Molecular characteristics and gene segment usage in IGH gene rearrangements in multiple myeloma

David González
Marcos González
Ana Balanzategui
Maria E. Sarasquete
Ricardo López-Pérez
M Carmen Chillón
Ramón García-Sanz
Jesús F San Miguel

Background and Objectives. Analysis of IgH rearrangements in B-cell malignancies has provided clinical researchers with a wide range of information during the last few years. However, only a few studies have contributed to the characterization of these features in multiple myeloma (MM), and they have been focused on the analysis of the expressed IgH allele only. Comparison between the expressed and the non-functional IgH alleles allows further characterizion of the selection processes to which pre-myeloma cells are submitted.

Design and Methods. We analyzed a cohort of 84 untreated MM patients in order to characterize their functional VDJH and non-functional DJH rearrangements. The pattern of mutations and gene segment usage for both types of rearrangements was analyzed by polymerase chain reaction and sequencing.

Results. VH3 and VH1 family members were over- and under-represented, respectively. VH3-30 and VH3-15 segments were the most frequently used, whereas VH4-34 was found only in non-functional or heavily mutated VDJH rearrangements. DH2 and DH3 family members were over-represented in both VDJH and DJH repertoires, while the DH1 family was under-represented only in the productive VDJH rearrangements. Finally, DH3-22 and DH2-21 gene segments were found to be over-represented in the functional repertoire while segments commonly used by less mature B-cell malignancies, such as DH6-19 or DH3-3, were under-represented.

Interpretation and Conclusions. Data reported here help to identify the clonogenic MM cell as a post-germinal center B cell that has undergone selection processes during the germinal center reaction.

Keywords: multiple myeloma, IgH rearrangements, incomplete rearrangements, gene usage, somatic hypermutation.

Haematologica 2005; 90:906-913

From the Hematology Service, University Hospital of Salamanca and Center for Cancer Research, University of Salamanca, Spain.

Correspondence: Marcos Gonzalez, Hematology Department, University Hospital of Salamanca, Paseo San Vicente 58-182, 37007, Salamanca, Spain. E-mail: margondi@usal.es

The recombination of the variable (V), diversity (D) and joining (J) segments of the immunoglobulin heavy chain genes (*IGH*) occurs early during B-cell differentiation.[1] These rearrangements will eventually provide the coding sequence for the IgH variable domain of the B-cell receptors.[2] In humans, there are between 39-51 functional VH (depending on individual haplotype),[3-5] 27 DH and 6 JH segments. VH and DH segments are grouped into seven different families, each containing members sharing more than 80% homology. During pro-B and pre-B stages of B-cell differentiation, a DH segment is joined to one of the 6 JH segments, generating an incomplete DJH rearrangement. This is followed by addition of a VH segment to the DJH joint, generating a complete VDJH rearrangement.[1] During VDJH recombination, insertion, deletion and/or duplication of a variable number of nucleotides in the joining regions confers the variable IgH domain an enormous combinatorial potential.[6-10] B cells undergoing affinity maturation in the germinal centers further diversify their Ig genes by the processes of somatic hypermutation and class-switch recombination.[11-14] During this germinal center reaction, B cells reactive to foreign antigens will be positively selected to become plasma or memory B cells, while those reactive to self-antigens will eventually undergo apoptosis.

Gene segment usage, complementarity-determining region-3 (CDR3) composition and somatic hypermutation rates have been described for some B-cell malignancies and B-cell subtypes, in which usage of particular VH and DH families and gene segments is often biased.[15-23] The most interesting finding to date has been made

in B-cell chronic lymphocytic leukemia (B-CLL), in which the presence of somatic hypermutation in the IgH genes is clearly correlated with a more favorable prognosis than that of unmutated cases.[24,25] Furthermore, the pattern of somatic hypermutation and CDR3 composition has been associated with particular VH families and gene segments in B-CLL.[26,27] A few studies have contributed to the characterization of these features in MM,[21,28] although no correlation between prognosis and gene segment usage or somatic hypermutation rates has been achieved.

MM is a B-cell malignancy produced by the accumulation of plasma cells in the bone marrow. Plasma cells represent the end stage of B-cell development, secreting large amounts of monoclonal antibody codified by mature *IGH* genes that have undergone the processes of somatic hypermuation and class-switch recombination. Hence, it has been assumed that the MM *malignant stem cell* is a post-germinal center B cell that has acquired one or more genetic lesions after exiting the germinal centers of the secondary lymphoid organs.[15] Previous molecular studies of *IGH* genes in MM have revealed a high rate of somatic hypermutation, and peculiar features of gene segment usage such as the complete absence of the VH4-34 segment from the functional VDJH repertoire.[21,28] However, these analyses were focused on the expressed IgH rearrangement only and did not take into account the non-expressed/non-functional allele. Recently, our group has reported a 60% incidence of incomplete, non-functional DJH rearrangements in MM samples.[29] Analysis of DH segments is of particular interest because these segments provide a major source for antigen recognition by antibodies. Differences in the coding sequence between the seven DH families are responsible for the main variability in CDR3 composition, which will eventually determine the affinity potential of particular antibodies to a given antigen.[2] Here, we compare for the first time the molecular characterization and gene segment usage between functional VDJH and non-functional DJH rearrangements in a large series of 84 untreated MM patients.

