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ARTICLE

Analysis of the Expressed Immunoglobulin Variable Region Heavy Chain Gene Products in Paraproteins from Iranian Patients with Multiple Myeloma

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The frequency of expression of immunoglobulin (Ig) variable region heavy (V_H) chain gene products was studied in 43 Iranian patients with mutiple myeloma (MM). The expressed V_H gene families and associated cross-reactive idiotypes (CRI) were analysed by immunoblotting and ELISA, using peptide-induced polyclonal antibodies specific for V_H1-V_H6 gene families and monoclonal antibodies (MAb) recognising CRI linked to theV_H1, V_H3, V_H4 and V_H6 gene families. The results revealed that the V_H3 family (60.5%) was the most predominant gene family. In contrast, no paraproteins were encoded by genes from the V_H2 gene family and only 2.3% were encoded by the V_H5 family. The panel of paraproteins tested rarely expressed the probed V_H-associated CRI. Our results suggest that: 1-The Ig V_H genes, may not be randomly expressed in the malignant plasma cells from Iranian patients with MM. 2- Some of the genes seem to be negatively selected or highly mutated, as evidenced by the lack of expression of the probed CRI. (Pathology Oncology Research Vol 6, No 3, 185–190, 2000)

Keywords: multiple myeloma, V_H genes, paraprotein, cross-reactive idiotype

Introduction

Multiple myeloma (MM) is a chronic lymphoid malignancy characterized by massive infiltration of the bone marrow by terminally differentiated malignant B-cells leading to progressive bone lesions. Although etiology of the disease is currently obscure, a variety of genetic defects have recently been found to be associated with MM.[1] Activating mutations within the proto-oncogene *ras* and inactivating mutations within the tumor suppressor gene p53 and retinoblastoma (Rb) have been reported by different investigators, accounting for approximately 39% and 50% of the MM cases studied, respectively.[2,3] Altered expression of the pax-5 gene, which is involved in regulation of B-cell development, and in CD19 expression, which is a key member of the B-cell receptor signal transduction complex have also been observed in MM cells.[4,5] Different patterns of structural and numeric chromosomal abnormalities have been observed in myeloma cells isolated from MM patients.[6] Using new molecular techniques, it was recently found that translocations involving chromosome 14 on which the Ig heavy chain genes are located, occur in almost all MM cases.[7,8] These complex genetic defects, and the fact that malignant cells are derived from germinal center cells subsequent to antigen challenge[1,9,10,11] may influence selection and expression of the immunoglobulin (Ig) variable region heavy (V_H) or light (V_L) chain genes by the malignant plasma cells. Due to the clonal origin of the myeloma cells and their potency to produce and secrete a large amount of monoclonal Ig in serum, analysis of the expressed Ig V_H or V_L genes can be easily performed on protein or DNA/RNA levels, using specific antibodies or nucleotide probes and primers. Nucleotide or amino acid mutations within the variable region can also be studied directly or indirectly by the same techniques. Molecular approaches have been

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employed in limited number of MM patients from different ethnic groups with inconclusive results.[9-13] In this study, the expressed V_H gene products were investigated in paraproteins isolated from Iranian MM patients, using polyclonal and monoclonal antibodies recognizing V_H subgroups and associated cross-reactive idiotypes (CRI).

Materials and Methods

Polyclonal and monoclonal antibodies

A panel of peptide-induced anti-subgroup antibodies (V_H1 to V_H6) generated by immunization with subgroup specific peptides was kindly provided by Dr. G. Silverman.[14]

The production and characterization of anti-CRI MAbs employed in this study have been described in detail elsewhere.[15-18] These antibodies are listed in *table 1.* Monoclonal anti-heavy and light chain isotypes and subclasses antibodies, AF6 (IgM) ,8a4 (IgG), 2D7 (IgA), JA11 (IgD), C4 (λ), 6e1 (λ), JL512 (IgG1), Gom2 (IgG2), ZG4 (IgG3) and RJ4 (IgG4), were kindly provided by Dr. M. Goodall (Dept. of Immunology, University of Birmingham, UK).

