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## Myeloma V<sub>L</sub> and V<sub>H</sub> Gene Sequences Reveal a Complementary Imprint of Antigen Selection in Tumor Cells

By Surinder S. Sahota, Regine Leo, Terry J. Hamblin, and Freda K. Stevenson

In multiple myeloma, sequence studies of V<sub>H</sub> genes used to encode clonal Ig in neoplastic plasma cells have shown a common pattern of extensive somatic hypermutation. A further consistent feature of these V<sub>H</sub> sequences is a complete lack of intraclonal variation. These findings indicate that the malignant cell arises at a mature, postfollicular stage of B-cell development. However, only a minority of cases have a distribution of somatic mutations in V<sub>H</sub> consistent with a prior role for antigen in selecting the B cell of origin. To complement these studies, and to take further the investigation of a role for antigen in the clonal history of myeloma, we have investigated tumor-derived V<sub>L</sub> sequences from bone marrows of 15 patients. All sequences (9V<sub>κ</sub> and 6V<sub>λ</sub>) were potentially functional and 5 of 15 had evidence for N-region

additions. All had undergone extensive somatic hypermutation, and showed no intraclonal variation. In 4 of 15 cases, the distribution of mutations revealed a significant ( $P < .05$ ) clustering of replacement mutations in the CDR sequences, indicating a role for V<sub>L</sub> in selection by antigen. Comparison with the V<sub>H</sub> sequences used by the same tumor cells showed that, if significant clustering was present, it was in either V<sub>H</sub> or V<sub>L</sub>, but not both. Altogether, 10 of 15 V-regions showed evidence for antigen selection, suggesting that the B cell of origin has behaved as a normal germinal center B cell. Deductions concerning a role for antigen selection may require both V<sub>H</sub> and V<sub>L</sub> sequences for validation.

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**M**ULTIPLE MYELOMA is a malignant tumor involving plasma cells. Neoplastic cells are found in the bone marrow (BM), and typically secrete a monoclonal Ig of IgG or IgA isotype. Although the major identifiable tumor population consists of plasma cells, there has been a great deal of debate concerning the nature of the malignant cell, with some early indications that this may be a less mature B cell capable of "feeding" the plasma cell compartment.<sup>1,2</sup> The advent of genetic technology aimed at Ig genes has allowed a more incisive investigation of the characteristics of myeloma clones. In fact, there have now been reports of a total of more than 50 sequences of V<sub>H</sub> genes used by tumor cells from patients' BM biopsies, and these have revealed common features.<sup>3-5</sup>

One conclusion is that usage of V<sub>H</sub> genes from the available repertoire appears to reflect no striking bias, with predominance of the large V<sub>H3</sub> family in line with serological analysis of myeloma proteins.<sup>6</sup> However, at the level of individual V<sub>H</sub> genes there may be some asymmetry in usage. For example, one gene, V<sub>4-34</sub>, commonly used by normal B cells, and mandatory for encoding IgM autoanti-red blood cell antibodies of I/i specificity in patients with cold agglutinin disease,<sup>7</sup> has so far not been found to be used by tumor cells in myeloma.<sup>5,8</sup> In all cases of myeloma, the V<sub>H</sub> genes have been found to be somatically hypermutated.<sup>3-5</sup> A further common feature is the lack of intraclonal variation in sequence, a finding that contrasts with the heterogeneity found in B-cell tumors of the germinal center.<sup>9,10</sup> This leads to the conclusion that the final tumorigenic event in myeloma has occurred at a postfollicular stage, when the cell is no longer influenced by the somatic hypermutation mechanism.<sup>11</sup> It argues against the concept that there is a "feeder" B cell, unless that cell has escaped the mutator before isotype switching.

However, IgM<sup>+</sup> B cells with V<sub>H</sub> sequences indicating a clonal relationship with the neoplastic plasma cells have been detected in some cases of myeloma.<sup>12,13</sup> Although there is some controversy about their frequency,<sup>14</sup> it appears that such cells do exist and presumably continue to proliferate. There is uncertainty as to their contribution to malignancy, and it is possible that these cells have undergone some, but not all, of the events leading to malignant behavior.<sup>4</sup>

A further problem in understanding development of myeloma lies in the role of antigen in selecting the V<sub>H</sub> sequences

of the tumor cell. If the cell of origin has been through somatic hypermutation, and antigen selection, before neoplastic transformation, this experience should be reflected in the V-gene sequences. For V<sub>H</sub> regions of antibody molecules, it is known that recognition of antigen can involve several sites, with CDR3 having a major influence.<sup>15</sup> However, replacement mutations in CDR1 and CDR2 have a significant role in affinity maturation.<sup>11,16</sup> For B-cell tumors, where the putative antigen is generally unknown, it is difficult to estimate involvement of CDR3 in recognition. In contrast, the possible clustering of replacement mutations in CDR1 and CDR2 which could be involved in affinity maturation can be analyzed. Rules to assess the significance of apparent clustering of replacement mutations compared with silent mutations have been developed.<sup>17</sup> When these rules were applied to the large panel of V<sub>H</sub> sequences from myeloma cells, only a minority of cases (10 of 52) showed statistically significant clustering in CDRs.<sup>5</sup> However, the antigen-binding site is known to involve both V<sub>H</sub> and V<sub>L</sub>,<sup>18</sup> and we have investigated V<sub>L</sub> sequences from a group of 15 patients both to extend our knowledge of V<sub>κ</sub> and V<sub>λ</sub> gene usage in myeloma, and to assess the role of V<sub>L</sub> in the selection of the cell of origin by antigen.