Design and Methods

Genomic DNA preparation, PCR amplification and heteroduplex analysis

High molecular weight DNA was isolated from bone marrow aspirates of 84 untreated MM patients by standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation.[30] For amplification of complete VDJH rearrangements three different sets of family-specific primers and one JH consensus primer were used in three different multiplexed PCR reactions covering the three framework regions.[31] Amplification of incomplete DJH rearrangements was performed in two different reactions using family-specific primers for DH1 to DH6 and DH7 families, respectively, together with the consensus JH primer.[31] All primers had been newly designed and tested during the BIOMED-2 Concerted Action *PCR-based clonality studies for early diagnosis of lymphoproliferative disorders* (BMH4-CT98-3936).[31] All reactions were carried out in 50 μL mixture containing 0.1 μg DNA and 10 pmoL of each primer.

For heteroduplex analysis, PCR products were denatured at 94°C for 10 min and subsequently cooled at 4°C for 60 min to induce duplex formation.[30,32] The hetero and/or homoduplexes generated were immediately loaded on a 10% non-denaturing polyacrylamide gel in 1× Tris-Acetate-EDTA (TAE) buffer, run at room temperature, and visualized by ethidium bromide staining.

Sequencing and analysis of Ig genes

PCR products were eluted from the polyacrylamide gels and directly sequenced in an automated ABI 377 DNA sequencer using Big-Dye terminators (Applied Biosystems, Foster City, CA, USA). Germline VH, DH and JH segments from complete VDJH rearrangements were identified by comparison with the V base[3] and IGMT database.[33] As both databases differ in the assignment of complementarity-determining and framework regions, mutational analysis of VH segments was made using only the closest germline gene segment found in V base. DH and JH germline segments from incomplete DJH rearrangements were identified using BLAST search in the DH-JH germline locus sequence (accession number EMB/X97051). The criteria of Corbett *el al.*[4] (i.e., a requirement of ten consecutive nucleotides identical to the germline sequence) were used to identify DH members from the longer DH families (D2 and D3) in the complete VDJH rearrangements. For the shortest DH families (D1, D4, D5, D6 and D7) the criteria of Fais *et al.*[34] were applied (seven consecutive germline nucleotides with no more than two differences). The DIR segments, *minor* DH segments and inverted DH segments were excluded from this analysis, as suggested.[4]

Sequences containing more than 2% deviation from the germline sequence were considered as somatically mutated rather than genomic polymorphisms. In such cases, the algorithm described by Chang and Casali[35] was applied in order to discriminate replacement (R) and silence (S) mutations in complementarity-determining and framework regions derived from antigen selection or acquired by random choice.

Table 1. VH, DH and JH gene segment usage and percentage of mutations in complete VDJH and incomplete DJH rearrangements in 84 MM patients.

