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Development of the Human Antibody Repertoire

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Abbreviations

CDR, complementarity determining region
D, diversity segment
Hib, *Haemophilus influenzae* type b
J, joining segment
V, variable segment
J_H, heavy chain joining segment
V_H, heavy chain variable segment

Humans are confronted with a vast array of foreign antigens, necessitating the generation of a large number of protective Ig molecules. It is estimated that the immune system is capable of giving rise to 10^{14} or more B cell clones, each synthesizing a unique antibody species with a distinct antigen specificity. In recent years, the molecular basis of this remarkable diversity has become increasingly clear. However, much of this information is derived from studies of the immune response of experimental animals to simple antigens, and it is apparent that significant differences exist among higher vertebrates both in the structure of Ig genes and in mechanisms used to generate diversity. This review will summarize advances in the understanding of the molecular basis for antibody diversity with particular attention to the developmental expression of the antibody repertoire. It will also focus on how molecular analysis of the human antibody response to Hib may further our understanding of the process by which humans develop a protective immune response.

The human immune response to Hib is an excellent model with which to study the development of the human antibody repertoire. This organism is an important cause of serious bacterial infections, and antibody directed against its capsular polysaccharide protects against invasive infection (1, 2). Similar to the response to some other polysaccharide antigens, antibody to Hib polysaccharide develops relatively late in ontogeny. Thus, children less than 5 y of age demonstrate poorer antibody responses to Hib colonization, to infection, and to vaccination with plain Hib polysaccharide vaccine than older children or adults and therefore are most susceptible to infection (2). Coupling Hib polysaccharide to carrier proteins significantly increases immunogenicity of Hib vaccines (3). In addition to the age-dependent acquisition of anti-Hib polysaccharide antibody, certain ethnic groups and individuals have an increased risk of Hib infection (4–6). It has been hypothesized that both phenomena may be related to restricted Ig variable region gene usage (7, 8).

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PRIMARY Ig GENE DIVERSIFIERS

Antibody molecules are composed of an identical pair of heavy and light chains, which together create two functional domains (9, 10). The constant region is responsible for effector functions such as complement activation and opsonization by attachment to receptors on neutrophils. Ig of the same class or subclass share identical constant regions (*e.g.* IgM, IgA, IgG). The amino termini of individual heavy and light chains are structurally diverse and are responsible for antigen-antibody interaction. This "variable region" of the molecule is responsible for the molecule's unique antigen specificity. Variable regions of individual heavy and light chains contain three areas of amino acid hypervariability, called CDR, which are principally involved in antigen contact. CDR are separated by "framework" regions, which are highly homologous among individual members of variable region gene families.

The heavy chain variable region locus has been localized to band 14q32.33 of chromosome 14 (Table 1) (11). The κ light chain locus is on the long arm of chromosome 2. However, a number of functional and nonfunctional V_κ segments have been found on other chromosomes (22). The λ light chain locus is located on chromosome 22 and is the least well characterized (24). Variable regions of heavy and light chain Ig genes are encoded in discontinuous segments along the chromosome (Fig. 1). In the process of forming a functional variable region gene, individual members of two or three groups of "germline" gene segments recombine with one another to form a continuous coding sequence. In the assembly of a heavy chain variable region gene, for example, one of about 30 D segments combines with one of six J_H segments and subsequently with one of a larger number (100–300) of V_H elements (13, 14, 19, 20). Intervening DNA is usually excised during the recombination process. After a functional heavy chain variable region gene is generated, a light chain variable region gene undergoes similar rearrangement, although D segments are not used (10). Two of the three CDR are encoded by the V_H or $V_{\kappa/\lambda}$ segment, whereas the third (CDR-3) is created by the junction of V, (D), and J segments. Considerable potential exists for the generation of diversity at V(D)J and VJ junctions as a result of both this combinatorial joining of germline segments and other molecular events. For example, the exact joining site between segments may be imprecise due to the action of enzymes removing nucleotides at segment boundaries (exonucleases) and/or the addition of single nucleotides ("N addition") by terminal deoxynucleotidyl transferase or a similar enzyme (9, 29, 30). Also, the addition of germline-encoded mono- or dinucleotides has been postulated to occur by "P addition"—the nicking of the terminal 5′ coding sequence followed by its inversion and joining to 3′ ends of complementary strands (31). These primary diversifiers: combinatorial diversity, junctional diversity, and the pairing of heavy and light

chains may potentially give rise to a preimmune repertoire of up to 10^{14} pre-B lymphocytes with distinct antigen specificities (30).

