Germline Variable Region Gene Segment Derivation of Human Monoclonal Anti-Rh(D) Antibodies

Evidence for Affinity Maturation by Somatic Hypermutation and Repertoire Shift

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Abstract

To date, there has been no systematic study of the process of affinity maturation of human antibodies. We therefore sequenced the variable region genes (V genes) of 14 human monoclonal antibodies specific for the erythrocyte Rh(D) alloantigen and determined the germline gene segments of origin and extent of somatic hypermutation. These data were correlated with determinations of antibody affinity. The four IgM antibodies (low affinity) appear to be derived from two germline heavy chain variable region gene segments and one or two germline light chain variable region gene segments and were not extensively mutated. The 10 IgG antibodies (higher affinity) appear to be derived from somatic hypermutation of these V gene segments and by use of new V gene segments or V gene segment combinations (repertoire shift). Affinity generally increased with increasing somatic hypermutation; on average, there were 8.9 point mutations in the V gene segments of the four IgM antibodies (K_a = 1–4 × $10^7/M^{-1}$) compared with 19 point mutations in the V gene segments of the 10 IgG antibodies. The four highest affinity antibodies (K_a = 0.9–3 × $10^9/M^{-1}$) averaged 25.5 point mutations. The use of repertoire shift and somatic hypermutation in affinity maturation of human alloantibodies is similar to data obtained in inbred mice immunized with haptens. (*J. Clin. Invest.* 1992. 90:2481–2490.) Key words: polymerase chain reaction • immunoglobulin heavy chain variable region • immunoglobulin light chain variable region • human hybridomas • affinity maturation

Introduction

The variable regions of the heavy (VH)[1] and light (Vκ or Vλ) chains of antibodies secreted by B lymphocytes are coded for by genes that have been derived by rearrangement of germline gene segments. The rearranged VH gene is derived from a germline V gene segment, diversity (D) segment, and joining (J) segment, and the rearranged Vκ or Vλ from a V gene segment and J segment. In humans, it has been estimated that there are ~ 75–100 VH germline gene segments (1, 2), ≥ 32 D segments (3), 6 JH segments (4), ~ 50 Vκ gene segments (5), 5 Jκ segments (6), and probably 4 functional Jλ segments (7, 8). There is little information on the total number of Vλ light chain gene segments but seven germline sequences have been reported (9–15).

Analysis of human V gene segment usage indicates that VH and Vκ gene families are used approximately in proportion to the number of germline members in unselected B cells (16) but that V gene segment usage may be restricted in response to self-antigens (17, 18). In three different murine models using inbred mice immunized with haptens (19–25), there is restricted V gene segment usage in the primary antibody response with few mutations in the V genes. The secondary and tertiary responses in mice are associated with higher affinity antibodies and somatic hypermutation of the original V gene partners together with a shift to new V gene partners (repertoire shift). However, there are no data available on the process of affinity maturation in a genetically diverse human population or in either mice or humans in response to protein or carbohydrate antigens.

Therefore, we have examined the process of affinity maturation of human antibodies generated in response to immunization with the erythrocyte rhesus (D) antigen [Rh(D)] (26). Specifically, we sequenced the V genes of 14 human monoclonal anti-Rh(D) antibodies and compared them to a database of germline sequences to determine V gene segment usage and extent of somatic hypermutation. The analysis indicates that there is preferential use of particular VH, VL, and JH germline gene segments, especially by the IgM antibodies, in this antigen-antibody system. By correlating affinity constant, isotype, germline gene of derivation, and extent of somatic hypermutation, we demonstrate that affinity maturation in this system occurs by somatic hypermutation and a repertoire shift to V genes or V gene combinations not seen in the IgM response.

Methods

Human monoclonal antibodies. The monoclonal anti-D antibodies were derived from hyperimmunized Rh-negative blood donors Fo (Fog-1, Fog-3, Fom-1, Fom-A), Ha (Ham-B), Hs (Gad-2, Mad-2), and Re (Reg-A) as previously described (27) and also from donor Oa (Oak-3, Og-31). All these antibodies were produced by EBV transformation and the formation of human–mouse heterohybridomas. The antibodies Brad-3 and Jac-10 were obtained from two different donors; they were derived from EBV-transformed B lymphocytes and were kindly supplied by Dr. Belinda Kumpel (United Kingdom Transplant

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1. *Abbreviations used in this paper:* PCR, polymerase chain reaction; Rh(D), erythrocyte rhesus (D) antigen; VH, heavy chain variable region; Vκ, kappa light chain variable region; VL, light chain variable region; Vλ, lambda light chain variable region.

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Service, Bristol, United Kingdom) (28). All the cell lines were clonal. All donors had been immunized ≥ 10 times with D-positive red cells at approximately yearly intervals. The functional affinity constants of all of these antibodies, except Jac-10, have been previously determined (references 29 and 30, and Gorick, B., and N. Hughes-Jones, unpublished data).