ID	VDJH rearrangements				DJH rearrangements			ID	VDJH rearrangements				DJH rearrangements		
	VH	% mutations	DH	JH	DH	% mutations	JH		VH	% mutations	DH	JH	DH	% mutations	JH
1215	3-11	11	4-17	4B	–	–	–	4311	3-49	10.4	5-12	4B	7-27	4	2
1248	3-30	12.4	1-01	3A	–	–	–	4313	–	–	–	–	4-04	0	6C
1287	3-15	4.4	2-02	6B	2-15	0	5B	4338	3-21	10	3-22	3B	–	–	–
1290	2-26	5	3-09	4B	5-12	0	4B		**3§**	§	**3-10**	**4B**	–	–	–
1384	3-15	5	3-10	4B	1-26	0	6B	4372	2-70	4	4-17	4B	2-08	0	5B
1390	3-48	10	7-27	4B	–	–	–	4411	4-34	20.8	–	6B	–	–	–
1481	3-11	12	3-03	6C	3-03	5	4B	4429	–	–	–	–	–	–	–
1646	3-33	13	7-27	4B	3-09	2.3	5B	4492	3-23	3.6	6-19	2	5-12	9	4B
1654	1-02	5.2	2-02	4B	2-21	0	5B	4526	5-51	7.2	6-19	4B	4-23	0.5	3B
1727	3-72	5.2	5-05*	4B	–	–	–	4527	4-39	13.2	2-21	5B	–	–	–
1742	4-31	5.8	3-22	3B	–	–	–	4572	2-26	4.4	4-17	5B	2-02	0.5	6C
1782	4-59	6.8	1-26	4B	–	–	–	4625	2-70	6.4	6-19	6B	–	–	–
1811	3-23	14	2-21	3A	5-18	0	J4	4726	3-21	6	6-06	6B	4-04	0	1
2173	4-59	7.5	–	5B	3-16	0	4B	4982	5-51	4.4	4-17	4B	6-19	0	3B
2216	3-30	4	5-05*	6B	–	–	–	4983	3-48	5.2	2-02	3B	2-15	0	6C
2334	3-13	7.2	3-10	4B	1-07	0	5B	4994	3-07	7.2	–	4B	1-26	0	4B
2359	–	–	–	–	2-21	0	4B	5172	3-30	10.8	3-03	4B	–	–	–
2374	–	–	–	–	3-09	1	6B	5177	5-51	3.2	2-21	4B	2-15	0	6B
2421	3-73	8.8	2-08	4B	7-27	6	3B	5179	1-18	13	2-02	4D	–	–	–
2446	5-a	6.4	2-15	5B	1-07	0	4B	5220	1-24	6	3-22	3B	–	–	–
2536	4-30-2	7.2	1-26	5B	–	–	–	5223	3-09	6.4	–	4B	3-03	2	4B
2609	1-69	10	4-11*	4B	–	–	–	5303	1-03	11	3-22	4B	–	–	–
2694	3-23	8.4	3-10	4B	–	–	–	5304	3-30	10.4	–	4B	3-03	0	4B
	3-23	**13**	**5-24**	**3B**	–	–	–	5401	4-39	9.6	6-13	4B	3-03	3	–
2846	2-05	6.4	2-21	3B	5-18	0.5	4B	5436	3-48	10	3-16	4B	–	–	–
2908	4-59	7.6	5-24	4B	–	–	–	5801	–	–	–	–	–	–	–
2933	3-30	2.2	2-02	6B	–	–	–	5877	1-24	5	5-12	3A	7-27	0	6C
	3-48	*9*	*2-02*	*4B*	–	–	–	6130	4-59	10.4	1-26	4B	2-21	0	4B
3053	3-21	5.6	6-06	6B	–	–	–	6445	–	–	–	–	–	–	–
	3-53	**11.2**	**6-19**	**4B**	–	–	–	7496	5-51	4	5-12	4B	2-02	1.3	5B
3125	3-30	8	6-19	3A	–	–	–	7509	4-61	10	3-10	4B	3-22	1.5	6
3196	3-23	19	–	4B	3-22	0	6B	7622	3-30	15.2	–	4B	4-23	0	1
3203	3-09	10	6-13	3A	–	–	–	8960	4-39	8	5-05*	1	–	–	–
3205	–	–	–	–	–	–	–	9069	–	–	–	–	4-04	1	4B
3395	3-15	5.2	1-01	4B	2-02	0.5	5B	9112	4-04	14	3-09	6C	6-19	0	4B
3420	3-15	5.6	7-27	6B	6-06	0	4B	9340	**3-07**	**22**	–	**6B**	–	–	–
3543	–	–	–	–	–	–	–	9366	–	–	–	–	3-09	1	4B
3793	4-34	10	2-21	3B	4-23	0	4B	9476	4-30-4	4.8	5-24	4B	–	–	–
3854	3-33	11	3-10	3B	6-19	1	4B	9585	3-48	5.2	3-22	6B	–	–	–
3913	3-43	8.4	2-21	4B	5-24	0	4B	9901	3-73	5.6	2-15	4B	1-26	0	4B
4156	3-15	6.8	2-15	6B	–	–	–	9985	3-11	3.6	7-27	3A	1-26	1	4B
4249	4-04	4.4	3-22	5B	3-10	2	4B	10144	3-21	6.8	3-22	4B	2-21	0	5B
4263	–	–	–	–	5-24	0	4B	10181	3-11	13	4-17	6B	4-17	1.5	6B
4280	3-43	5.2	5-05*	4B	1-07	0	5B	11000	3-30	5.6	4-23	4B	1-07	0	4B

*A dash indicates that no clonal allele was detected. Bold represent out-of-frame rearrangements or rearrangement containing stop codons. *D4-04 and D5-05 in the VDJH repertoire are indistinguishable from D4-11 and D5-18, respectively. §This rearrangement was only amplified with FR3 primers so the VH gene segment and the level of mutations could not be obtained.*

Results

Detection of clonal rearrangements in MM by PCR

The overall detection rate of clonality by amplifying VDJH and DJH rearrangements was 94% (79/84). VDJH rearrangements were detected in 73 out of 84 patients (84%) and DJH rearrangements were seen in 50/84 patients (60%) (Table 1). Three out of 6 patients in whom only one DJH rearrangement was detected had Bence-Jones MM, whilst the remaining three DJH-only patients secreted a functional immunoglobulin. As described previously in myeloma patients, mismatches of the consensus primers with the hypermutated VH and JH regions were more likely to account for these latter cases in which VDJH rearrangements could not be amplified by PCR.

VH family and gene segment usage in VDJH rearrangements

VH, DH and JH gene segment usage, as well as the rate of somatic hypermutation for each MM patient

Table 2. Distribution of VH families and somatic hypermutation in productive VDJH rearrangements from MM patients.