Recombination of V, D, and J segments appears to be mediated by enzymes found only in lymphoid cells, which are active at discrete stages in maturing B cells. These enzymes are targeted to recombination signal sequences that flank V, D, and J segments. These sequences are composed of conserved segments of 7 and 9 bp separated by a spacer of 12 or 23 bp (10). The recombination signal sequence of a light chain chain V_κ or V_λ segment binds to complementary nucleotides adjacent to the J_κ or J_λ in light chain variable region genes, bringing the two segments into juxtaposition. A similar strategy is used in the recombination of heavy chain genes, with DJ rearrangement preceding V to DJ recombination.

The enzymes responsible for Ig gene rearrangement are not fully characterized. A number of proteins are likely to participate in recombination signal recognition, site-specific DNA cleavage, nucleotide addition and deletion at segment boundaries, and ligation of segment junctions. It appears that identical or very similar enzymes rearrange T cell receptor genes in developing T cells (32). Two closely linked "recombination activating" genes, RAG-1 and RAG-2, have been isolated and shown to synergistically activate V(D)J recombination (33, 34). Both genes are highly conserved between mice and humans, and are expressed concordantly only in pre-B and pre-T cells. The proteins encoded by these genes are not structurally similar to one another, and the exact role they play in the recombination process remains to be elucidated. They may comprise distinct subunits of a "recombinase" complex or may act at different steps in the recombination pathway. Another enzyme, the murine J_κ recombination signal-binding protein, may play an important role in V(D)J rearrangement, based on its binding specificity and structural similarity to the integrase family of microbial recombinases (35, 36).

Table 1. *Ig loci**

	Chromosomal location	Number	Families/numbers
Ig V_H locus (Refs. 11–21)			
V_H	14q32.33	100–300	V_H1 (16–200)
			V_H2 (5–10)
			V_H3 (24–150)
			V_H4 (6–14)
			V_H5 (3)
			V_H6 (1)
			V_H7 (NK)†
D		~30	
J_H		9 (3ψ)	
C_H		11 (3ψ)	
Ig V_κ locus (Refs. 22, 23)			
V_κ	2p2‡	~80	$V_\kappa1$ (~50)
			$V_\kappa2$ (~35)
			$V_\kappa3$ (~10)
			$V_\kappa4$ (1)
J_κ		5	
C_κ		1	
Ig V_λ locus (Refs. 24–28)			
V_λ	22q11	NK	$V_\lambda1$ (NK)
			$V_\lambda2$ (NK)
			$V_\lambda3$ (NK)
			$V_\lambda4$ (NK)
			$V_\lambda5$ (NK)
			$V_\lambda6$ (1)
			$V_\lambda7$ (10)
J_λ		7	
C_λ		7 (2ψ)	

* Columns show members of the three Ig linkage groups, chromosomal location of Ig loci, total estimated number of V (variable), D (heavy chain diversity), J (joining), and C (constant region) elements in each group (ψ indicates pseudogenes), known variable region families, and estimated number of variable region family members.

† NK, not known.

‡ Twenty-five to 40 "orphan" V_κ segments are located outside the κ locus.