Cloning and sequencing of antibody V genes. The cloning and sequencing of the V genes of the antibodies Fog-1, Fom-1, and Fom-A was carried out as previously described (31). For the antibodies Brad-3, Fog-3, Gad-2, Ham-B, Jac-10, Mad-2, Oak-3, Og-31, and Reg-A, total RNA was prepared from ~ 10^7 cells using the method of Favaloro et al. (32). For first-strand cDNA synthesis, 10 μg of total RNA in 20 μl water was heated at 70°C for 3 min, cooled on ice, and added to a 30-μl reaction mixture containing 140 mM KCl, 50 mM Tris·HCl (pH 8.1, at 42°C), 8 mM $MgCl_2$, 10 mM DTT, 500 μM deoxythymidine triphosphate, 500 μM deoxycytidine triphosphate, 500 μM deoxyadenine triphosphate, and 500 μM deoxyguanine triphosphate (nucleotide triphosphate [500 μM dNTPs]), 80 U of human placental RNAse inhibitor, and 10 pmol of HuVH1FOR (5′-CTT GGT GGAG/TGC TGA G/TGA GAC GGT GAC C-3′) (33) or HuVκ1FOR (5′-GGT GCA GCC ACA GTA CGT TAG ATC TCC A-3′) (33) or HuVλFOR (5′-GGA ATT CTT ATG AAG ATT CTG TAG GGG CCA C-3′) as appropriate. 2 μl (50 U) of avian myeloblastosis virus reverse transcriptase was added, the mixture was incubated at 42°C for 1 h, heated to 100°C for 3 min, cooled on ice, and centrifuged for 2 min. For polymerase chain reaction (PCR) amplification, a 50-μl reaction mixture was prepared containing 5 μl of the supernatant from the cDNA synthesis; 20 pmol of HuVH1FOR, HuVκ1FOR, or HuVλFOR as appropriate; 20 pmol of an equimolar mixture of HuVH1/5BACK (5′-CAG CTG CAG CTG CAG CAG TCT GG-3′), HuVH2BACK (5′-CAG GTC AAC CTG CAG GAG TCT GG-3′), HuVH3BACK (5′-GAG GTG CAG CTG CAG GAG TCT GG-3′), HuVH4BACK (5′-CAG GTG CAG CTG CAG GAG TCG GG-3′), and HuVH6BACK (5′-CAG GTA CAG CTG CAG CAG TCA GG-3′) (33), or HuVκl/4BACK (5′-GAC ATC CAG CTG ACC CAG TCT CC-3′), HuVκ2BACK (5′-GAT ATT CAG CTG ACT CAG TCT CC-3′), and HuVκ3BACK (5′-GAA ATT CAG CTG ACG CAG TCT CC-3′) (33); or HuVλ1BACK (5′-AAC CAG CCA TGG CCC AGT CTG TGT TGA CGC AGC C-3′), HVλ3ABACK (5′-AAC CAG CCA TGG CCT CCT ATG TGC TGA CTC AGC C-3′), and HVλ3BBACK (5′-AAC CAG CCA TGG CCT CTG AGC TGA CTC AGG ACC C-3′) as appropriate; 250 μM dNTPs; 50 mM KCl; 100 mM Tris·HCl (pH 8.3); 1.5 mM $MgCl_2$; 175 μg/ml BSA; and 1 μl (5 U) Thermus aquaticus (Taq) DNA polymerase (Cetus Corp., Emeryville, CA). The reaction mixture was overlaid with paraffin oil and subjected to 30 cycles of amplification. The cycle was 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension). The product was analyzed by running 5 μl on a 1.8% agarose gel. The remainder was run on a 1.8% agarose gel in Tris-acetate EDTA buffer, purified with GeneClean (BIO 101, Inc., Vista, CA) and the amplified DNA was end-filled, phosphorylated, ligated into Sma1-cut dephosphorylated pUC18 vector, and used to transform DH5α-competent *Escherichia coli* (34). At least two clones from separate PCR amplifications for each hybridoma were sequenced and in every case the duplicates gave identical results (apart from nucleotides within the primer region in some cases).

Determination of the germline origin of the VH, D, JH, VL, and JL segments and extent of somatic hypermutation. Computer analysis of DNA sequencing data was performed using the sequence analysis software package of the Genetic Computer Group (version 6.2) of the University of Wisconsin (35). The VH genes were compared with the 84 germline gene segments present in the VH directory compiled by Tomlinson et al. (36); VL genes were compared with 28 published kappa germline gene segments (37–53), seven published lambda gene segments (9–15), and one unpublished lambda gene segment (J. D. Marks and G. Winter). The germline gene with the closest homology was considered to be the origin of the rearranged gene. D regions, defined as the nucleotides not clearly derived from the VH or JH genes, were compared with the 32 D segments and two DIR segments that have been described (3, 54–60) using Bestfit and Gap programs. The JH genes were compared with the six JH segments (4). The JL genes were compared with either the published Jκ (6) or the Jλ (7, 8) segments.

Results

Assignment of germline gene segments. Heavy chain germline gene segment assignments could be confidently made for all the IgM antibodies and 7 of the 10 IgG antibodies (Table I). In these antibodies, the next most homologous germline gene segment differed by at least five additional nucleotides. Furthermore, the additional differences were at exactly the same position for the multiple antibodies derived from either VH4-21 (61) or hv3019b9 (62). In three of the IgG antibodies, Oak-3, Pag-1, and Fog-B, the germline VH segment assignment was less certain since the germline gene segment with the next closest homology differed by only 1 or 2 bp. Light chain germline gene segment assignments could be confidently made for all the IgM antibodies and for all but three of the IgG antibodies (Table I).

Anti-Rh(D) VH gene sequences: germline gene segment usage and extent of somatic hypermutation. The nucleotide and deduced amino acid sequences of the expressed VH genes of the 14 anti-Rh(D) antibodies are shown in Figs. 1, 2, and 3. The VH genes belong to both the VH3 (seven antibodies) and the VH4 families (seven antibodies) and were derived from a small number of germline gene segments. The VH genes from the four IgM antibodies appear to be derived from only two germline VH gene segments, hv3019b9 (VH3) and VH4-21 (VH4) (or its polymorphic allelic form, Tou-VH4-21 [63]), with an average of only 3.3 point mutations per gene. In contrast, the VH genes from the 10 IgG antibodies appear to be derived not only from the original two germline gene segments used by the IgM antibodies (six antibodies) but from an additional four germline gene segments as well (hv3005 [64], V71-4 [65], V2-1 [66], and H11 [67]). In addition, the VH genes of the IgG antibodies were more somatically mutated, with an average of 9.6 point mutations per gene.

JH segment usage also appears to be restricted with 11 of the VHs using the JH6 segment and three using the JH4 segment (Fig. 4). Two polymorphic forms of the JH6 segment were used that correspond to the JH6b and JH6c forms (68) and that differ from the JH6 gene originally described (4).

There does not appear to be an obvious restriction in D segment usage. All 14 D regions could be partially matched to one of 17 different D segments (Fig. 5), and the only D segment used more than once was DN1 (four times).