### MATERIALS AND METHODS

*Patients and cell preparation.* Heparinized BM aspirates from unselected patients with multiple myeloma at different stages of

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**Table 1. Characteristics of Myeloma Patients**

Patient	Stage*	Status	Monoclonal Ig		% Plasma Cells in BM
			Class	Level (g/L)	
1	IIIA	Relapsed progressive	IgG $\kappa$	24.0	11.0
2	IIA	Stable	IgG $\kappa$	18.0	21.0
3	II	Progressive	IgG $\kappa$	43.0	5.0
4	II	Stable	IgG $\kappa$	28.0	30.0
5	III	Progressive	IgG $\kappa$	15.0	18.0
6	IIA	Presentation	IgG $\kappa$	59.0	14.0
7	IIIA	Progressive	IgG $\kappa$	71.0	30.0
8	IB	Presentation	IgA $\kappa$	40.7	60.0
9	III	Progressive	IgG $\kappa$	48.0	30.0
10	IIA	Progressive	IgG $\lambda$	20.0	19.0
11	I	Stable	IgG $\lambda$	16.0	4.0
12	III	Progressive	IgG $\lambda$	60.0	5.0
13	III	Progressive	IgG $\lambda$	21.0	10.0
14	I	Progressive	IgA $\lambda$	3.0	1.8
15	IIIA	Presentation	IgG $\lambda$	79.0	50.0

\* Durie and Salmon staging.

disease from the Hematology (UK) or Immunology (Germany) clinics were taken for investigation. All patient material was obtained with consent, and with permission from local Ethical Committees. Clinical and laboratory features are shown in Table 1. All patients had an identifiable monoclonal Ig in serum (13 IgG, 2 IgA) of the same light chain type (9 $\kappa$ , 6 $\lambda$ ) as the major plasma cell population. Mononuclear cells (MNC) were isolated by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and plasma cell involvement was assessed by direct immunofluorescent staining for surface CD38 and cytoplasmic  $\kappa$  or  $\lambda$  light chains using the FACS-SCAN (Becton Dickinson, CA).<sup>19</sup> In some cases, cytocentrifuged MNC preparations were stained with the same reagents, and assessed by fluorescence microscopy.

**Analysis of V<sub>L</sub> genes.** For preparation of cDNA, total RNA (2 to 10  $\mu$ g) was isolated from the MNC fraction (1 to 6  $\times$  10<sup>6</sup> cells) using RNAzol B (Cinna Biotech Labs Inc, Houston, TX). Reverse transcription was carried out with an oligo dT primer, using a first-strand cDNA synthesis kit (Pharmacia). A sample of the cDNA (1/3 to 1/5) was then amplified by polymerase chain reaction (PCR) using a mixture of 5' oligonucleotide FWR1 primers specific for the expressed V $\kappa$  or V $\lambda$  families together with a mixture of downstream 3' primers specific for J $\kappa$  or J $\lambda$  genes as appropriate (Table 2). Amplification conditions were as described,<sup>4,20</sup> except that annealing temperature was 65°C for 1 minute. Amplified products were cloned and sequenced as described<sup>4</sup>; alignment was made to current EMBL/GenBank and V-BASE sequence directories<sup>21</sup> using MacVector 4.0 sequence analysis software (International Biotechnologies Inc, New Haven, CT). At least two independent PCR amplifications were performed from each sample.

## RESULTS

**V<sub>L</sub> gene usage by tumor cells.** The V<sub>L</sub> genes used by the tumor cells were identified as repeated identical V<sub>L</sub>-J<sub>L</sub> sequences obtained after cloning of PCR products.<sup>4</sup> Remaining sequences, presumably from normal B cells in the aspirates,<sup>20</sup> were different from each other. Repeated sequences were seen in all cases, at variable frequency, as indicated in Tables 3 and 4 for V $\kappa$  and V $\lambda$ , respectively. The profile of V $\kappa$  genes used by the 9  $\kappa$ -positive tumors indicates that 5 of 9 use V $\kappa$ 1 and 4 of 9 V $\kappa$ III frequencies

in line with normal B cells.<sup>22,23</sup> Among the V $\kappa$ 1 group, 4 of 5 use the O8/18 gene, which is commonly rearranged in B-cell tumors,<sup>24</sup> and 3 of 4 of the V $\kappa$ III group use the A27 gene, found to be used frequently in chronic lymphocytic leukemia.<sup>25</sup> The V $\lambda$  genes (Table 4) used three different families. There appeared to be no preferential use of particular J<sub>L</sub> genes for either light chain type.