	Known functional VH genes per family[a]	*MM VDJH rearrangements*	*%SHM in VH*[b]
V_H1	11 (21.6%)	6 (8.2%)[c]	8.0±3.4
V_H2	3 (5.9%)	5 (6.8%)	5.0±1.1
V_H3	22 (43.1%)	42 (57.5%)[c]	7.6±4.2
V_H4	11 (21.6%)	15 (20.5%)	8.0±4.2
V_H5	2 (3.9%)	5 (6.8%)	4.4±1.7
V_H6	1 (2.0%)	0 (0%)	–
V_H7	1 (2.0%)	0 (0%)	–
Total	51	73	

[a]The number of VH genes per family was obtained from data on VDJH recombinants contained in the V-BASE Sequence Directory (Cook & Tomlinson, 1995). [b]Median values ± standard deviations are shown. SHM=somatic hypermutation. [c]Significant difference between observed and expected frequency ($p<0.01$).

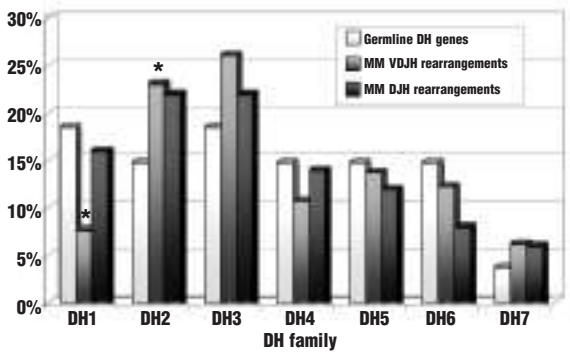

Figure 1. Distribution of DH families in VDJH and DJH rearrangements from MM patients. The number of germinal DH genes segments per family was obtained from Corbett *et al.*, including non-expressed DH segments. *Significant difference ($p<0.05$) between observed and expected frequency.

are shown in Table 1. The nomenclature used for the VH segments has been adapted from Matsuda *et al.*, who described 39 functional VH segments.[5] However, some VH segments corresponding to alleged haplotype variants or non-functional segments were found in this series and have been included in Table 1 (i.e. VH5-a, VH4-30-2 and VH4-30-4).[3] Table 2 shows the expected and observed frequencies of VH gene family usage according to the number of VH gene segments per family. Under-representation of VH1 family and over-representation of VH3 family are statistically significant ($p<0.01$). The distribution of the remaining VH families resembles that theoretically expected from random choice. In our series, a total of 30 out of the 39 functional expressed VH segments[5] were found. Overall, VH3-30 and VH3-15 gene segments were the most frequently used (11% and 6.8%, respectively), and their frequency differed significantly from that expected from random choice (2.3%; $p<0.01$). They were followed by VH3-11, VH3-21, VH3-23, VH3-48, VH4-59 and VH5-51, each comprising 5.5% of the 73 VDJH rearrangements. In contrast, several VH segments which are frequently found in normal early and/or circulating B cells, such as VH3-20, VH3-49 and VH3-53, were under-represented or even completely absent in this series. Interestingly, VH4-34 gene segment, which is normally depleted from the expressed VDJH repertoire in MM, was found in two VDJH rearrangements at the DNA level in our series.

DH and JH gene segments and family usage in VDJH rearrangements

Identification of the DH gene segment by the above criteria[4,34] was possible in 65 of the 73 VDJH rearrangements (89%). Overall, DH3 and DH2 families predominated (26% and 23%, respectively; Figure 1). There was a statistically significant over-representation of DH3-22 (10.8%) and DH2-21 (9.2%) gene segments as compared to the theoretically expected value of 3.7% ($p<0.01$; Figure 2). By contrast, there was a complete absence of six DH gene segments: DH1-7, DH1-14, DH1-20, DH4-4, DH5-18 and DH6-25, with three of them (DH1-14, DH1-20 and DH6-25) also missing in the incomplete DJH repertoire (*see below*).

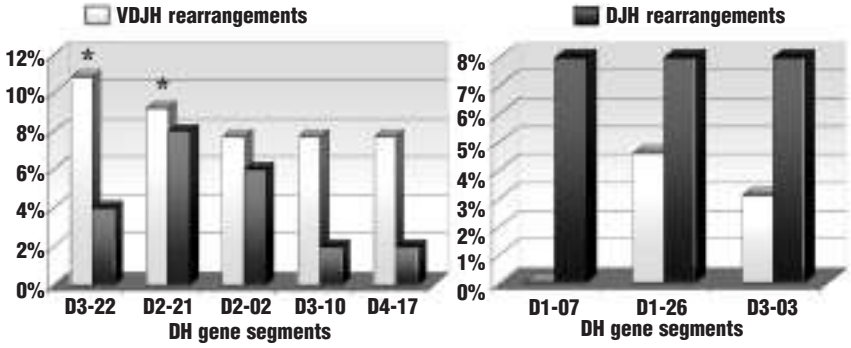

Figure 2. Comparison of the most frequently used DH gene segments in VDJH and DJH rearrangements in MM patients. Left: DH families most frequently found in VDJH rearrangements, with overall frequencies for VDJH and DJH rearrangements. Right: DH gene segments most frequently found in DJH rearrangements, with overall frequencies for VDJH and DJH rearrangements. *Significant difference ($p<0.01$) between observed and expected frequency (3.7% assuming DH random choice).