Clinical samples

Serum samples were isolated from 15 ml non-heparinized peripheral blood from 43 Iranian MM patients (22 males, 21 females, mean age 53 years). Diagnosis of MM was based on clinical criteria as outlined elsewhere.[19]

Protein purification

Paraproteins were purified from serum by a combination of gel chromatography, ion exchange and affinity chromatography. Ion exchange chromatography was performed using DEAE-cellulose (DE-52, Whatmann, England) equilibrated with 0.01 M phosphate buffer PH 7.2. IgG paraproteins were usually eluted as breakthrough fractions with the equilibration buffer. IgA paraprotein purification was accomplished by ammonium sulfate precipitation followed by ion exchange chromatography. IgA was eluted with 0.02M−0.05 M phosphate buffer. The purified samples were mostly contaminated with a small proportion of normal polyclonal Igs, transferrin and albumin. Contaminating polyclonal Igs were mostly removed by affinity chromatography on Sepharose 4B-protein-A column (Sigma, UK) equilibrated with 0.1 M Tris-HCl buffer (PH 8.0). Further enrichment of IgA was achieved by passage over a column of Sephadex G-100. Purity of the isolated preparations was determined by SDS-PAGE, under denaturing conditions in 10% polyacrylamide gel as described elsewhere.[20]

Table 1. **Monoclonal anti-CRI antibodies employed in this study**

Antibody	*Specificity*	*Frequency in normal B cells*	*Reference*
G6	V_H1-associated	1.7	15
G8	V_H1-associated	3.2	15
D12	V_H3-associated	19.5	15
Lc1	V_H4-associated	5.7	16
9G4	V_H4-associated	6.9	17
JE6	V_H6-associated	0.1	18

Zone electrophoresis, Immunoelectrophoresis (IEP) and Immunofixation (IF)

The procedure has been described elsewhere.[20] Sera were electrophoresed on cellulose acetate (Helena, France) in barbital buffer (0.03 M, PH 8.6) for 30 minutes. For IEP and IF, sera were first separated by electrophoresis on 1% agarose gel (L-Agarose, Behring, Germany) in barbital buffer (0.03 M, PH 8.6). Monospecific polyclonal anti-isotype antibodies (Dakopatts, Denmark) were then added to the troughs (IEP) or were directly applied to the separated bands (IF). Following incubation and several washings, the gel was stained with amidoblack (Merck, Germany) and finally destained.

Determination of immunoglobulin V_H subgroups

Heavy chain variable region subgroups were determined by immunoblotting using rabbit antisera directed against synthetic peptides, corresponding to consensus first framework sequences.[14] The technique has been described in detail elsewhere.[20] Briefly, approximately 5 μg of the purified paraproteins or diluted serum in disruption buffer (Tris-Hcl 0.2 M, PH 7 containing 20% SDS, 1% bromophenol blue and 6% sucrose) were loaded on to a10% polyacrylamide slab gel. Electrophoresed paraproteins were transfered on to nitrocellulose membrane (Schleicher & Schuell, Germany) in transfer buffer (Tris-Glycin, PH 8.3) at 60 mA, overnight. The membrane was then blocked with 2% bovine serum albumin and incubated with appropriate dilutions of polyclonal anti-subgroup antibodies for 2 hours at room temperature. Following extensive washes with PBS, bound proteins were revealed with horseradish peroxidase- conjugated goat anti-rabbit (Dakopatts, Denmark) and finally visualized with diaminobenzidine tetrahydrochloride (DAB) (Sigma) substrate.

Detection of cross-reactive idiotypes (CRI)

A capture ELISA was employed for determination of the CRI as previously described.[21] Briefly, microtiter polystyrene plates (Maxisorp, Nunc, Denmark) precoated with

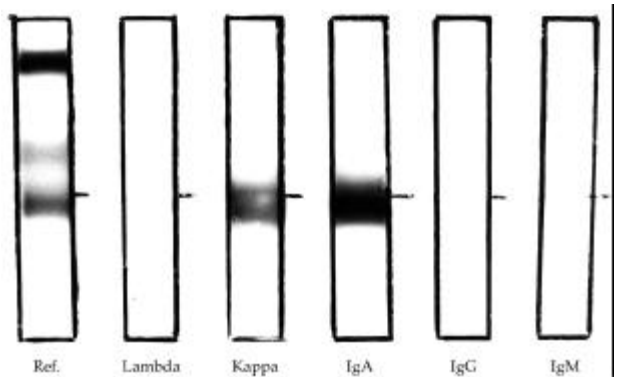

Figure 1. Immunofixation pattern of a serum sample from one of MM patients.