**V-J joining region.** Clonality of tumor-derived sequences was confirmed by analysis of the V-J junction (Fig 1), which showed intraclonal identity. In 9 of 15 sequences, there were base additions at the junction which were not encoded by the V or J genes. In some cases, these appeared to be derived from flanking regions of the genes, and could therefore be accounted for by imprecision at the joint. In 5 of 15, there were additional nucleotides which may represent N-region additions, contributed by TdT activity. In a majority (11 of 15) of cases, nucleotides had been lost by trimming from either V or J genes.

**Somatic mutation.** Nucleotide sequences of all V<sub>L</sub> genes have been submitted to the EMBL/GenBank database (accession numbers Z70253-255; Z70258-261; Z70263-264; Z75558; X98894-898). To assess the degree of somatic hypermutation, comparison with the closest counterparts in the database of germline sequences has been made. This does not take into account any polymorphisms in V<sub>L</sub>, but these are known to be insignificant in V $\kappa$ .<sup>26</sup> Less information is available for V $\lambda$  genes, but again suggests only limited polymorphic variation.<sup>27</sup> The V<sub>L</sub> sequences obtained deviated significantly from the closest germline genes in the database (Tables 3 and 4), with a mean percent mutation of 5.3 for V $\kappa$  and 6.2 for V $\lambda$ . There was evidence for block mutations, involving two or more adjacent nucleotides, in both light-chain types. For V $\kappa$ , the high incidence of block mutations (6 of 9 sequences [67%]) compares with the reported figure of ~50%.<sup>28</sup> The numerical distribution of the mutations in FWRs and CDRs, and the ratio of replacement to silent mutations are shown in Tables 3 and 4. Deduced amino acid sequences are shown in Figs 2 and 3. In all cases, somatic mutations were identified in the V<sub>L</sub> sequences, with 9 of 15 having additional identifiable mutations in J<sub>L</sub>, even though events at the 3' end of J<sub>L</sub> are obscured by the primer sites. Several sequences derived from the same V<sub>L</sub> family member were available for the V $\kappa$  genes O8/18 and A27. Comparison of these showed no evidence for common sites or "hot-spots" of mutational activity. Analysis of the distribution of somatic mutations in each sequence (Table 5) has been car-

**Table 2. Oligonucleotide V<sub>L</sub> PCR Primers**

Primer	Location	Orientation	Sequence (5'-3')
V $\kappa$ 1&4	FR1	Sense	GACATCSWGATGACCCAGTCTCC
V $\kappa$ 2&6	FR1	Sense	GAWRRTGTGMTGACTCAGTCTCC
V $\kappa$ 3	FR1	Sense	GAAATTGTGTTGACGCACTCTCC
V $\kappa$ 5	FR1	Sense	GAAACGACACTCACGCACTCTCC
J $\kappa$ 1-4	FR4	Anti-sense	ACGTTTGATHITCCACYTTGGTCCC
J $\kappa$ 5	FR4	Anti-sense	ACGTTTAATCTCCAGTCGTGTCCC
V $\lambda$ 1	FR1	Sense	CAGTCTGTSBGTGACKCAGCCRCCTC
V $\lambda$ 2	FR1	Sense	CAGTCTGCCCTGACTCAGCTCSSYT
V $\lambda$ 3	FR1	Sense	TCYMTGWGCTGACTCAGSMM
V $\lambda$ 7&8	FR1	Sense	CAGRCTGTGGTGACYCAGGAGCCMTC
V $\lambda$ 9	FR1	Sense	CAGCCTGTGTGACTCAGCCACCTTC
J $\lambda$ C	FR4	Anti-sense	ACCKAGGACGGTSASCTKGGTSCC

**Table 3. Analysis of V<sub>κ</sub> Genes From Myeloma Patients**

Patient No.	Ig Light Chain	V <sub>L</sub> Family	GL Donor	% Homology	R/S Mutations		J <sub>L</sub>	Tumor-Derived Sequences/ Clones Sequenced
					FWR	CDR		
1	κ	V <sub>κ</sub> I	O8/18	93.2	5/8	4/1	J <sub>κ</sub> 5	8/11
2	κ	V <sub>κ</sub> I	O8/18	93.8	6/2	5/3	J <sub>κ</sub> 2	8/12
3	κ	V <sub>κ</sub> I	O8/18	94.6	4/5	3/2	J <sub>κ</sub> 4	9/11
4	κ	V <sub>κ</sub> I	O8/18	94.7	3/5	2/4	J <sub>κ</sub> 4	7/9
5	κ	V <sub>κ</sub> I	A30	95.0	5/3	5/0	J <sub>κ</sub> 1	8/11
6	κ	V <sub>κ</sub> III	A27	93.6	6/2	6/4	J <sub>κ</sub> 1	8/8
7	κ	V <sub>κ</sub> III	A27	95.0	4/1	6/2	J <sub>κ</sub> 4	10/12
8	κ	V <sub>κ</sub> III	A27	93.9	4/1	11/0	J <sub>κ</sub> 2	12/12
9	κ	V <sub>κ</sub> III	L6	98.5	0/0	3/1	J <sub>κ</sub> 3	6/11

ried out by the method of Chang and Casali.<sup>17</sup> In this method, each V<sub>L</sub> or V<sub>H</sub> gene sequence is assessed codon by codon for significance of deviation from germline sequence. A modification of the binomial distribution model is then used to calculate whether the probability (*P* in Tables 5 and 6) of an excess (in CDRs) or scarcity (in FWRs) of replacement mutations resulted by chance alone.<sup>17</sup> For the FWRs, there were generally fewer replacement (R) mutations than expected due to chance, with significant (*P* < .05) conservation of sequence in 10 of 15 sequences, a feature commonly seen for V<sub>H</sub>.<sup>14</sup> For the CDRs, there were more R mutations than expected in 14 of 15 sequences, with significant (*P* < .05) clustering indicative of antigen selection in 4 of 15 (3Vκ and 1Vλ).