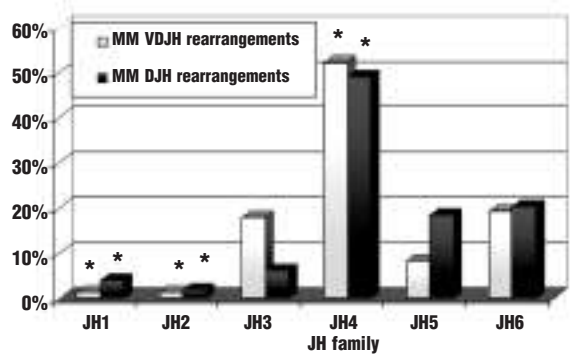

Figure 3. Distribution of JH gene segments in VDJH and DJH rearrangements from MM patients. *Significant *p* value (<0.01) between observed and expected frequencies assuming a random choice for JH (16.7%).

The hierarchy of JH gene usage in the 73 VDJH rearrangements in this series reveals the same pattern that has been observed in normal circulating B cells,[36,37] B-CLL,[34] pre-B cells and B-ALL[38] (Figure 3). Thus, a statistically significant bias of JH4 usage (52.1%; $p<0.001$) was observed, followed by usage of JH6 (19.2%) and JH3 (17.8%), which would seem to be consistent with theoretical random expectations (i.e., 16%). In contrast, most VH-proximal JH2 and JH1 segments were under-represented, since they were both present in only one VDJH rearrangement each (1.4%; $p<0.01$).

DH and JH gene segments and family usage in DJH rearrangements

DH2 and DH3 were the most frequently observed families, each representing 22% of the samples (Figure 1). However, the pattern of DH gene segment usage in DJH rearrangements was slightly different from the one observed in VDJH rearrangements. The most significant difference was the relatively high frequency of DH1-07 (8%; Figure 2), since this was completely absent in the productive repertoire. By contrast, DH1-14, DH1-20 and DH6-25 were absent in both the VDJH and DJH repertoire. Interestingly, DH1-01 and DH6-13, which were present in the VDJH repertoire, were not seen in DJH rearrangements. On the other hand, there was no significant bias in the usage of any particular DH gene segment, although DH1-07, DH1-26, DH2-21 and DH3-03 were more commonly used (Figure 2).

Figure 3 shows the distribution pattern of the JH gene segments. JH4 was statistically over-represented (49%; $p<0.001$), followed by JH6 (20.4%), resembling the pattern of complete VDJH rearrangements. Interestingly, the JH5 was represented more than twice as frequently in DJH as in VDJH (18.4% vs. 8.2%), while the JH3 gene was under-represented in the incomplete repertoire (6.1% vs. 17.8%). Finally, as in the complete rearrangements, most VH-proximal JH1 and JH2 gene segments were significantly under-represented in DJH rearrangements (4.1% and 2%, respectively: $p<0.001$).

Somatic hypermutation and CDR3 composition

Somatic hypermutation was present in all 73 patients who had VDJH rearrangements (median 7.8%; range 2.2-20.8) (Table 1). Analysis of the distribution of replacement and silent mutations suggested antigen-driven somatic hypermutation in 67% of the patients. On the other hand, only 12% of incomplete DJH rearrangements had more than 2% deviation from the germline sequence. The rate of somatic hypermutation was not significantly different between VH families (Table 2). Analysis of N-nucleotide addition and exonuclease activity at the DH-JH junctions of complete and incomplete rearrangements showed no significant differences between both types of rearrangements.[29]

Discussion

Preferential usage of particular VH gene segments and the pattern of somatic hypermutation in VDJH rearrangements have brought new insights into the clonal history and clinical-biological implications of different types of malignant B cells. We analyzed the configuration of *IGH* genes in a large series of 84 untreated MM patients looking for significant over- and under-representation of VH, DH and JH family and individual members, which might suggest a potential biological and clinical role in the development of the disease. As opposed to previous studies, we chose to use genomic DNA instead of cDNA because it allows the analysis of both the productive and non-productive *IGH* allele. It is essential to compare the family and gene segment distribution between functional and non-functional alleles in order to understand the source of a biased repertoire. While the biased usage of particular gene segments in the non-functional allele mainly reflects favored or impaired molecular recombination due to the absence of selective pressure, the distribution of families and gene segments in the productive VDJH rearrangements would be dependent on positive and negative selection mechanisms during B-cell development (e.g. encounter with self or foreign antigens). VH family usage resembled the relative complexity of the germline distribution (i.e. VH3>VH4>VH1>VH2>VH5>VH6>VH7), although a significant over-representation of VH3 members, as well as an under-representation of the VH1 family was observed (57.5% and 8.2%, respectively; $p<0.01$). These results agree with previous reports on

both B-cell malignancies and normal B cells.[17,19,34,39] Thus, these findings cannot be considered as a hallmark of malignant plasma cells, but as a reflection of the selection mechanisms occurring during normal B-cell differentiation.