Anti-Rh(D) VL gene sequences: germline gene segment usage and extent of somatic hypermutation. The nucleotide and deduced amino acid sequences of the expressed VL genes of 12 of the 14 anti-Rh(D) antibodies are shown in Figs. 3, 6, and 7 (two of the Vλ genes, from hybridomas Ham-B and Oak-3, could not be amplified). The eight Vλ genes and four Vκ genes sequenced appear to be derived from a small number of germline gene segments. Thus the VL genes from three IgM antibodies were derived from a single germline Vλ gene segment, IGLV3S1 (14), with an average of 6.0 point mutations per gene. The VL genes from nine IgG antibodies appear to be derived from IGLV3S1 (two antibodies) and from an additional five germline gene segments as well (Humkv325 [48], L11 [52], 02 [53], VλIII.1 [13], and DPλ1A). In addition, the VL genes of the IgG antibodies were more somatically mu-

Table I. Germline V Gene Segment Derivation of Human Anti-Rh(D) Antibodies

Assigned germline gene	Antibody	Isotype	Additional nucleotide differences of V genes with next closest homology			
			Germline gene	Additional differences	Germline gene	Additional differences
Heavy chain						
VH4-21	Fom-A	IgM	V58	13	VH4-11	21
	Mad-2	IgM	V58	13	VH4-11	18
	Fom-1*	IgM	V58	14	VH4-11	22
	Og-31	IgG	V58	13	VH4-11	18
	Fog-1	IgG	V58	13	VH4-11	21
3019b9	Ham-B	IgM	1.9III	5	hv3005f3	7
	Reg-A	IgG	1.9III	5	hv3005f3	7
	Gad-2	IgG	1.9III	5	hv3005f3	7
	Fog-3	IgG	1.9III	5	hv3005f3	7
	Brad-3	IgG	1.9III	5	hv3005f3	7
H11	Jac-10	IgG	DP-58	25	DP-51	26
hv3005‡	Oak-3	IgG	GL-SJ2	2	1.9III	4
V71-4	Pag-1	IgG	VH4-11	1	VH4-16	2
V2-1	Fog-B	IgG	VH4-18	1	DP-65	12
Light chain						
IGLV3S1	Fom-A	IgM	VλIII.1	69	IGLV1S2	83
	Mad-2	IgM	VλIII.1	61	IGLV1S2	79
	Fom-1	IgM	VλIII.1	67	IGLV1S2	87
	Og-31	IgG	VλIII.1	64	IGLV1S2	77
	Gad-2	IgG	VλIII.1	67	IGLV1S2	81
VλIII.1	Pag-1	IgG	IGLV3S1	26	IGLV1S2	44
	Jac-10	IgG	IGLV3S1	63	Huml v117	65
02	Fog-3	IgG	L11	13	Va′	15
Humkv325	Reg-A	IgG	Humkv305	6	Vh	17
	Brad-3	IgG	Humkv305	6	Vh	15
DPλ1A	Fog-B	IgG	Huml v117	2	Vλ1.1	19
L11	Fog-1	IgG	HK137	4	Vd	4

References for germline genes are as follows: heavy chains: 1.9III (1). DP-51, DP-58, and DP-65 (36); hv3005 (61); V71-4 (62); V58 and V2-1 (63); VH4-21, VH4-11, VH4-18, and VH4-16 (64); 3019b9 and hv3005f3 (65); H11 (67); GL-SJ2 (76). Light chains: Vλ1.1 (9); IGLV1S2 (11); VλIII.1 (13); IGLV3S1 (14); huml v117 (15); HK137 (38); Va′ and Vd (39); Vh (41); Humkv305 (46); Humkv325 (48); L11 (52); 02 (53).
* Fom-1 is derived from Tou-VH4-21, which is an allele of the gene VH4-21.
‡ hv3005 and hv3005f3 are polymorphisms.

tated, with an average of 9.4 mutations per gene. Pag-1 was excluded from these calculations since the number of nucleotide differences from the germline gene with the closest homology, VλIII.1 (52 differences), suggests a different and unknown germline gene segment of origin.

All of the Jλ segments were derived from the Jλ2 or 3 segment, which have identical sequences, except for Og-31, which was derived from the Jλ1 segment (Fig. 4). Three of the Jκ segments were derived from the Jκ2 segment and one was derived from the Jκ3 segment (Fig. 4).

Correlation between antibody affinity, isotype, germline derivation, and extent of somatic hypermutation. The four IgM anti-Rh(D) antibodies (lowest affinity) appear to be derived from only two VH germline gene segments and one or two germline Vλ gene segments, and on average there were 15.7 mutations/1,000 bases (Tables II and III). In contrast, the IgG antibodies were of higher affinity, were more somatically mutated (34.6 mutations/1,000 bases) (Tables II and III), and showed evidence of repertoire shift in V gene segment usage (an additional four VH and five VL germline gene segments used). The mutations occurred with greater frequency in the CDRs (Table III) than in the framework regions and frequently resulted in amino acid changes in the antigen-binding loops (Fig. 3).

The change in the derivation of VH and VL genes, which is characteristic of repertoire shift, is illustrated by the five antibodies from the donor Fo. The two IgM antibodies (Fom-A and Fom-1) appear to be derived from VH4-21 and IGLV3S1 (Table IV). The heavy chains of the three IgG antibodies appear to be derived either from the same VH gene (Fog-1), the hv3019b9 VH gene (Fog-3), or a VH gene (V2-1) not seen in the IgM response (Fog-B) (Table IV). The light chains of the three IgG antibodies appear to be derived from a Vλ gene (Fog-B) and two Vκ genes (Fog-1 and Fog-3) not seen in the IgM response (Table IV).

