antibodies are structurally heterogeneous, as all individuals had L chains from at least three VL families. In general, V κ 3- and V κ 2-derived L chains dominated the responses of all 10 subjects. In agreement with past reports, we found that six individuals had anti-Hib PS antibodies with L chains derived from the A2 gene (or a closely related gene). Significantly, because these circulating antibodies were isolated by precipitation with antigen, the VH and VL regions identified in these anti-Hib PS antibodies should be representative of physiologically important antibodies.

The serologic reagents used in these studies were shown to be highly sequence specific, and in one instance could detect single conservative amino acid differences. However, these immunoblot studies cannot assign the exact genes from which these antibodies originate. Due to the degeneracy of the nucleic acid code and the possibility that sequences with conservative amino acid replacements are also identified, several related genes could encode for the recognized V region sequence. Even so, having identified characteristic features of the dominant clones of anti-Hib PS antibodies, the isolation and sequence analysis of physiologically representative clones will be greatly simplified, and the contribution of somatic mutation and junctional diversity can be assessed.

Past studies have described anti-peptide antibodies that recognize primary sequences in immunoglobulin HV regions (21, 32, 33), and we have also previously described anti-peptide antibodies that are V gene family specific (12, 14). However, previously anti-peptide reagents have been applied only to the study of monoclonal Ig from cell lines or lymphoproliferative disorders, and this was the first application to the survey of a physiologic humoral response. This approach allows for the simultaneous analysis of induced antibodies of all isotypes.

Our studies were designed to qualitatively analyze the diversity of V regions present in anti-Hib PS antibodies. Quantitations of antibody content were also performed to ensure that the precipitation method removed a significant proportion of the anti-Hib PS antibodies. These determinations of anti-Hib PS content were also in accordance with relative band intensities within an immunoblot. It is unlikely that major antibody subpopulations were undetected. Extensive experience has documented that these anti-peptide reagents can identify the V gene family origins of > 95% of monoclonal Ig from cell lines and lymphoproliferative syndromes ([12], unpublished observations). Moreover, if prolonged autoradiographic exposures are used, this method can detect minor subsets of polyclonal populations that represent < 0.5% of a band of 5 μ g (20).

The human VH locus has been estimated to contain from 50 to 200 gene segments (17). To date, six VH families have been described based on cross-hybridization and > 80% nucleic acid sequence homology within a family, and less than ~ 70% homology between families. The VH3 family has the greatest genetic complexity. Restriction fragment analyses with full-length genes detect 25–30 VH distinct bands, that represent an estimated 50–100 members, with perhaps 30–40% pseudogenes (34, 35). However, the extent of polymorphism is unknown, and the determination of whether minor sequence differences represents allelic differences or distinct genes is an area of active investigation (36–43).

The products of several genes related to 30P1 may be recognized by the anti-VH3-HV2b reagent. Of the nine distinct VH

genes isolated in the first reported fetal repertoire, the VH3-HV2b sequence is present in both the 30P1 and the 60P2/63P1 clones (18). The 30P1 clone and the VH26 germline gene are identical, and they are > 99% homologous with a related germline gene, J00236 (44). To provide an estimate of complexity of 30P1/VH26 related genes, Southern analysis was performed on genomic digests, and only five to seven bands were detected by hybridization at high stringency with a 5' VH26 flanking probe, or one to five bands when probed with oligonucleotides with sequences that overlap the VH3-HV2b region (36, 37).

The 30P1/VH26 and related genes have been shown to encode polyreactive antibodies with a variety of autoreactivities including anticardiolipin, antiplatelet membrane, insulin, vimentin, thyroglobulin, Fc, and anti-DNA (23, 44, 45). The 18/2 IgM hybridoma was made from the peripheral cells of a leprosy patient, and it is directly encoded by the germline VH26 gene (23). This antibody has binding capacity for both double stranded DNA and the *Klebsiella* capsular polysaccharide, K30, and we have used it as a positive control for 30P1/VH26 encoded proteins. These antibodies also often bear the H chain associated 16/6 idiotype that is increased in the circulation of both lupus patients with active disease, and patients with systemic *Klebsiella* infection (46, 47). Therefore, genes in the 30P1/VH26 subfamily are likely to be important in a variety of pathologic as well as physiologic responses.

Kabat and others have hypothesized that phosphodiester groups with a given spacial separation may constitute a cross-reacting, immunodominant determinant present in DNA, phospholipids, and certain bacterial PS (48, 49). Perhaps this common motif can be recognized by a conserved type of antigen binding site that can be encoded, in part by 30P1/VH26 related genes. In preliminary studies, the antibodies to several polyanionic pneumococcal PS also use related H chains (Silverman, G. J., unpublished observations). This model is consistent with the finding of structurally diverse L chains and restricted VH expression in anti-Hib PS antibodies, and would explain the high levels of VH26-associated idiotypes derived from anti-DNA antibodies that are often present in the circulation of patients with *Klebsiella* infections (50).