**Comparison with V<sub>H</sub> genes.** For 6 cases (patients 1, 3, 6, 8, 14, and 15), tumor-derived V<sub>H</sub> gene sequences were known.<sup>4,29</sup> V<sub>H</sub> sequences from the remaining 9 patients were obtained as described<sup>4</sup> and deduced amino acid sequences are shown in Fig 4. Nucleotide sequences have been submitted to EMBL/GenBank database (accession numbers: Z70256-257; Z75556-5557; X98899-99003). Although the closest germline gene has been obtained from the database, rather than from the patients' DNA, it appears that in general polymorphism in V<sub>H</sub> is not sufficient to require this approach.<sup>21</sup> In fact, where we<sup>4</sup> and others<sup>5</sup> have analyzed the patients' germline V<sub>H</sub> genes, the sequence has been found in the majority of cases to correspond exactly to that obtained from the database. However, in 1 of 9 cases of myeloma, a 2-bp difference from the published sequence of a VII-5 germline gene was found also in the patient's germline sequence, indicating that this was probably caused by a polymorphism.<sup>5</sup> The distribution of somatic mutations in V<sub>H</sub> of 6 of 15 of

these cases indicated a significant clustering in CDRs (Table 6). Comparison of patterns in V<sub>H</sub> with those in V<sub>L</sub> (Table 6) showed that clustering in CDRs of V<sub>H</sub> was not paralleled by clustering in CDRs of V<sub>L</sub>. In addition, the clustering in V<sub>L</sub> observed in 4 of 15 cases was not paralleled by clustering in V<sub>H</sub>. Therefore, from the 10 cases where clustering was evident, it was localized in either V<sub>H</sub> or V<sub>L</sub>, but not both. However, in 5 of 15 cases, there was no significant clustering in either V<sub>H</sub> or V<sub>L</sub>.

#### DISCUSSION

Analysis of V-genes used by neoplastic B cells is extending our understanding of the origin and progression of B-cell tumors. Now that a complete map of the V<sub>H</sub> gene germline repertoire is available,<sup>21,30</sup> it is possible to compare a V<sub>H</sub> sequence from a tumor cell to the germline gene of origin with confidence. Although some nucleotide changes may reflect polymorphic variation, particularly for certain V<sub>H</sub>3 genes,<sup>31</sup> it can be assumed that the majority of deviations from germline sequence in V<sub>H</sub> genes of a B cell represent somatic mutations.<sup>21</sup> In some cases this has been proved by comparing the tumor-derived sequence with the germline counterpart in the patient.<sup>4,5</sup> Accumulation of such mutations indicate that the cell of origin has been exposed to the hypermutation mechanism in the germinal center.<sup>11,18,32</sup> Heterogeneity of mutations within the tumor clone indicates that the tumor cell is still under the influence of the mutation mechanism, subsequent to neoplastic transformation.<sup>9,10</sup> Finally, concentration of replacement mutations in CDRs can suggest a role for antigen in selection of the B cell.<sup>17,33</sup>

In the case of multiple myeloma, V<sub>H</sub> gene analyses from several laboratories have shown that the malignant cell has

**Table 4. Analysis of V<sub>λ</sub> Genes From Myeloma Patients**

Patient No.	Ig Light Chain	V <sub>L</sub> Family	GL Donor	% Homology	R/S Mutations		J <sub>L</sub>	Tumor-Derived Sequences/ Clones Sequenced
					FWR	CDR		
10	λ	V <sub>λ</sub> I	DPL2	95.6	3/3	4/2	J <sub>λ</sub> 7	12/12
11	λ	V <sub>λ</sub> I	DPL3	91.0	3/6	9/6	J <sub>λ</sub> 1	4/10
12	λ	V <sub>λ</sub> III	IGLV3S2	96.5	0/2	6/1	J <sub>λ</sub> 7	8/8
13	λ	V <sub>λ</sub> II	DPL11	95.9	2/4	5/0	J <sub>λ</sub> 2	6/9
14	λ	V <sub>λ</sub> II	HSLV2046	94.4	4/3	6/2	J <sub>λ</sub> 2	9/11
15	λ	V <sub>λ</sub> III	DPL23	92.9	3/6	7/2	J <sub>λ</sub> 2	8/8