Regarding the particular usage of VH family members, we found a significant over-representation of the VH3-30 and VH3-15 gene segments (11% and 6.8%, respectively), as well as a marked bias for the usage of VH3-23 and VH4-59 (5.5% each), among others. All but VH3-15 are normally over-represented in the normal B-cell compartment and in MM samples.[15,19,28,40] However, VH3-15 over-representation has not been reported in normal B cells or B-cell malignancies other than myeloma, suggesting that positive selection of VH3-15-containing VDJH rearrangements may occur at the germinal center stage of B-cell maturation prior to the acquisition of the malignant phenotype in MM.

VH4-34 gene segment, which is known to be associated with auto-immune diseases,[41] is markedly over-represented in the VDJH repertoire of normal circulating B cells,[17,19,42] B-CLL,[34,43] B-ALL[43] and diffuse large B-cell lymphoma (DLBCL).[22] By contrast, VH4-34 is excluded from the normal plasma cell repertoire[44] and has not been observed in other large MM series.[28] However, we found the VH4-34 gene segment in 2 out of 73 VDJH rearrangements (2.7%). Interestingly, one of these patients had Bence-Jones MM, thus, the VDJH rearrangement involving VH4-34 was non-functional. In the second patient, the frequency of VH mutations was much higher than the median observed in our series (20.8% vs. 7.3%; $p<0.05$), with clustering of replacement mutations in complementarity-determining regions (replacement : silent=14:1; $p<0.05$), indicating strong germinal center reaction and antigen selection. This suggests that the original autoantibody encoded by VH4-34 would have completely changed its auto-affinity and had become reactive to a foreign antigen, avoiding programmed cell death during the germinal center reaction. If this were the case, however, we should expect a higher frequency of somatically hypermutated VH4-34 rearrangements in MM, as frequently occurs in subgroups of B-CLL and DLBCL. Hence, these data indicate that different molecular mechanisms of selection must take place in the germinal center in pre-malignant MM cells compared to *IGH*-mutated B-CLL and DLBCL precursor cells.

DH gene segment usage differed between functional VDJH and incomplete DJH rearrangements, although the DH2 and DH3 families were clearly over-represented in both subsets, suggesting a more favorable recombination ability of segments belonging to these two families. By contrast, the DH1 family was found to be under-represented in the productive VDJH rearrangements compared to the non-functional DJH repertoire. Thus, DH1 family members recombine at a normal frequency in non-productive rearrangements, but they are somehow excluded from the functional repertoire, probably at the pre-B cell stage.[38] Usage of specific DH gene segments was also biased when comparing VDJH and DJH rearrangements. In particular, DH3-22 and DH2-21 were significantly over-represented in functional VDJH rearrangements, while they are found at expected frequencies in DJH rearrangements. Remarkably, DH1-07 was found in 8% of incomplete DJH but in none of the VDJH rearrangements. DH segments such as DH1-14, DH1-20 and DH6-25 were completely absent from the VDJH and DJH repertoires. Furthermore, DH1-14 and DH6-25 were also not observed in a series of 451 identified DH genes from 893 VDJH rearrangements;[4] this supports the assumption that aberrant recombinant signal sequences exist in these DH gene segments, preventing them from D to JH recombination. Other DH segments commonly found in VDJH rearrangements from normal and malignant less mature B-cells, such as DH6-19 or DH3-03,[4,34,38] were also under-represented in this series. These data indicate that particular DH gene segments are positively and/or negatively selected after VDJH recombination at the level of pre-B cells or, alternatively, during the germinal center reaction. However, whether this is characteristic of MM only, or normal post-germinal center B cells, remains unknown.

The pattern of JH usage was similar to that in pre-B cells and acute lymphoblastic leukemia (over-usage of JH4 and JH6), which has been justified by recombination bias rather than by positive selection.[38,45] This can be explained by the existence of a higher similarity to consensus sequences in the recombinant signal sequences from JH4 and JH6 segments than in the remaining JH gene segments.[45] Interestingly, JH5 was represented in DJH at more than double its frequency in VDJH (18.4% vs. 8.2%), suggesting negative selection at the protein level of VDJH rearrangements containing JH5 during or after the germinal center reaction, since this segment is frequently found in a subgroup of unmutated class-switched B-CLL.[46,47] Conversely, the JH3 gene was under-represented in the incomplete repertoire (6.1%) while it accounted for 17.8% in VDJH rearrangements, indicating positive selection of B cells carrying JH3-containing VDJH rearrangements. In summary, the pattern of *IGH* gene rearrangements in MM cells is particular and consistent and can be distinguished from that in less mature B-cell malignancies. This pattern includes a high rate of somatic hypermutations, preferential usage of VH and DH gene segments and a strong bias against particular gene segments in the