SECONDARY Ig GENE DIVERSIFIERS

Further "second order" diversification of variable regions can occur even after V(D)J rearrangement is complete. After a functional V(D)J rearrangement occurs on one chromosome, recombination on the other usually is silenced, a property referred to as "allelic exclusion." However, we and others have demonstrated new V to (D)J recombination at both light and heavy chain loci after an initial rearrangement event (37–39). Such V segment replacement can occur independently on both alleles and may lead to the expression of new antigen binding domains. In heavy chain variable region genes, an upstream V_H segment recombines with the existing rearrangement at a seven-nucleotide sequence near the 3′ end of the original V_H segment, thus preserving the initial DJ rearrangement (37). Ongoing rearrangement of the light chain loci allows an upstream V segment to recombine with a downstream J segment, thereby replacing the original rearrangement entirely (38, 39). The extent to which this process contributes to the generation of antibody diversity *in vivo* is uncertain.

Somatic hypermutation of variable region genes contributes substantially to the maturation of the mammalian B cell immune response (10, 40). Ig mutation rates approach one mutation per thousand bases in each cycle of cell replication, at least 10 000-fold greater than the spontaneous mutation rate for other eukaryotic genes (41). Single base substitutions predominate and occur in both productively and nonproductively rearranged genes (42–46). This hypermutation is tightly focused to the rearranged V(D)J segment and immediate flanking regions (47, 48). The onset of hypermutation appears to occur after contact with antigen (49). Substitutions that result in greater antigen affinity are selected for during the late primary and secondary immune response, a phenomenon known as "affinity maturation."

B lymphocyte malignancies represent cells arrested at discrete stages of development and are useful models to study Ig gene diversification. Extensive variable region gene mutation has been demonstrated within the cells of human follicular lymphoma (surface Ig-positive cells with morphologic characteristics of germinal center lymphocytes) (50, 51), but not pre-B acute lymphocytic leukemia, sporadic Burkitt's lymphoma, or chronic lymphocytic leukemia (52–54). This suggests that Ig hypermutation is activated at a well-defined stage in B cell development. Also, ongoing hypermutation has been demonstrated in the variable region genes of murine B cells isolated from individual germinal centers (55, 56). Positive selection of germinal center lymphocytes with high affinity for antigen bound to follicular dendritic cells may result in their differentiation into memory cells or plasma cells. Conversely, B cells whose mutated variable region genes have lower affinity for antigen appear to be removed by a process termed apoptotic cell death (57).

Although several mechanisms for Ig hypermutation have been proposed, two general models have received the most support and prompted the most investigation: *1*) repair of variable region genes by an error-prone DNA polymerase (43, 58) and *2*) error-prone replication (59, 60). We have found no evidence of error-prone repair in human lymphoid cell lines representing intermediate stages in B cell development (61), although Valles-Ayoub *et al.* (62) demonstrated altered repair efficiency in germinal center B cells of human tonsils. Rogerson *et al.* (60) have recently developed a replicative model of hypermutation in the mouse, postulating the transient presence in mutating B cells of a factor characterized by high affinity for a putative "mutation initiation region" located upstream of variable region gene pro-