PATIENT	LIGHT CHAIN	V			N			J			J <sub>L</sub>	TRIMMING			ADD <sup>N</sup> BASES
		V	N	J	V	N	J	V	J	TOTAL					
1	κ	TAT	GAT	AA <u>T</u>	CTC	CCT	CC	G	ATCACCTTCGG	5	0	0	0	3	
2	κ	TAT	GAT	GAT	CTC	CCC		G	TACACTTTTGG	2	0	0	0	0	
3	κ	TAT	GAT	AGT	CTC	CC		G	CACTTTCGG	4	1	2	3	0	
4	κ	TAT	GAT	AGT	CTC	CCT	C*	G	TCACCTTTGG	4	0	1	1	1	
5	κ	TAT	AA <u>A</u>	AGT	TAC	CCT	C	T	GACGTTTGG	1	0	2	2	2	
6	κ	TAT	GGG	AGT	TTA	CCT	C		GGACGTTTCGG	1	0	1	1	1	
7	κ	TAT	GGG	AGC	TC			G	CTCACTTTCGG	4	4	0	4	1	
8	κ	CAT	GTT	ACC	GAA	C	A	G	TGCACCTTTGG	2	2	0	2	2	
9	κ	CGT	AGC	AAC	TGG	CCT	CC	GA	TCACTTTCGG	3	0	1	1	4	
10	λ	GAC	AGC	CTG	AAT	GGT	CC	G	GTGTTCGG	7	0	3	3	3	
11	λ	GAC	AGC	CTG	AGA	GGT*			TATGTCTTGCC	1	0	0	0	0	
12	λ	AGT	AGT	AA <u>N</u>	GAT	CAG		GGG	GTGTTCGGCGG	7	0	3	3	3	
13	λ	AGC	AGC	AGC	ACT	CTC			CTGGTATCCGC	2	0	0	0	0	
14	λ	GGC	AGC	AAC	AAT				GTGATATTCGG	2	3	0	3	0	
15	λ	GAC	CGC	AA <u>C</u>	ACT				TTGATATTCGG	2	3	0	3	0	

Base additions  
from V gene 3'  
flanking region

Base additions  
from J gene 5'  
flanking region

\* nucleotide encoded by either V<sub>L</sub> or J<sub>L</sub> gene segment  
N denotes mutation event

**Fig 1. Nucleotide sequences of the V-J junctional regions. Junctional regions are identical in the repeated sequences from each individual case (Tables 3 and 4). Base additions from flanking regions and losses by trimming are indicated. Remaining bases are presumed to have arisen via N-region addition.**

	5'primer	---CDR1---	---CDR2---	---CDR3---	3'primer	
08/18 P1	DIQMTQSPSSLSASVGDRTTTC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSSTDFFTTISLQPEDFATYYC	QQYDNLP
	-----P-a-----	-----T-----	---q--g--k-----	V---QP	--p-----G--tH-t--G-----Fa-----	-----P
						ITFGQGTKVEIKR Jk5
08/18 P2	DIQMTQSPSSLSASVGDRTTTC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSSTDFFTTISLQPEDFATYYC	QQYDNLP
	-----H--N-H--	-F-V-pg-	-a-I--	-----A-----	-----F-----	-----D-p
						YTFGQGTKVEIKR Jk2
08/18 P3	DIQMTQSPSSLSASVGDRTTTC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSSTDFFTTISLQPEDFATYYC	QQYDNLP
	V-E-----v-----t-	-----s-----	---qH-----Q-----	---H---	---r-----L--N-l-----	-----S-
						P TFGGQTKVDIKR Jk4
08/18 P4	DIQMTQSPSSLSASVGDRTTTC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSSTDFFTTISLQPEDFATYYC	QQYDNLP
	--E-----a-----I--	-----i--F--	---I-----k-l--	-----et	-----Ift-----	---q--s---
						L tFGGQTKVEIKR Jk4
A30 P5	DIQMTQSPSSLSASVGDRTTTC	RASQGIKNDLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSSTDFFTTISLQPEDFATYYC	LQHNSYP
	--E-----D-G-A--	---R-E-----R--	-----g--sT-----g-----	-----YK---		
						L TFGQGTKVEIKR Jk1
A27 P6	EIVLTQSPGTLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSSTDFLTISRLEPEDFAVYYC	QQYGSSP
	D--Mt-----V--	-----RNSV-	---k-----G--F	---N---	-V-----V-----	-q-gsL-
						R TFGQGTKVEIKR Jk1
A27 P7	EIVLTQSPGTLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSSTDFLTISRLEPEDFAVYYC	QQYGS
	-----L-N--	---NA-----	S-N---	-----S-----	-----S1-----	-L-g-
						S LTFGGQTKVDIKR Jk4
A27 P8	EIVLTQSPGTLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSSTDFLTISRLEPEDFAVYYC	QQYGS
	-----L-	-----N--	---P-----	RTF--P	-----a--F--	--HVTE
						Q CTFGQGTKLEIKR Jk2
L6 P9	EIVLTQSPATLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	DASNRTAT	GIPARFSGSGSSTDFLTISRLEPEDFAVYYC	QQRSNWP
	---t-----GN---	-----Y-----			-----y-----	-----PI
						TFGPGTKVDIKR Jk3