Within the IgG antibodies, the V genes tended to be more somatically mutated and/or paired with a different V gene partner as their affinity increased (Table II). This pattern is clearly seen in both the five antibodies derived from the donor Fo (Table IV) and also in the four highest affinity antibodies;

Figure 1. Nucleotide sequences of VH3 family genes of anti-Rh(D) antibodies compared with the most homologous germline gene. Dashes indicate identity, and nucleotide differences shown in lower case were coded for by the primer used in the PCR. w, A or T; r, A or G; and s, C or G. CDRs are underlined. References for germline genes are as follows: hv3019b9 (65), hv3005 (61), and H11 (67). These sequence data are available from EMBL/Genbank/DDBJ under accession numbers: X64149, X64151, X64158, X64154, X64157, X64148, and X64155.

the Fog-3, Fog-B, Fog-1, and Brad-3 VHs are heavily mutated derivatives (48 mutations/1,000 bases) of either the VH genes used in the IgM response (Fog-3, Fog-1, and Brad-3) or a VH gene not used in the IgM response (Fog-B) whereas the VLs are heavily mutated derivatives (40 mutations/1,000 bases) of VL genes not used in the IgM response.

Discussion

V H, JH, and VL gene segment usage is restricted in the human anti-Rh(D) response. There are ~ 75–100 human germline VH gene segments (2), 50 human germline Vκ gene segments (5), and ≥ 8 human germline Vλ gene segments (9–15, and

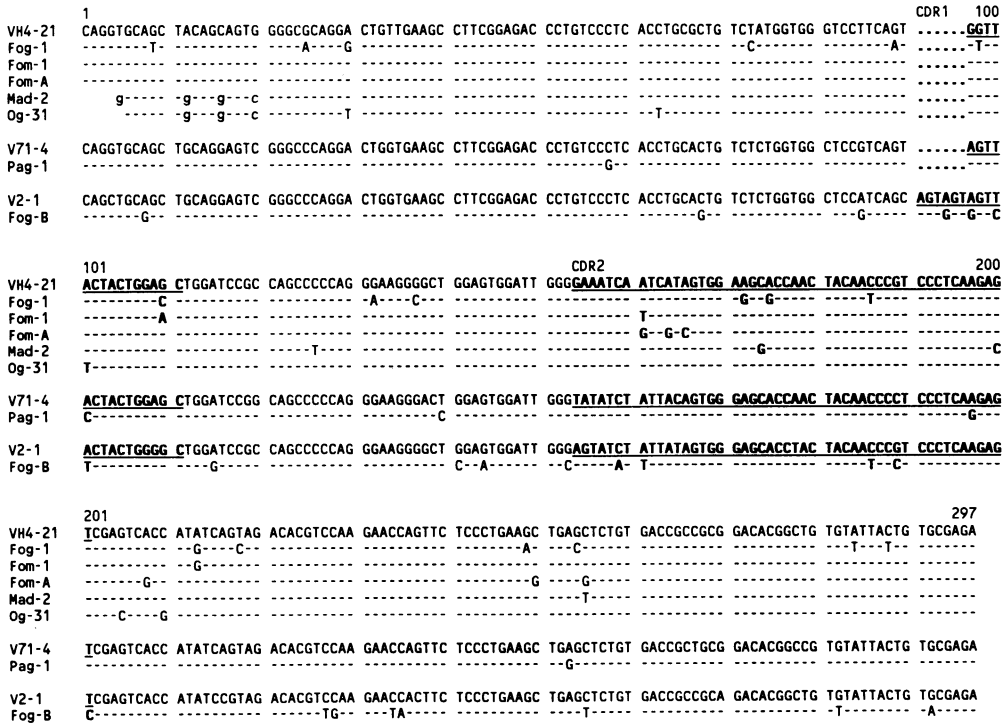

Figure 2. Nucleotide sequences of VH4 family genes of anti-Rh(D) antibodies compared with the most homologous germline gene. A gap of six nucleotides has been introduced at positions 91–96 where necessary to align the sequences. Dashes indicate identity, and nucleotide differences shown in lower case were coded for by the primer used in the PCR. CDRs are underlined. References for germline gene are as follows: VH4-21 (64), Tou-VH4-21 (66), V71-4 (62), and V2-1 (63). These sequence data are available from EMBL/Genbank/DDBJ under accession numbers: X64150, X64153, X64159, X64156, X64152, X52111, and X52110.