Our study also found that a majority of subjects had circulating antibodies that are identified by reagents directed at sequences from the 20P1 gene from the fetal repertoire. By sequence comparison the 20P1 gene is a distant relative to other elements in the VH3 family, and it has been designated a separate subfamily (25). Clones with sequences identical to 20P1 have been isolated from the germline, termed 9-1 (17), from three fetal libraries of rearranged VH genes (51), and from the 4B4 cell line (25). In a sensitive gel hybridization method, only a single monomorphic band was identified when the genomic digests from 15 individuals were probed with an oligonucleotide with a sequence that overlaps the region used in our VH3-HV2a peptide (38). Therefore, although not definitive, the cumulative data suggest that the 20P1/9-1 gene is highly conserved in outbred populations.

The IgM 4B4 is the direct expression of the germline 9-1 VH gene, and it was used as a control in our studies. It has the fine binding specificity for the Sm/RNP complex that is associated with human lupus, but this antibody also binds retrovirus gag protein (52). Therefore, in addition to our characterization of anti-Hib PS responses, antibodies derived from 20P1/9-1 related genes may also be expressed in response to other microbial pathogens.

The identification of specific VH3 region determinants that are conserved in human IgM, IgA, and IgG anti-Hib PS antibodies has significant genetic and structural implications. In addition to the evidence of restricted V gene usage, the retention of hypervariable region sequences in IgA and IgG antibodies may suggest that antigenic selection has not systematically altered these recognition sites. Hib PS immunization may select B cell clones that express these VH genes either, because these germline genes express optimal antigen binding capacity, or due to regulation at the level of V gene rearrangements or B cell clonal expansion.

Our study of anti-Hib PS antibodies parallels recent findings concerning the human antibodies to the Ii system of related oligosaccharide determinants on membrane glycoconjugates of autologous red blood cells. Monoclonal anti-I and anti-i autoantibodies from lymphoproliferative diseases are identified by both a VH4 family specific and a VH4 second HV region directed antipeptide reagent (21, 53). More recently, anti-I and anti-i autoantibodies from lymphoblastoid cell lines were shown to be encoded by a single germline VH4 gene with little or no somatic point mutations, but can be associated with diverse L chains (53a). Presumably, these VH4 encoded autoantibodies have binding sites that are distinct from those of VH3 anti-Hib PS antibodies, which is reflective of the structural dissimilarity of these two carbohydrate targets, i.e., the Ii determinants are present on neutral oligosaccharides, while the Hib PS expresses a simple anionic polymeric structure of ribosylribitol phosphate.

In murine strains, high levels of induced antibodies to bacterial dextran requires expression of certain VH alleles (54, 55), which may only be inducible after a defined point in development (54). In humans, the anergy of infants to purified Hib PS can often be circumvented if the Hib antigen is conjugated to a protein carrier (56). These antibody responses have been shown to share the idiotype expressed on anti-Hib PS antibodies in adults induced by purified Hib PS (9), but otherwise it is unknown whether these groups produce antibodies with the same V region biases. By application of our immunoblot technique we will be able to extend these studies to comparisons of the structural diversity of the anti-Hib PS antibodies that are induced during different stages of development by various Hib PS vaccine formulations.

A study has recently shown that immunization of certain Native American infants with a Hib PS conjugate vaccine induced only limited responses that were ineffective in protecting from Hib infection (5). The mechanism for this form of nonresponse to Hib PS immunization is unknown. However, twin studies have documented that high responder status to many pneumococcal PS and to Hib PS is less dependent on environmental factors than on genetic factors (57). In our study, all of the adult Caucasian subjects were shown to have responses that were dominated by structurally homogeneous VH regions. Therefore, this study presents a testable hypothesis. After confirmation that 20P1/9-1 and 30P1/VH26 related genes encode for anti-Hib PS antibodies, VH gene-specific probes can be designed for restriction fragment analysis. Genomic DNA can then be studied to determine whether nonresponders have VH gene locus deletions or absent VH genes, compared to high responders. This approach may indicate which exact alleles (or genes) are necessary for optimal anti-Hib PS response. Future investigations will also provide evidence of whether rearrangement of these genes is the structural basis for the postulated

developmentally staged acquisition of important antigen binding activities. Due to the simple epitopic structure of carbohydrates and the presumed limited role of somatic diversification in these antibody responses, it is apparent that the study of anticarbohydrate responses will reveal much about the forces that mold the preimmune repertoire, and the mechanism by which antigenic selection results in restricted V gene recruitment.

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