functional VDJH repertoire used by other B-cell malignancies such as B-ALL, B-CLL or DLBCL. In addition, the usage of DH and JH gene segments differs between productive VDJH and unproductive DJH rearrangements, supporting positive and negative selection at the immunoglobulin protein level in MM. Collectively, our data support the origin of the MM clonogenic cell as a post-germinal center B cell, which has already undergone processes of positive selection during B-cell development and the germinal center reaction.

DG, MG and R G-S were responsible for the conception of the study and interpretation of results. DG analyzed the results and wrote the manuscript. AB, MS, RL-P participated in the molecular analysis of the samples. The order in which the names of the authors appear is based on their contribution to the study. All authors critically revised the paper and give the final approval for its submission. JFS is the head of the department and is cited last. The authors declare that they have no potential conflicts of interests.

Manuscript received February 14, 2005. Accepted June 7, 2005.

References

1. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M et al. Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J 1984;3:1209-19.
2. Delves PJ, Roitt IM. The immune system. First of two parts. N Engl J Med 2000;343:37-49.
3. Cook GP, Tomlinson IM. The human immunoglobulin VH repertoire. Immunol Today 1995;16:237-42.
4. Corbett SJ, Tomlinson IM, Sonnhammer EL, Buck D, Winter G. Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. J Mol Biol 1997; 270:587-97.
5. Matsuda F, Ishii K, Bourvagnet P, Kuma K, Hayashida H, Miyata T, et al. The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. J Exp Med 1998; 188:2151-62.
6. Alt FW, Oltz EM, Young F, Gorman J, Taccioli G, Chen J. VDJ recombination. Immunol Today 1992;13:306-14.
7. Lewis SM. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv Immunol 1994;56:27-150.
8. Gellert M. V(D)J recombination: RAG proteins, repair factors, and regulation. Annu Rev Biochem 2002;71:101-32.
9. McBlane JF, van Gent DC, Ramsden DA, Romeo C, Cuomo CA, Gellert M, et al. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell 1995;83:387-95.
10. Kirch SA, Rathbun GA, Oettinger MA. Dual role of RAG2 in V(D)J recombination: catalysis and regulation of ordered Ig gene assembly. EMBO J 1998; 17: 4881-6.
11. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol 2002;20:165-96.
12. Kelsoe G. V(D)J hypermutation and receptor revision: coloring outside the lines. Curr Opin Immunol 1999;11:70-5.
13. Klein U, Goossens T, Fischer M, Kanzler H, Braeuninger A, Rajewsky K, et al. Somatic hypermutation in normal and transformed human B cells. Immunol Rev 1998;162:261-80.
14. Stavnezer J. Antibody class switching. Adv Immunol 1996;61:79-146.
15. Kosmas C, Stamatopoulos K, Stavroyianni N, Zoi K, Belessi C, Viniou N, et al. Origin and diversification of the clonogenic cell in multiple myeloma: lessons from the immunoglobulin repertoire. Leukemia 2000;14:1718-26.
16. Sakai A, Marti GE, Caporaso N, Pittaluga S, Touchman JW, Fend F, et al. Analysis of expressed immunoglobulin heavy chain genes in familial B-CLL. Blood 2000;95:1413-9.
17. Rao SP, Riggs JM, Friedman DF, Scully MS, LeBien TW, Silberstein LE. Biased VH gene usage in early lineage human B cells: evidence for preferential Ig gene rearrangement in the absence of selection. J Immunol 1999;163:2732-40.
18. Szczepanski T, Pongers-Willemse MJ, Langerak AW, Harts WA, Wijkhuijs AJ, van Wering ER, et al. Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant DH6-19 and DH7-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor αβ lineage. Blood 1999; 93: 4079-85.
19. Brezinschek HP, Foster SJ, Brezinschek RI, Dorner T, Domiati S, Lipsky PE. Analysis of the human VH gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral $CD5^{(+)}/IgM^{+}$ and $CD5^{(-)}/IgM^{+}$ B cells. J Clin Invest 1997; 99: 2488-501.
20. Chapman CJ, Mockridge CI, Hamblin TJ, Stevenson FK. Tracking of the V4-34 (VH4-21) gene in human tonsil reveals clonal isotype switch events and a highly variable degree of somatic hypermutation. Clin Exp Immunol 1996;105:360-8.
21. Kiyoi H, Naito K, Ohno R, Naoe T. Comparable gene structure of the immunoglobulin heavy chain variable region between multiple myeloma and normal bone marrow lymphocytes. Leukemia 1996;10:1804-12.
22. Hsu FJ, Levy R. Preferential use of the VH4 Ig gene family by diffuse large-cell lymphoma. Blood 1995;86:3072-82.
23. Kipps TJ, Rassenti LZ, Duffy S, Johnson T, Kobayashi R, Carson DA. Immunoglobulin V gene expression in CD5 B-cell malignancies. Ann NY Acad Sci 1992;651:373-83.
24. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999; 94:1840-7.
25. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999; 94: 1848-54.
26. Tobin G, Thunberg U, Johnson A, Eriksson I, Soderberg O, Karlsson K, et al. Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted Vlambda2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope. Blood 2003;101:4952-7.
27. Chiorazzi N, Ferrarini M. B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. Annu Rev Immunol 2003; 21:841-94.
28. Rettig MB, Vescio RA, Cao J, Wu CH, Lee JC, Han E, et al. VH gene usage is multiple myeloma: complete absence of the VH4.21 (VH4-34) gene. Blood 1996;87:2846-52.
29. Gonzalez D, Balanzategui A, Garcia-Sanz R, Gutierrez N, Seabra C, van Dongen JJ, et al. Incomplete DJH rearrangements of the IgH gene are frequent in multiple myeloma patients: immunobiological characteristics and clinical implications. Leukemia 2003; 17:1398-403.
30. Gonzalez M, Gonzalez D, Lopez-Perez R, Garcia-Sanz R, Chillon MC, Balanzategui A, et al. Heteroduplex analysis of VDJ amplified segments from rearranged IgH genes for clonality assessments in B-cell non-Hodgkin's lymphoma. A comparison between different strategies. Haematologica 1999; 84:779-84.
31. van Dongen JJ, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17:2257-317.
32. Langerak AW, Szczepanski T, van der Burg M, Wolvers-Tettero IL, van Dongen JM. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. Leukemia 1997; 11: 2192-9.
33. Lefranc MP. IMGT, the international ImMunoGeneTics database. Nucleic Acids Res 2001;29:207-9.
34. Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL, et al. Chronic lymphocytic leukemia B cells express