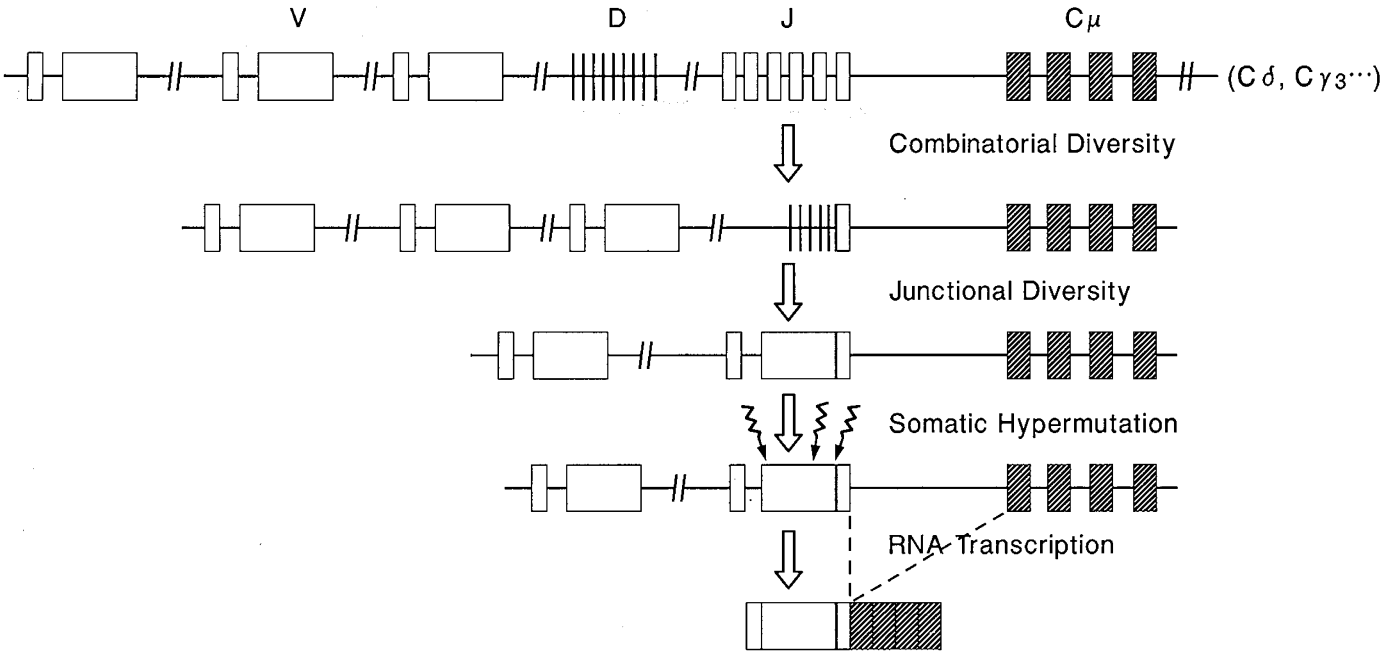

Fig. 1. Rearrangement of an Ig V_H gene. During the process of recombination, one of about 30 D segments is joined to one of six J_H segments, and subsequently to one of 100–300 V_H segments. Intervening DNA between recombined segments is usually excised but may be retained if individual segments are in opposite transcriptional orientation to one another. Rearranged Ig V_H genes may be further diversified by somatic hypermutation.

moters and by dependence upon an active upstream origin of replication.

STRUCTURAL ORGANIZATION OF Ig GENES IN HIGHER ORGANISMS

Although a substantial amount of information is available regarding the molecular mechanisms of antibody gene diversity, the relative contributions of individual molecular diversifiers to the final makeup of the human immune response to a specific antigen is unknown. The overall structure of human heavy and light chain Ig loci has not been completely characterized. The heavy chain locus is estimated to contain up to 300 V_H segments, divided into six or seven families on the basis of >80% nucleic acid sequence homology (Table 1) (13, 63). Members of individual families are interspersed rather than being grouped together on the chromosome (12). The κ light chain variable region gene locus is estimated to contain about 50 functional V_κ genes composing four families (22). A large portion of the locus is duplicated and a small number of V_κ genes are not located in association with the J_κ segments and κ light chain constant region gene segments on chromosome 2 (64–66). The human λ light chain variable region gene locus is less well characterized but appears more complex than that of the mouse, with an unknown number of V_λ segments belonging to seven families (25).

Although the structural organization of Ig loci is generally similar in vertebrate species, important differences exist and, further, each species appears to rely on alternative molecular mechanisms to diversify its antibody repertoire. The murine heavy chain variable region gene locus is estimated to contain a larger number of V_H segments than the human locus (up to 1000 or more germline elements) and, in contrast to the highly interspersed rearrangement of human V_H gene families, members of murine families are clustered together (12, 67, 68). Despite these differences, the generation of antibody diversity in mice may most closely resemble that of humans (10). In contrast, although several hundred V_H gene segments are present in the rabbit heavy chain variable region gene locus, 80–90% of expressed antibody is encoded by a single V_H gene segment and diversity in rabbit heavy chain variable region genes appears to be generated largely by secondary diversifiers (somatic mutation and gene conversion) (69). The chicken λ light chain variable region gene locus is composed of a single functional V_λ gene and 25 nonfunctional "pseudogenes." Only the functional segment is rearranged initially, and diversification is achieved subsequently through gene conversion, with pseudogenes providing donor sequences (70). These species-specific differences emphasize the importance of using a human model to examine the development of human specific immunity.