2.5–10 μg/ml of monoclonal anti-CRI antibodies were incubated with appropriate concentrations of the purified paraproteins. Following addition of isotype specific HRP-conjugated goat anti-human Ig (Dakopatts), the reaction was revealed with o-phenylenediamine dihydrochloride (OPD) (Sigma) substrate.

Results

Detection and isotypic characterization of the paraproteins

Paraprotein was detected as a sharp band in the serum electrophoresis pattern of all samples tested (43/43). Isotype and subclass of paraproteins were determined by IEP and/or IF and ELISA techniques. Typical results obtained by IF is illustrated in *Figure 1*.

Of all samples tested, 70% (30/43) and 30% (13/43) belonged to IgG and IgA isotypes, respectively. The frequency of IgG and IgA subclasses was as follows: IgG1 73.3% (22/30), IgG2 10% (3/30), IgG4 16.7% (5/30), IgA1 92.3% (12/13) and IgA2 7.6% (1/13). None of the IgG paraproteins belonged to IgG3 subclass.

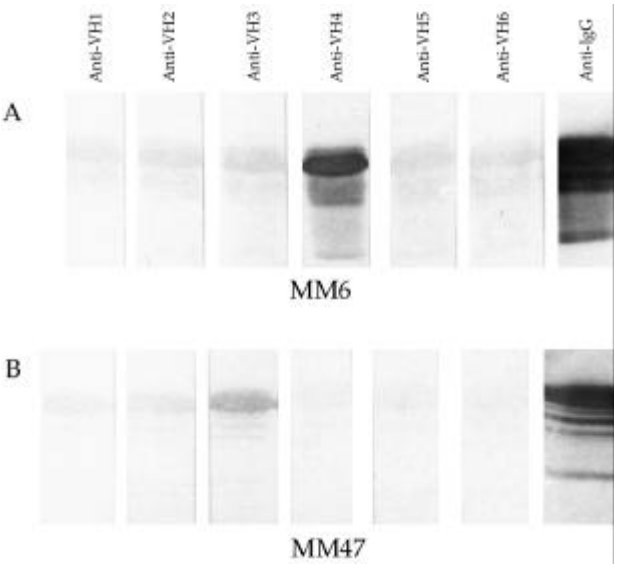

Figure 2. Western blot analysis of V_H expression of two paraproteins.

Distribution of V_H gene family

V_H gene families were identified by Western blotting. *Figure 2* illustrates the immunobloting profile of two paraproteins. The results of the V_H expression are summarized in *Table 2*. Of 43 paraproteins tested, 26 (60.5%) belonged to V_H3 family (39.5% IgG and 20.9% IgA). The frequency of other V_H families was as follows: 28%, 4.6%, 4.6% and 2.3% for V_H4, V_H6, V_H1 and V_H5, respectively.

None of the paraproteins reacted with the anti-V_H2 family specific reagent. Distribution of V_H gene families within IgG and IgA subclasses is given in *Table 3*. All V_H6 and V_H5 gene family expressing IgG paraproteins were from the IgG1 subclass. While the majority of the IgG1 myelomas (63.6%) belonged to V_H3 family, none were from the V_H1 family. The V_H1, V_H3 and V_H4 families**,** were similarly represented within IgG2 and IgG4 subclasses.

Detection of V_H-associated CRIs

V_H-associated CRIs were identified by capture ELISA. Of the paraproteins tested, 7% (3/43) expressed the V_H3-associated CRI (D12), of which 1/30 and 2/13 were from the IgG and IgA isotypes, respectively (*Table 4*). All the CRI^+ samples belonged to the V_H3 gene family. The other V_H-associated CRI identified by G6, G8, Lc1, 9G4 and JE6 MAbs were negative in all the samples tested.