**Fig 2. Deduced amino acid sequences of the V<sub>κ</sub> regions of the tumor-derived clones from patients with myeloma. Comparisons are made with the closest germline V<sub>κ</sub> genes. Uppercase, replacement mutations; lowercase, silent mutations. Replacement mutations in J<sub>κ</sub> are underlined.**

```

      5'PRIMER          CDR1          CDR2          CDR3          3'PRIMER
      |-----|          |-----|          |-----|          |-----|          |-----|
DPL2  QSVLTQPPSASGTPGQRTVISC  SGSSSNIGSNMIVN  WYQQLPGTAPKLLIY  SNNQRPS  GVPDRFSGSKSGTSASLAISGLQSEDEADYYC  AAWDDSLNG  |-----|
P10   -P-----a-----s-----  -----n--G-----  ---H-----V---  TD-----  -----t-----g-----  T-----  P  VFgGGTQLTVLG JA.7

DPL3  QSVLTQPPSASGTPGQRTVISC  SGSSSNIGSNYVY  WYQQLPGTAPKLLIY  RNNQRPS  GVPDRFSGSKSGTSASLAISGLRSEDEADYYC  AAWDDSLSG
P11   -----G-----sK-n--TY-I-I-  -F-qI-----i-  WTD-R--  g-----s-a-----y-  aa-d---R-  YVLPGGTKLTVLG JA.1

GLI*  SYVLTQPPSVSVAPGKTARITC  GGNIGSKSVH  WYQQKPGQAPVLIY  YSDRPS  GIPERFSGSNGNTATLTISRVEAGDEADYYC  QWWDSSDH
P12   -----ET-----  -----V-----  F-T-----e-----  -----d--N-Q  G  VFgGGTQLTVLG JA.7

DPL11 QSALVTPASVSGSPGQSITISC  TGTSSDVGGYNYVS  WYQQHPGKAPKLMY  EVSNRPS  GVSNRFSGSKSGNTASLTISGLQAEDEADYYC  SSYTSSSTL
P13   -----q-----I-A-D-----  -yE-Q-----  D-N-----f-----  -----c-----  LVSAGGNMLTVLG JA.2

GL2** QSALVTPASVSGSPGQSITISC  TGTSSDVGGYNYVS  WYQQHPGKAPKLMY  EVSKRPS  GVPDRFSGSKSGNTASLTISGLQAEDEADYYC  SSYAGSNN
P14   -----A--G-----A-tsG-I--FD---  ---q-p-----V---  --T-----F--A-----  -----  VIFGGTQLTVLG JA.2

DPL23 SYELTQPPSVSVSPGQTASITC  SGDKLGDKYAC  WYQQKPGQSPVLIY  QDSKRPS  GIPERFSGSNGNTATLTISGTQAMDEADYYC  QAWDSST
P15   -----E-----c--g-----VS  ---q-----w-v-y  --AN-s  W-----s-----a-----  ---RN-  LIFGGTQLTVLG JA.2
    
```

GL1\* is IGLV3S2  
 GL2\*\* is HSLV204

**Fig 3. Deduced amino acid sequences of the V<sub>L</sub> regions of the tumor-derived clones from patients with myeloma. Comparisons are made with the closest germline V<sub>L</sub> genes. Uppercase, replacement mutations; lowercase, silent mutations. Replacement mutations in JA are underlined.**

undergone extensive somatic hypermutation.<sup>3-5</sup> There is further wide agreement that there is no intraclonal heterogeneity among the tumor cell population,<sup>3-5</sup> and there is evidence that the V<sub>H</sub> sequence is stable from diagnosis through plateau

phase.<sup>34</sup> These findings strongly suggest that the malignant cell has exited from the germinal center, and is no longer susceptible to the mutation mechanism.<sup>4,5</sup>

The germline repertoire of V<sub>L</sub> genes has also been mapped,<sup>27,35,36</sup> but there have been fewer studies of usage in B-cell tumors. In myeloma, using DNA as a source, 7 V<sub>κ</sub> sequences were obtained from 29 cases, with 4 of 7 potentially functional.<sup>37</sup> Sequences were somatically mutated, with a hint of antigen selection from R:S ratios.<sup>37</sup> A second study investigated V<sub>κ</sub>-gene usage in 3 cases of myeloma.<sup>24</sup> Together, these studies showed that 3 of 7 functional genes were derived from the O8/18 gene,<sup>24,37</sup> and we have confirmed this incidence (4 of 9 cases). Although the V<sub>κ</sub>1 family is often used by normal B cells,<sup>22,23</sup> the level of usage of the O8/18 gene appears high in myeloma. However, frequency