DEVELOPMENTALLY RESTRICTED Ig GENE DIVERSIFICATION

Although the structure of Ig loci and recombinatorial mechanisms theoretically may yield an almost unlimited number of Ig molecules, considerable constraints may be placed on the use of variable region gene segments during development. In both mice and humans, a small group of V_H genes is preferentially expressed in early development (71–76). In mice, preferential use of certain V_H genes is related in part to their location proximal to the J_H segment on the chromosome. The most J_H-proximal members of the human V_H locus are the single member of the V_H6 gene family, a V_H4 and a V_H2 segment, and several V_H1 gene family members (17, 71, 77, 78). V_H6 and V_H2 gene segments are among those preferentially expressed in the human fetus, perhaps disproportionately so in view of the small size of these families (76). Over 60% of "fetal" transcripts, however, are encoded by V_H3 genes, which are not among the most 3′ V_H segments. Further, V_H5 genes may also be over-represented in the early fetal repertoire and, again, are not among the most J_H-proximal V_H segments (17, 75–78). Thus, mechanisms other than geographic location within the heavy chain variable region gene locus are likely to play a role in preferential use of human V_H genes in fetal development. Disproportionately frequent expression of some murine V_κ families has been observed in both fetal B cells and in adult mice, suggesting that constraints on V_κ segment use may persist beyond the neonatal period (79, 80). In mice there is evidence that preferential expression of certain V_H gene family members is related to the regulated expression of these segments by different B cell subpopulations (81).

The use of junctional diversifiers may also be developmentally regulated. In humans, expression of diversity gene segments appears to differ with age, with preferential use of DHQ52 or DXP families in early development and more stochastic usage in adults (19, 30, 76, 82). J_H segment use may also differ, with J_H3 and J_H4 segments predominating in fetal and J_H4 and J_H6 in adult B cell populations (76, 82). N addition is absent in murine fetal B cells and increases with time. A similar increase in the frequency of N and P insertions with age is observed in murine $\gamma\delta$ T cell receptors (31, 83). The average length of human fetal and neonatal CDR-3 regions is shorter than that of adults, independent of N addition (30).

These developmental constraints on variable region gene use may have important implications for the maturation of the immune response. In mice, the antibody response to certain antigens appears in a well-defined temporal pattern that is highly conserved among different murine strains (84, 85). A similar pattern of responsiveness exists in humans, with the appearance of antibodies to protein antigens in the first several months of life followed later by the acquisition of the ability to produce protective levels of antibody directed against carbohydrate antigens at 12 mo of age or older (86). Additionally, each individual appears to generate anticarbohydrate antibodies from a limited number of B cell clones and, in contrast to antibody directed against protein antigens, these antibodies are not characterized by extensive somatic hypermutation (7, 8, 87). Programmed changes in variable region gene use and other diversifiers described above may explain the age-related acquisition of specific immunity.