Table 2. **Frequency of V_H subgroups in the panel of paraproteins**

Paraprotein	*Total*	*V_H1*	*V_H2*	*V_H3*	*V_H4*	*V_H5*	*V_H6*
IgG	30	2	0	17	8	1	2
IgA	13	0	0	9	4	0	0
Total	43	2	0	26	12	1	2

Table 3. **Distribution of V_H subgroups within different subclasses of myeloma proteins**

Paraprotein	*Total*	*V_H1*	*V_H2*	*V_H3*	*V_H4*	*V_H5*	*V_H6*
IgG1	22	0	0	14	5	1	2
IgG2	3	1	0	1	1	0	0
IgG4	5	1	0	2	2	0	0
IgA1	12	0	0	9	3	0	0
IgA2	1	0	0	0	1	0	0

Table 4. **Frequency of V_H-associated CRIs in MM parproteins**

Paraprotein	*V_H-CRI*					
	G6	*G8*	*D12*	*Lc1*	*9G4*	*JE6*
IgG	0	0	1 / 30	0	0	0
IgA	0	0	2 / 13	0	0	0
Total	0	0	3 / 43	0	0	0

Discussion

Biased utilization of Ig V_H and/or V_L genes has been reported in a variety of B-cell malignancies, including acute lymphoblastic leukemia (ALL),[22] chronic lymphocytic leukemia (CLL),[15,23] prolymphocytic leukemia (PLL),[24] Burkitt's lymphoma (BL),[25,26] lymphoproliferation associated with autoimmune Sjogren's syndrome,[20] and diffuse large cell lymphoma (DLCL).[27] These malignancies represent B-cell precursors arrested at different stages of the differentiation pathway, from the early pre-B cell (ALL) to the activated germinal center derived B-cell stage (BL and DLCL).

The clonal origin of the neoplastic cells in MM is derived from the most terminally differenctiated B-lymphocytes, i.e. the plasma cells. At this stage of development, the cells are no longer selectable by antigen and are committed to produce and secrete a large amount of Ig in the bone marrow. This could suggest stochastic rearrangement of V_H genes similar to that of the mature naïve peripheral blood lymphocytes. This issue however, has not been extensively studied in MM, with limited unconclusive data being reported in very small cohorts of patients.[9-13] There has been only one extensive investigation on the use of V_H genes in a large cohort of MM patients which has recently been reported by Rettig et al.[28] This limitation seems to be largely due to limited access to bone marrow samples required for extraction of RNA/DNA from the malignant plasma cells which do not circulate in peripheral blood. One simple way of assessment of the frequency of V_H and/or V_L gene expression in MM is the use of polyclonal and monoclonal anti-subgroup and anti-CRI antibodies.

In the present study a comprehensive panel of V_H-associated monoclonal anti-CRI antibodies which have already been extensively characterized together with polyclonal anti-V_H antibodies, were employed for the first time to characterize paraproteins isolated from 43 Iranian patients.

Our results regarding the frequency of V_H gene use show a predominance of the V_H3 and V_H4 gene families. The over-representation of these two families seems to correspond to the total number of functional germline genes within theV_H3 and V_H4 families (*Table 5*), though the figures are slightly higher in our MM samples, accounting for 60.5% and 28% of the total V_H, respectively. However, complete lack of V_H2 family and particularly substantially decreased expression frequency of the V_H1 family (4.6% vz 21.5%), together with slightly higher frequency of the V_H6 gene products (4.6% vz 2%), suggest non-random usage of these V_H families in our MM samples. This is more evident in the IgA paraproteins where only V_H3 and V_H4 genes were selectively expressed with complete absence of the other gene families (*Table 2*).

The results of V_H1, V_H2 and V_H6 gene family distribution, do not comply with previous data reported by Rettig and colleagues,[28] who have demonstrated random expression of all the V_H families in 72 MM patients by RT-PCR and nucleotide sequencing.

The difference in the frequency of V_H family usage is more signified when analyzed in association with the IgG subclass profiles. Although the sample size is too small to allow firm conclusions, absence of V_H1 within 22 IgG1 paraproteins and expression of this V_H family in 2 out of 8(25%) IgG2 and IgG4 subclasses (P=0.06) (*Table 3*), implies non-random distribution of V_H1 family in a subset of MM. A large cohort of MM paraproteins with a variety of heavy chain isotypes and subclasses needs to be studied to confirm and extend our observation.