**Table 5. R and S Mutations in Myeloma V<sub>L</sub> Genes**

Patient	Ig Class	Germline Gene	R:S (CDR) <sub>obs</sub>	R (CDR) <sub>exp</sub>	P (CDR)*
			R:S (FWR) <sub>obs</sub>	R (FWR) <sub>exp</sub>	P (FWR)
1	IgG <sub>κ</sub>	O8/18	4.00 (4:1)	4	.22
			0.63 (5:8)	10	.02
2	IgG <sub>κ</sub>	O8/18	1.70 (5:3)	4	.14
			3.00 (6:2)	9	.09
3	IgG <sub>κ</sub>	O8/18	1.50 (3:2)	3	.26
			0.80 (4:5)	8	.03
4	IgG <sub>κ</sub>	O8/18	0.50 (2:4)	3	.20
			0.60 (3:5)	8	.01
5	IgG <sub>κ</sub>	A30	5.00 (5:0)	3	.09
			1.00 (4:4)	7	.19
6	IgG <sub>κ</sub>	A27	1.50 (6:4)	4	.12
			3.00 (6:2)	10	.16
7	IgG <sub>κ</sub>	A27	3.00 (6:2)	3	.03
			4.00 (4:1)	7	.06
8	IgA <sub>κ</sub>	A27	∞ (11:0)	3	.0001
			4.00 (4:1)	8	.02
9	IgG <sub>κ</sub>	L6	∞ (3:0)	1	.04
			0.00 (0:1)	2	.00
10	IgG <sub>λ</sub>	DPL2	2.00 (4:2)	3	.20
			1.00 (3:3)	6	.06
11	IgG <sub>λ</sub>	DPL3	1.50 (9:6)	6	.08
			0.50 (3:6)	12	.0002
12	IgG <sub>λ</sub>	IGLV3S2	6.00 (6:1)	2	.006
			0.00 (0:2)	0	.00
13	IgG <sub>λ</sub>	DPL11	5.00 (5:0)	3	.08
			0.50 (2:4)	6	.03
14	IgA <sub>λ</sub>	HSLV2046	3.00 (6:2)	4	.09
			1.33 (4:3)	8	.04
15	IgG <sub>λ</sub>	DPL23	3.50 (7:2)	4	.14
			0.50 (3:6)	10	.0012

\* Probability calculations according to Chang and Casali.<sup>17</sup>

**Table 6. Comparison of Antigen-Driven R Mutations Locating to Myeloma V<sub>H</sub> or V<sub>L</sub> Genes**

Patient	P (CDR)*	
	V <sub>H</sub>	V <sub>L</sub>
1	.10	.22
2	.03	.14
3	.06	.25
4	.18	.20
5	.11	.09
6	.0009	.12
7	.13	.03
8	.21	.0001
9	.25	.04
10	.0029	.20
11	.0098	.08
12	.10	.006
13	.049	.08
14	.18	.09
15	.04	.14

\* Probability calculations according to Chang and Casali.<sup>17</sup>

	CDR1	CDR2	CDR3		
V2-26 P2	QVTLEKESGFLVLPKPTETLTLCTVSGFSLN ---V---Q-----S-----EG-----	NARMGVS WIRQPPGKALEWLA	HIFSNDKSYSTSLKS -----S---ISS--Rs	RLTISKDTSKSQVVLIMTNMDFVDTATYYCA r-----N--G-----KV-pT-t---y-V	RVVQLRVP KYHFDHWGGTLVTVSS JH4b
V3-11 P4	QVQLVESGGGLVLPKPGGSLRLSCAASGFTFS -Aq-----D--k-----V--R--R -H---	DYHMS WIRQAPGKGLEWVS	YISSSGSTIYADSVKG -l-----g-----F-R---ATF---s---	RFTISRDNKNSLYLQMNSLRAEDTAVYYCAR -----D-nTl-----VD---v-----	GRYSTSPR <u>TFNLWGH</u> GLTLVTVSS JH4a
8M27 P5	QVQLVQSGAEVVKPKGSSVVKSCAKSGFTFS --Ni-----A---M-----C-----	SYTIS WVRQAPGQGLEWVG	RIIPILGTANYAQKFGQ --L-----T---v-----T-----S---	RVTITADKSTSTAYMELSSLRSEDVAVYYCA --t---R---a-----N-y-----v-y---	VYYFDSNR YDYWQQGLTVTVSS JH4b
V3-7 P7	EVQLVESGGGLVQPGGSLRLSCAASGFTFS --l-----AEp-----E-----t--b N--H	SYHMS WVRQAPGKGLEWVA	NIKQDGSEKYYVDSVKG -----gk-----R--M--L---	RFTISRDNKNSLYLQMNSLRAEDTAVYYCAR -----VNL-I---C---v-----r	DGSSYARP YWYFDLWGRGTLVSVSS JH2
V3-9P P9	EVQLVESGGGLVQPGGSLRLSCAASGFTFD G-----v-----G-----SN-----	DYAMH WVRQAPGKGLEWVS	GISWNSGSGYADSVKG -----A- ---ET-----	RFTISRDNKNSLYLQMNSLRAEDTALYYCAKD -----Te---yF-v---QARGYSYGNLFDYWGQGLTVAVSS	JH4b
DP-66 P10	QVQLQESGPELVKPESETLTLCTVSGGSVS --q-----A---M-----C-----V---	SGSYHMS WIRQPPGKGLEWIG	YIYSGSTNYNPSLKS FF--T-H---Qs	RVTISVDTSKNTQFSLKLSVTAADTAVYYCAR --A-v---n-Ls-R-T-----GRQPD	YYYGMDVWVGGQGLTVISVSS JH6b
VHVMW P11	EVQLVQSGAEVVKPKGPELRLSCKGSGYFT -----N-----	SYWIS WVRQMPGKGLEWVG	RIDPDSYTYNPSPSFQG K-G-I-----	HVTISADKSISTAYLQWSSLKASDTAVYYCAR -----T-----	HPTYYDSSGYNFDHWGGTLVTVSS JH4b
DP-38 P12	EVQLVESGGGLVLPKPGGSLRLSCAASGFTFS -----I---TG--s	NAWMS WVRQAPGKGLEWVG	RIKSKTDGGTIDYAAPVKG -----v- r-----g-I---E---	RFTISRDDSKNTLYLQMNSLKTEDTAVYYCTT -----n-f-----v-----ALTRYFFDSSGYPHFDHWGGTLVTVSS	JH4a
b28e P13	QVQLVESGGGVVQPGGSLRLSCAASGFTFS L-----S---	SYGMH WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG -----g-Q-MV Y--S--NT-y-----	RFTISRDNKNSLYLQMNSLRAEDTAVYYCAR -----RS--F-----G---v-----	DPG LDYWQQGLTVSVSS JH4b