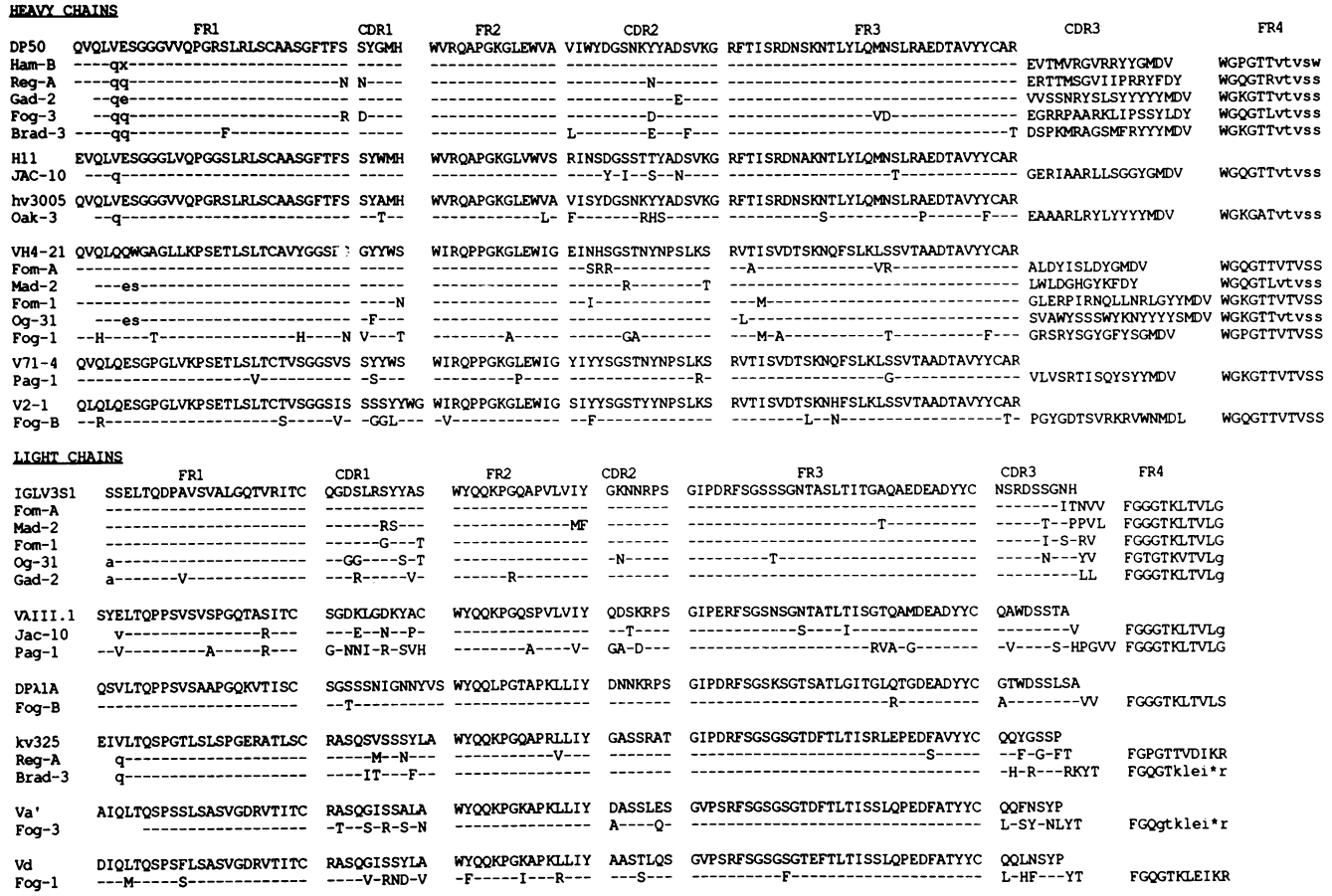

Figure 3. Deduced amino acid sequences of VH and VL regions of anti-Rh(D) antibodies compared with the most homologous germline gene. Dashes indicate identity, and differences shown in lower case were coded for by the primer used in the PCR reaction. References for the germline genes can be found in Figs. 1, 2, 6, and 7.

our unpublished data). Despite the large number of germline V gene segments, we have shown that V gene segment usage in the human antibody response to the Rh(D) alloantigen is restricted. This repertoire restriction seems remarkable considering that the donors were hyperimmunized and antibodies were made from seven different donors; however, the true heterogeneity of an antigen-specific immune response is unknown. 10 of the 14 VH genes appear to be derived from one of two VH germline gene segments (hv3019b9 and VH4-21) and these germline gene segments were used by the antibodies from five of the seven donors. 5 of the 12 VL genes were derived from one Vλ germline gene segment (IGLV3S1) and this germline gene segment was used by antibodies from three of the seven donors. The V gene segment usage of the IgM antibodies is even more restricted with all four VH genes using hv3019b9 or VH4-21 and the three Vλ genes that were sequenced using IGLV3S1. The restriction does not result from EBV transformation of B cells nor is it present in V genes not subjected to antigen stimulation (69).

Our results confirm and clarify previous observations on VH gene segment usage of anti-Rh(D) antibodies. Natvig et al. (70) used family-specific antisera to determine that polyclonal anti-Rh(D) from 40 of 45 donors used the VH2 family; however, the serologic reagents would not have distinguished between VH2s and the closely related VH4s, a family whose existence was unknown at that time. More recently, Thompson et al. (71) isotyped human monoclonal anti-Rh(D) antibodies and analyzed VH gene segment usage with a monoclonal antibody (9G4) against the VH4-21 idiotype. All 13 IgM anti-Rh(D) antibodies expressed lambda light chains and 9 of the 13 expressed the 9G4 idiotype (VH4.21 V gene segment). In contrast, only 3 of 13 IgG antibodies expressed lambda light chains and, unlike our results, none expressed the 9G4 idiotype. However the use of an anti-idiotype antibody to determine V gene segment usage of IgG antibodies is unreliable since somatic hypermutation might abolish the interaction with the monoclonal antibody. Thus, Thompson et al. (71) found that Fog-1 lacks the 9G4 idiotype whereas our sequence analysis indicates that the Fog-1 antibody is derived from the VH4.21 germline gene segment. Presumably, the large number of mutations in the VH gene of Fog-1 has led to a loss of the idiotype recognized by the 9G4 monoclonal antibody.

The expressed VH genes selectively use the JH6 segment (11/14, 79%). This contrasts with other examples of expressed VH genes where only 15/99 (22%) (68) and 10/52 (19%) (33) used the JH6 segment. Preferential JH6 segment usage could be due to structural requirements since in 9 of 11 instances it results in the protein sequence tyr-x-met-glu-val in the CDR3 loop (Fig. 3).

We found no evidence for restricted Jκ, Jλ, or D segment usage. It appears that 17 different D or DIR segments may have been used and although the DN1 segment was used four times,