ANTIBODY RESPONSE TO Hib

To examine variable region gene use during the human immune response to Hib polysaccharide, we have generated from immunized subjects a panel of hybridomas that secrete monoclonal human antibodies specific for this antigen. The polymerase chain reaction was used to clone variable region genes from these cell lines (7, 8) (Table 2). The most striking feature of this immune response is the degree of restriction evident in both heavy and light chain variable region gene use. The majority of heavy chain variable region genes encoding anti-Hib polysaccharide antibodies are members of the V_H3 gene family. All anti-Hib polysaccharide V_H genes sequenced to date are members of the V_H3 gene family (7). However, serologic analysis indicates that some individuals also express a small portion of antibodies encoded by V_H1 or V_H4 family members after immunization (92). We have obtained 11 heavy chain variable region genes from a total of nine subjects (unpublished manuscript) (Fig. 2). All these V_H segments are encoded by members of the V_H3 gene family, and eight are members of the smaller V_H3b subfamily. The V_H gene segments are 70–100% homologous to one another, and also highly homologous to two candidate germline V_H3 genes. The high homology shared by hybridoma gene segments and close identity with unrearranged germline genes suggests that these genes are derived from a very small subset of the total available V_H repertoire. Although these antibodies are likely to represent a secondary immune response and subjects were immunized with either "T-independent" plain Hib polysaccharide or "T-dependent" polysaccharide-protein conjugate vaccines, somatic mutation appears to be infrequent in many of these heavy chain variable region genes. Interestingly, the anti-Hib polysaccharide heavy chain variable region gene segments are highly homologous to segments preferentially expressed in the fetus and to those encoding autoantibodies (75, 76, 93, 94).

At least four V_κ and two V_λ gene families encode anti-Hib polysaccharide light chain variable region genes (8, 89, 95). A large proportion of these antibodies, however, as in the heavy chain variable region response, appear to be encoded by a limited number of germline elements. Approximately 60% of serum antibody of older children and adults is encoded by a single $V_\kappa II$ gene, A2, and at least a portion of these antibodies are encoded in an unmutated form, irrespective of antigen presentation as plain polysaccharide or polysaccharide-protein conjugate form (8, 89, 96). The majority of λ anti-Hib polysaccharide antibody cross-reacts with the structurally related antigen *Escherichia coli* K100 polysaccharide, and gastrointestinal colonization with this bacteria may induce protective anti-Hib polysaccharide antibodies before exposure to Hib itself (97, 98). *E. coli* K100 cross-reactivity of λ anti-Hib polysaccharide antibody also appears to be mediated by a single germline $V_\lambda VII$ element (8). The paucity of sequence differences in $V_\lambda VII$ segments obtained from four unrelated subjects suggests that these genes also are relatively unmutated.

CDR-3 regions of both heavy and light chain variable region genes, created by VDJ and VJ joints, respectively, also appear to be relatively restricted. Although a variety of D and J segments are used, preferential use of certain DJ combinations is seen and some heavy chain variable regions, obtained from unrelated individuals, share identical VDJ joints (unpublished manuscript). VJ joints of the majority of anti-Hib polysaccharide antibody light chain variable region genes, including all λ antibodies and the predominant $V_\kappa II$ A2-encoded κ antibodies, are notable for the presence of an arginine residue. In at least some of these antibodies, this residue must have arisen by N addition, an uncommon event in light chain variable region gene rearrangement (8, 89).

Table 2. *Hybridoma cell lines**

Cell line	Isotype	Age	Vaccine form	Ig V_H	Ig V_L
SB5/D6	IgA1-λ	Adult	PS-D	V_H3b LSG6.1†	$V_\lambda VII$ 4A (Ref. 24)
RAY4	IgA2-λ	Adult	PS-D	V_H3b LSG6.1	$V_\lambda VII$ 4A
JB32	IgA1-λ	Adult	PS	V_H3b LSG6.1	$V_\lambda VII$ 4A
JB21	IgG2-λ	Adult	PS	V_H3b LSG6.1	$V_\lambda II$ 2.1 (Ref. 88)
RC3	IgA-κ	Adult	PS-D	V_H3b LSG6.1	$V_\kappa II$ A2 (Ref. 89)
LSF2	IgA1-λ	Adult	PS	V_H3b LSG6.1	$V_\lambda VII$ 4A
SB1/D8	IgG1-κ	Adult	PS-D	V_H3b LSG6.1	NK‡
ED6.1	IgG2-κ	4 y	PS-D	V_H3b LSG6.1	$V_\kappa I$ clone KC-1 (Ref. 90)
ED8.4	IgM-κ	4 y	PS-D	V_H3a VH26 (Ref. 91)	NK
SD15	IgA2-κ	11 y	PS	V_H3a VH26	NK
16M3C8	IgG2-λ	Adult§	PS	V_H3a VH26	$V_\lambda II$ 2.1

* Columns show name of hybridoma cell line, isotype of secreted anti-Hib polysaccharide antibody, age of subject at immunization, vaccine formulation (PS is plain polysaccharide, PS-D is Hib PS covalently linked to diphtheria toxoid), and most closely homologous germline V_H and V_κ/V_λ segments identified. Ig V_L, light chain variable region Ig.