**Fig 4.** Deduced amino acid sequences of the  $V_H$  regions of tumor cells from patients with myeloma. Comparisons are made with the closest germline  $V_H$  genes. Uppercase, replacement mutations; lowercase, silent mutations. Replacement mutations in  $J_H$  are underlined. Patient identification numbers are indicated.  $V_H$  sequences from other patients are published.<sup>4,37</sup>

of this gene in other B-cell tumors has also been reported to be high, and it is not yet clear if there is a difference among the tumor categories.<sup>24</sup>

The current results have focused on functional  $V_L$  genes, obtained from RNA. Identification of repeated sequences in the cloned PCR product supports the derivation from tumor cells, which can be a problem otherwise. We have analyzed the pattern of both  $V_K$  and  $V_L$  sequences. These confirm the high level of somatic hypermutation, with the level of 5.8% mutation for  $V_L$  being comparable with that of 8.2% for  $V_H$ .<sup>5</sup> There is also a lack of intraclonal heterogeneity in  $V_L$  of all patients, again confirming findings in  $V_H$ , and supporting the concept that the final event in malignant transformation has occurred at a postfollicular stage.<sup>3,4,14</sup> In contrast, the  $V_H$  genes in the benign counterpart of myeloma (monoclonal gammopathy of undetermined significance or MGUS), showed intraclonal heterogeneity in 3 of 7 cases.<sup>29</sup> This could indicate that the clonal plasma cell in MGUS is less mature, and may have undergone some, but not all the events leading to malignant behavior.<sup>29</sup>

If the final neoplastic event is late in maturation of the B cell, it might be expected that the myeloma precursor cell will have been subjected to the same processes of development as a normal B cell. Even if there is an  $IgM^+$  clonal precursor, which has undergone some neoplastic event, the few cases available for analysis have indicated that it has a homogeneous  $V_H$  gene sequence identical to the isotype-switched plasma cell.<sup>12,13,38</sup> This would argue that neoplastic transformation in myeloma begins in a mature B cell immediately before isotype-switch. Because a B cell would have reached this point following antigen selection, the imprint of this procedure should remain as a clustering of mutations in CDRs of V-gene sequences.<sup>11,17,33</sup> In fact, analysis of the

stable sequences in myeloma should be particularly useful, because the selected sequence will not be obliterated by continuing posttransformation mutations. However, analysis of  $V_H$  sequences in myeloma has given mixed results, with only 21% of the tumor-derived sequences from a large series showing significant clustering in CDRs.<sup>5,14</sup> This leaves open the question of the clonal history of the tumor cells in the remaining 79% of the cases. Because  $V_L$  sequence is also known to be involved in recognition of antigen,<sup>18</sup> deductions from V-gene sequences that relate to a role for antigen in selection should be strengthened by including analysis of  $V_L$ .

In this study, significant clustering of mutations in CDRs of  $V_L$  was seen in 4 of 15 sequences. In contrast, a preliminary report of a study of  $V_K$  sequences in 9 cases of myeloma has indicated that clustering of replacement mutations in CDRs occurred in all cases, but more details of the analysis are required.<sup>39</sup> In our cases, comparison with  $V_H$  sequence in the same cell showed that clustering was in either  $V_H$  or  $V_L$ , but not both. This suggests that a role for antigen might be more common than estimated from  $V_H$  alone, reaching 67% in our study. There have been insufficient studies of normal human B cells to know if this is a typical finding. Investigation of the classical murine response to the hapten phenyl oxazolone has shown that affinity maturation is accompanied by somatic mutations in the CDRs of both  $V_H$  and  $V_K$ .<sup>40</sup> This is likely to be the case in human antibodies, and might suggest that a role for antigen is more common than estimated from  $V_H$  alone. For myeloma, the findings support the idea that the cell of origin has undergone the process of conventional antigen selection. However, there remains a minority of sequences which appear to have no clustering in either V-region. Even this pattern does not rule

out a role for antigen selection, because optimal binding may occur via CDR3. Clearly we need more information on how normal human B cells generate antibody, but this study would suggest that deductions concerning a role for antigen in the clonal history of neoplastic B cells should take into account mutational events in both V<sub>H</sub> and V<sub>L</sub>.

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