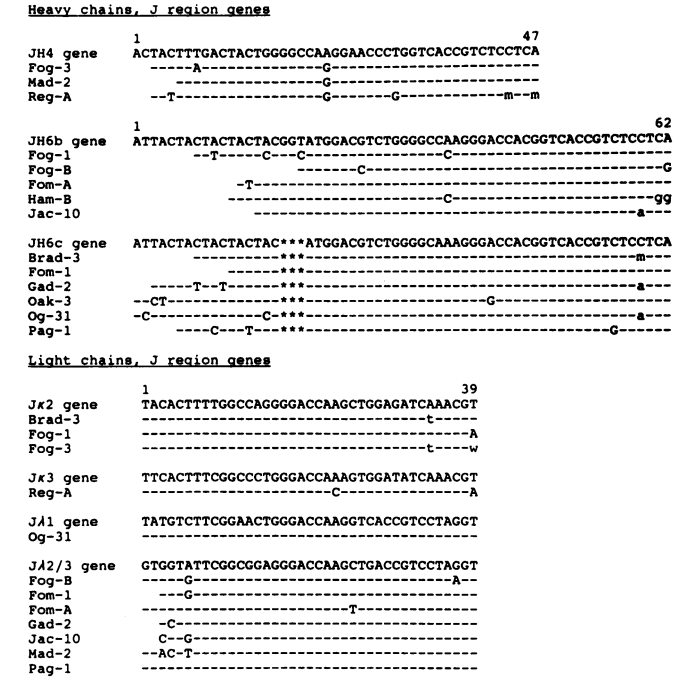

Figure 4. Nucleotide sequences of the JH and JL regions of anti-Rh(D) antibodies compared with the most homologous germline gene. w, A or T; and m, A or C. Asterisks indicate deletions, and nucleotide differences shown in lower case were coded for by the primer used in the PCR. References for the germline genes are as follows: JH4 and 6 (4), Jκ2 and 3 (6), Jλ 1 and 2 or 3 (7).

it is also overpresented in unselected VHs (72). However, assignation of an expressed D region to its germline D origin cannot always be made with certainty because of the high rate of somatic mutation, N segment addition, and the probability that not all of the D segments have been sequenced (72). There were seven instances where the D region may have been derived by the joining of two separate D segments (68, 72) and three instances where the expressed D regions may have been derived from inverted D–D fusions (72).

Affinity maturation of the human anti-Rh(D) response occurs by somatic hypermutation of V gene segments used in the IgM response and by a repertoire shift to V gene segments not seen in the IgM response. To date, there has been no analysis of the genetic mechanism of affinity maturation of antibodies generated in response to glycoprotein immunogens. The Rh(D) antigen is a protein complex known to be one of the most immunodominant structures on the erythrocyte membrane. We have shown that the maturation of the human alloantibody response to the Rh(D) antigen occurs by somatic hypermutation and by a repertoire shift to V gene partners not used in the IgM (primary) response. Since the donors had received at least 10 immunizations before the time B lymphocytes were obtained, it is not clear whether the IgM antibodies arose during an earlier primary response and were perpetuated as memory cells or whether they arose during the most recent antigen challenge (usually 14–28 d before harvesting the B cells). Whatever the mechanism, it is reasonable to suppose that they are representative of the primary response structure.

The IgM antibodies that were sequenced (low affinity) used only two germline VH gene segments (hv3019b9 and VH4-21) and one VL germline gene segment (IGLV3S1). Although we have sequenced only four IgM antibodies, our results are probably representative since an analysis of an additional nine anti-Rh(D) IgM antibodies using isotyping and an antibody recognizing the VH4-21 idiotype demonstrated that six heavy chains used the VH4-21 germline gene segment and all nine used a λ light chain (71).

The higher affinity IgG antibodies arose as a result of both a repertoire shift to V gene segments not used in the IgM response and by somatic hypermutation. Repertoire shift occurred for both the light chain (7 of 10 antibodies, including the 6 with the highest affinity) and the heavy chain (4 of 10 antibodies). The fact that repertoire shift can be observed within the five antibodies made from the donor Fo (Table IV) indicates that it is not an artifact resulting from the use of different donors.

Affinity generally increased with increasing somatic hypermutation: 8.9 point mutations in the V gene segments of the IgM antibodies (15.7 mutations/1,000 bases) compared with 19.0 point mutations in the V gene segments of the IgG antibod-