† Manuscript submitted for publication. This sequence is identical to the cDNA clone M85 reported by Schroeder and Wang (76).

‡ NK, not known.

§ Pool of three adult donors.

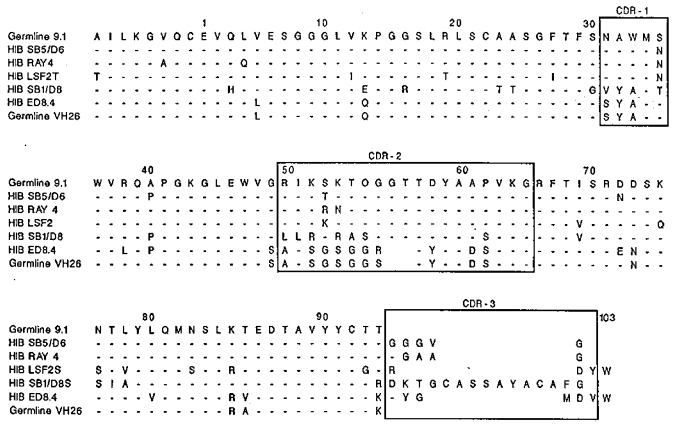

Fig. 2. Anti-Hib polysaccharide Ig V_H genes. Shown are the translated amino acid sequences of five anti-Hib polysaccharide specific Ig V_H genes and, for comparison, the translated amino acid sequences of two candidate germline genes, 9.1 (13) and V_H26 (92). CDR are boxed. Sequences illustrate the high homology of hybridoma V_H sequences and the differences produced within CDR-3 regions by combinatorial and junctional diversifiers. [From Adderson *et al.* (7), with permission.]

The restriction in both heavy and light chain variable region genes may explain the poor response to Hib vaccines and the increased incidence of invasive Hib disease in some individuals and ethnic groups. These subjects may lack one or more of these important gene segments, leading to the inability to produce antibody of optimal affinity or specificity. An individual with a deletion of the germline gene encoding the predominant κ light chain has been described. This individual produced anti-Hib polysaccharide antibody with other less commonly expressed light chains (89). In the more restricted heavy chain variable region gene response, it may not be possible to rely on alternative germline elements in the event of such a genetic polymorphism.

Although the V_H gene segments that appear most likely to be used in the anti-Hib polysaccharide antibody response are known to be expressed in the fetus, variable region gene use in the developing infant has not been closely studied. Polysaccharide-protein conjugate Hib vaccines are capable of eliciting antibody in children as young as 2 mo of age. These infants appear to use the V_κII gene segment encoding the predominant κ light chains identified in older subjects (96). Whether these antibodies are produced by an alternative B cell subset or whether B cells specific for Hib polysaccharide are relatively less responsive to plain polysaccharide antigen compared to T-dependent antigen formulations in early development is the subject of ongoing investigation.

Although significant progress has been made in understanding the molecular basis of antibody diversity, major questions still remain unanswered. Current research is focused on identification of the recombinase complex responsible for Ig gene rearrangement and the understanding of selective gene segment use both in individual B cell subsets and in different developmental stages of humans and other organisms. The molecular basis of somatic hypermutation, which is responsible for affinity maturation of the immune response, remains to be elucidated. Finally, the clinical impact of variable region gene deletion or polymorphisms on the development of the normal immune response and on disorders of immunodeficiency, autoimmunity, and malignancy is an active area of contemporary immunologic research (reviewed in Refs. 99–102).

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