restricted sets of mutated and unmutated antigen receptors. J Clin Invest 1998;102:1515-25.
35. Chang B, Casali P. The CDR1 sequences of a major proportion of human germline Ig VH genes are inherently susceptible to amino acid replacement. Immunol Today 1994; 15:367-73.
36. Yamada M, Wasserman R, Reichard BA, Shane S, Caton AJ, Rovera G. Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult human peripheral blood B lymphocytes. J Exp Med 1991;173:395-407.
37. Huang C, Stewart AK, Schwartz RS, Stollar BD. Immunoglobulin heavy chain gene expression in peripheral blood B lymphocytes. J Clin Invest 1992;89:1331-43.
38. Wasserman R, Ito Y, Galili N, Yamada M, Reichard BA, Shane S, et al. The pattern of joining (JH) gene usage in the human IgH chain is established predominantly at the B precursor cell stage. J Immunol 1992;149:511-6.
39. Brezinschek HP, Brezinschek RI, Lipsky PE. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. J Immunol 1995;155:190-202.
40. Kraj P, Rao SP, Glas AM, Hardy RR, Milner EC, Silberstein LE. The human heavy chain Ig V region gene repertoire is biased at all stages of B cell ontogeny, including early pre-B cells. J Immunol 1997;158:5824-32.
41. Pascual V, Victor K, Spellerberg M, Hamblin TJ, Stevenson FK, Capra JD. VH restriction among human cold agglutinins. The VH4-21 gene segment is required to encode anti-I and anti-i specificities. J Immunol 1992; 149: 2337-44.
42. Kraj P, Friedman DF, Stevenson F, Silberstein LE. Evidence for the overexpression of the VH4-34 (VH4.21) Ig gene segment in the normal adult human peripheral blood B cell repertoire. J Immunol 1995;154:6406-20.
43. Deane M, Baker BW, Norton JD. Immunoglobulin VH4 gene usage in B lymphoid leukaemias. Br J Haematol 1993;84:242-9.
44. Pugh-Bernard AE, Silverman GJ, Cappione AJ, Villano ME, Ryan DH, Insel RA et al. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. J Clin Invest 2001;108:1061-70.
45. Ramsden DA, Wu GE. Mouse κ light-chain recombination signal sequences mediate recombination more frequently than do those of λ light chain. Proc Natl Acad Sci USA 1991; 88: 10721-5.
46. Hashimoto S, Dono M, Wakai M, Allen SL, Lichtman SM, Schulman P, et al. Somatic diversification and selection of immunoglobulin heavy and light chain variable region genes in IgG+ CD5+ chronic lymphocytic leukemia B cells. J Exp Med 1995; 181: 1507-17.
47. Ghiotto F, Fais F, Valetto A, Albesiano E, Hashimoto S, Dono M, et al. Remarkably similar antigen receptors among a subset of patients with chronic lymphocytic leukemia. J Clin Invest 2004;113:1008-16.