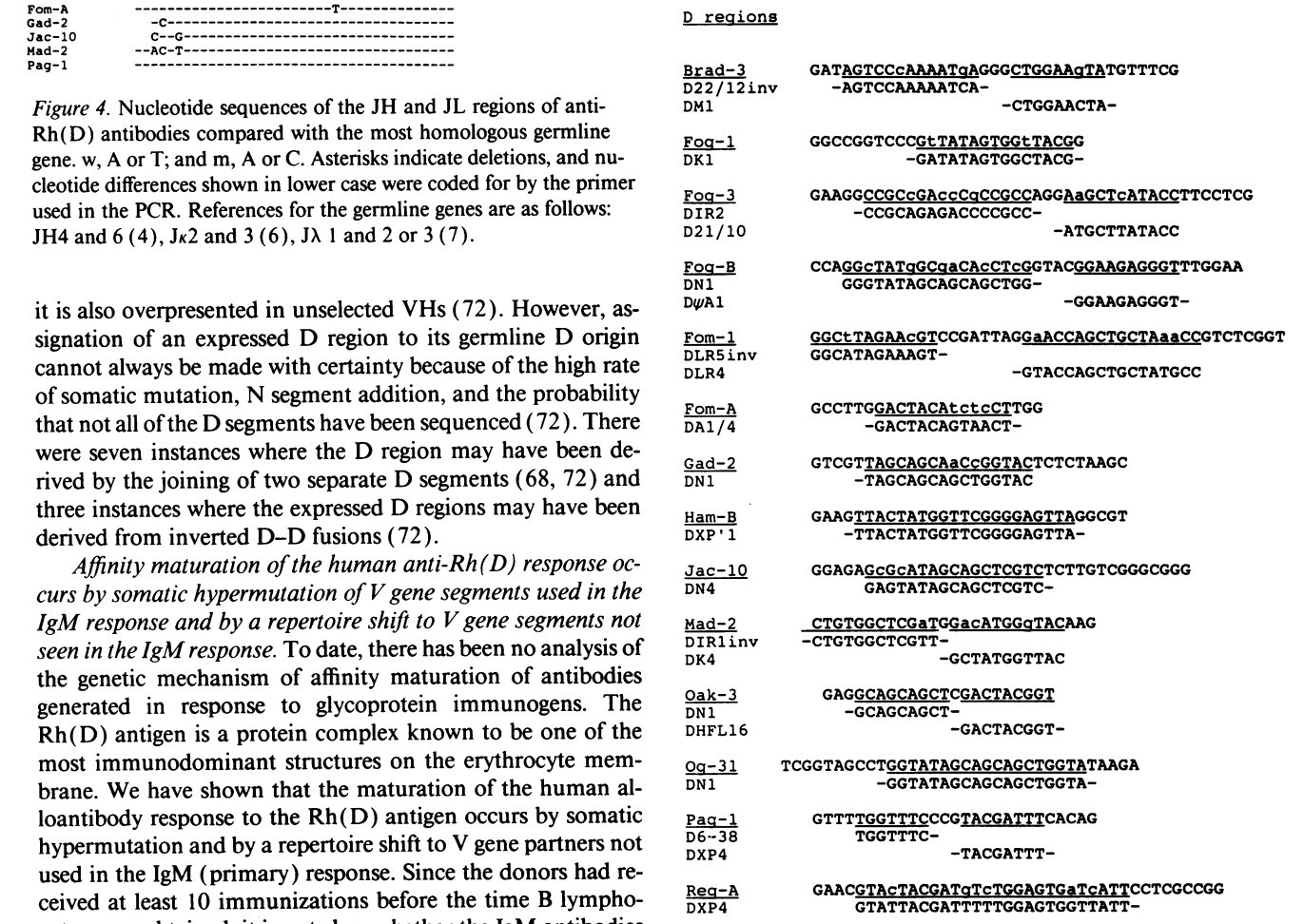

Figure 5. Nucleotide sequences of the D segments of anti-Rh(D) antibodies compared with the germline D gene segments that have the highest homology. Mismatches in the expressed gene are shown in lower case letters. Dashes at the end of the germline gene indicate that nucleotides have been omitted beyond this point. Inv indicates that the D gene is inverted. One of the matches for Fog-B, DψA1, may be a pseudogene but this assignment has been included as other matches show considerably less homology. References for the germline genes are 3, 55, and 56.

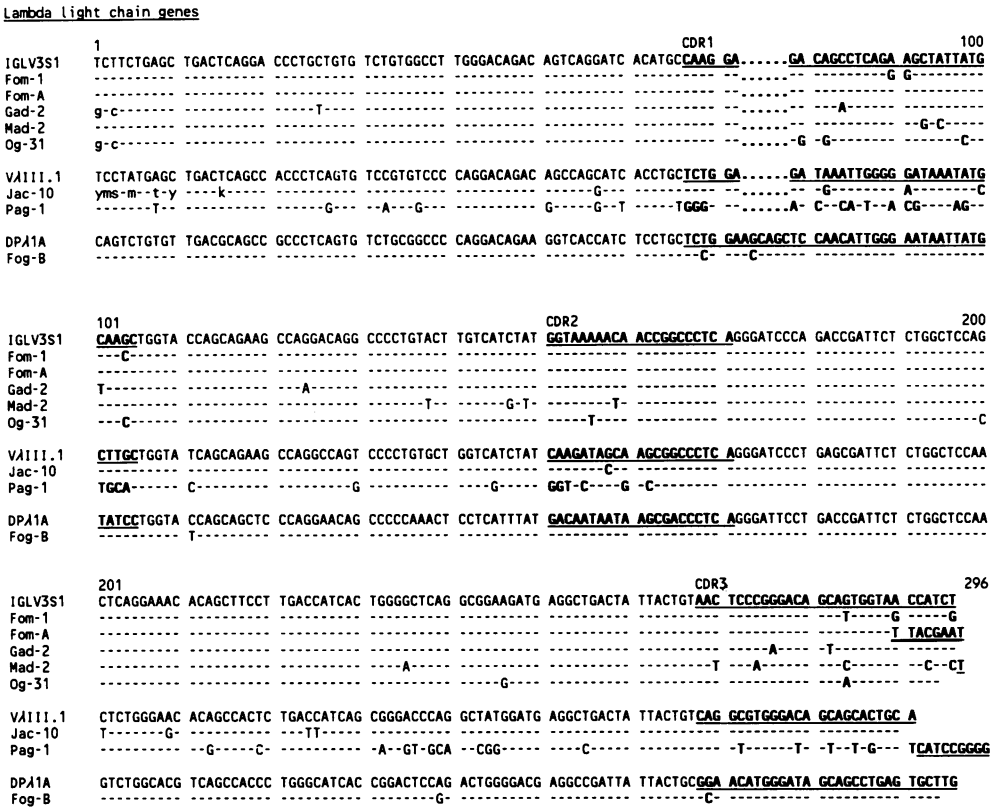

Figure 6. Nucleotide sequences of the Vλ genes of anti-Rh(D) antibodies compared with the most homologous germline gene. Dashes indicate identity, and nucleotide differences shown in lower case were coded for by the primer used in the PCR. m, A or C; s, C or G; and y, C or T. CDRs are underlined in the germline gene. Nucleotides probably derived from joining diversity or N segment addition are underlined in the rearranged gene. References for germline genes are as follows: IGLV3S1 (14), VλIII.1 (13), and DPλ1A (J. D. Marks and G. Winter, unpublished results). These sequence data are available from EMBL/Genbank/DDBJ under accession numbers: X64165, X64166, X64161, X64160, X64167, X64169, X52112, and X52109.

Figure 7. Nucleotide sequences of the Vκ genes of anti-Rh(D) antibodies compared with the most homologous germline gene. A gap has been introduced at positions 94–97 where necessary to align the sequences. Dashes indicate identity, and nucleotide differences shown in lower case were coded for by the primer used in the PCR. k, G or T; w, A or T; y, C or T; and x, ambiguous. CDRs are underlined in the germline gene. Nucleotides probably derived from joining diversity or N segment addition are underlined in the rearranged gene. References for germline genes are as follows: Humkv325 (48), L11 (52), and 02 (53). These sequence data are available from EMBL/Genbank/DDBJ under accession numbers: X64162, X64168, X64163, and X64164.

Table II. Correlation between Antibody Affinity, Isotype, Germline Derivation and Extent of Somatic Hypermutation

Antibody	Isotype	K_a	VH gene		VL gene		Total VH and VL point mutations
			Germline	Differences from germline	Germline	Differences from germline	
		M^{-1}					
Fom-A	IgM/λ	1.0×10^7	VH4-21	6	IGLV3S1	0	6
Mad-2	IgM/λ	1.4×10^7	VH4-21	4	IGLV3S1	12	16
Ham-B	IgM/λ	1.9×10^7	hv3019b9	1	ND*	ND*	1‡
Fom-1	IgM/λ	4.0×10^7	Tou-VH4-21	2	IGLV3S1	6	8
Oak-3	IgG/λ	1.3×10^8	hv3005	13	ND*	ND*	13‡
Reg-A	IgG/κ	2.0×10^8	hv3019b9	4	Humkv325	7	11
Og-31	IgG/λ	2.2×10^8	VH4-21	5	IGLV3S1	8	13
Gad-2	IgG/λ	3.9×10^8	hv3019b9	4	IGLV3S1	6	10
Pag-1	IgG/λ	6.0×10^8	V71-4	5	VλIII.1§	52§	57§
Fog-3	IgG/κ	9.0×10^8	hv3019b9	8	02	11	19
Fog-B	IgG/λ	2.0×10^9	V2-1	23	DPλ1A	5	28
Fog-1	IgG/κ	2.2×10^9	VH4-21	18	L11	18	36
Brad-3	IgG/κ	3.0×10^9	hv3019b9	8	Humkv325	11	19
Jac-10	IgG/λ	ND‖	H11	8	VλIII.1	9	17

* The sequences of Ham-B and Oak-3 could not be determined but the light chains were shown to be of the lambda class by serological means (our unpublished data).

‡ The total only includes the value for the heavy chain.

§ The number of point mutations is an overestimate since VλIII.1 is probably not the germline gene from which Pag-1 is derived.

‖ The K_a value for Jac-10 has not been determined.

ies (34.6 mutations/1,000 bases) and 25.5 point mutations for the four highest affinity antibodies. The higher rate of mutation seen in the antigen-binding CDRs (63.1 mutations/1,000 bases) compared with the framework regions (17.3 mutations/1,000 bases) is consistent with antigen-driven selection.

For individual V gene segments, however, there are exceptions to affinity increasing with an increasing number of mutations, presumably because of the effect of different V gene partners or neutral or detrimental point mutations (73). For example, the VH gene segments of Fom-A and Oak-3 have multiple replacement substitutions in CDR2 not seen in the VH gene segments of higher affinity antibodies, which might be detrimental to antigen binding. It is also possible that in some instances the extent of somatic hypermutation has been overestimated due to incomplete knowledge of the germline sequences or polymorphism. Although this may be true for the Vλ genes, where only eight germline gene segments have been sequenced (references 9–15; J. D. Marks and G. Winter, unpublished data), it is less likely for the Vκ and VH genes. Of the estimated 50 functional Vκ germline gene segments (5), 28 functional gene segments have been sequenced (37–53), and of the 75–100 estimated VH gene segments (1, 2), 83 have been sequenced (for review see reference 36). Although five of the IgG rearranged genes could have been derived from a different germline gene, reassignment to the second- or thirdmost homologous gene would not have affected the degree of repertoire shift and would have increased the degree of somatic hypermutation.

The process of affinity maturation we have observed in genetically diverse humans in response to a highly immunogenic protein appears to be similar to that observed in inbred mice immunized with haptens (19–25). For example, in the murine response to phenyloxazolone, the low affinity antibodies appearing early in the primary response are predominantly derived from only two germline genes (Vκ-Ox1 and VH-Ox-1) and are either unmutated or minimally mutated from germline (19). In contrast, antibodies of higher affinity arise during the secondary and tertiary responses and result from somatic hypermutation of the primary response Vκ-Ox1 and VH-Ox-1 genes and from a repertoire shift to different VH and Vκ germline genes than those dominating the primary response (20, 21).

Table III. Rates of Somatic Mutation in the Frameworks and CDRs of IgM and IgG anti-Rh(D) Antibodies

	Framework		CDR		Total	
	Mutations/ 1,000 bases	Mutations/ total bases	Mutations/ 1,000 bases	Mutations/ total bases	Mutations/ 1,000 bases	Mutations/ total bases
IgM	7.5	11/1,467	39.7	20/504	15.7	31/1,971
IgG	21.3	78/3,658	72.4	93/1,285	34.6	171/4,943

Data include the V gene only and not nucleotides contributed by the D or J segments.

Table IV. Correlation between Antibody Affinity, Isotype, Germline Derivation and Extent of Mutation of the Five Antibodies Obtained from the Donor Fo

Antibody	Isotype	K_a	VH gene		VL gene		Total VH and VL point mutations
			Germline	Differences from germline	Germline	Differences from germline	
		M^{-1}					
Fom-A	IgM/λ	1.0×10^7	VH4-21	6	IGLV3S1	0	6
Fom-1	IgM/λ	4.0×10^7	Tou-VH4-21	2	IGLV3S1	6	8
Fog-3	IgG/κ	9.0×10^8	hv3019b9	8	02	11	19
Fog-B	IgG/λ	2.0×10^9	V2-1	23	DPλ1A	5	28
Fog-1	IgG/κ	2.2×10^9	VH4-21	18	L11	18	36

In summary, we have demonstrated that a small number of V gene segments are used in the human antibody response to immunization with the Rh(D) protein and that affinity maturation occurs as a result of somatic hypermutation and repertoire shift. The applicability of this result to other protein immunogens is unknown since no other systems have been studied in detail; however, studies of human autoimmune antibodies indicate that IgG but not IgM V genes are somatically mutated (74). It is unclear why the V gene segments that appear later during the affinity maturation process are not seen in the primary response. One possible explanation is that the pool of unstimulated B cells is biased towards the V gene segments seen in the IgM response. For example, Walter et al. (75) have shown by deletion mapping that 77% of rearrangements in EBV-transformed human B cells occur within the D-J-C proximal third of the VH locus. Alternatively, bias could result from the presence of enhancer-like sequences associated with some V gene segments (61). More detailed mapping and sequencing of the human V gene locus may help clarify this question.

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