Lack of expression of the CRI linked to V_H1 (G6 and G8), V_H4 (9G4 and Lc1) and V_H6 (JE6) and low expression frequency of the V_H3-associated CRI (D12) in our samples *(Table 4)* may imply accumulation of somatic hypermutations within the V_H genes. Although extensive mutations have been reported in all V_H sequences isolated from MM patients,[9-13,28] the results obtained for each idiotope should be treated and interpreted separately, to avoid oversimplification. A rough estimate of the expression frequency of the gene(s) coding for the probed CRI in normal peripheral blood, normal bone marrow plasma cells and malignant plasma cells is essential to make precise correlations between CRI expression and somatic mutation. Recent

Table 5. **Comparative frequency of V_H gene family usage in 43 Iranian MM patients**

V_H family	*Germline genes[1]*	*Functional germline genes[2]*	*Frequency of V_H- genes in MM patients*
1	17 (17.9%)	11 (21.5%)	2 (4.6%)
2	4 (4.2%)	3 (5.9%)	0
3	51 (53.7%)	22 (43.1%)	26 (60.5%)
4	13 (13.7%)	11 (21.5%)	12 (28%)
5	3 (3.2%)	2 (3.9%)	1 (2.3%)
6	1 (1%)	1 (2%)	2 (4.6%)
7	1 (1%)	1 (2%)	–
Total	95 (100%)	51 (100%)	43 (100%)

[1,2] The frequency of V_H germline genes and functional germline genes has been adapted from refs. 29 and 30, respectively.

investigations have clearly demonstrated that expression of the functional V_H genes in human adult and fetal life is not random and some members of each V_H family rearrange more frequently than others and some have not been found in any functional rearrangements sequenced to date.[29,31-33]

Taking these considerations into account, our results concerning lack of expression of the V_H4-associated CRI identified by MAb 9G4 may not be easily attributed to somatic mutation. This CRI is encoded by a single gene (V_H 4.34) from the V_H4 family [34.] The idiotope has been localized to the first frame work region (FR1) of the variable region and therefore is expressed on germline encoded and somatically mutated proteins.[35] Almost all V_H proteins encoded by the V_H4.34 gene have so far been found to express the 9G4 CRI.[36] This gene is expressed at a high frequency in peripheral blood B-cells [31,32] and 3.5–6% of these cells were found to express the CRI.[31] Despite overexpression in a variety of B-cell malignancies,[36] surprisingly this gene was not rearranged in malignant plasma cells from a large number of MM patients.[28] Similar findings have also been reported in normal plasma cells isolated from the lamina propria.[37] Although, no data from the normal bone marrow plasma cells is available, expression of the 9G4 CRI in a minor proportion of normal polyclonal IgM (~0.7%),[31] presents indirect evidence for lower representation of the V_H 4.34 gene in normal bone marrow-derived plasma cells. Our results, therefore, could be interpreted to imply a negative selection for the V_H 4.34 gene in the myeloma cells, rather than somatic mutation.

This interpretation may also hold for the lack of expression the other V_H4-associated CRI identified by MAb Lc1 in Iranian MM patients. As opposed to the 9G4 CRI, the Lc1 idiotope was found to be encoded by multiple members of the V_H4 family, including V71-2 (V_H4-61), V71-4 (V_H4-59), V_H4.18 and V2-1 (V_H4-39) and V72-1.[35,38,39] This suggests that Lc1 identifies a sub-sub group of the V_H4 gene products and should be localized to the FR residues. Subsequent studies have proved this assumption and mapped the idiotope to the FR1.[35] Altogether, these observations argue for negative selection of the genes coding for Lc1 CRI, rather than somatic mutation, though the influence of the latter mechanism should not be totally discounted.

Lack of expression of the V_H1-associated CRI identified by the MAbs G6 and G8 could also be attributed to decreased rate of rearrangement of the V_H1 gene family members in our MM samples. Only two of the paraproteins tested (4.6%) belonged to the V_H1 family (*Table 2*). Considering that this gene family constitutes eleven functional members, of which only one (V_H1-69 or 51p1) seems to encode the G6 CRI,[23] inability to detect this CRI can not, therefore, be simply taken to indicate contribution of somatic mutation. This argument also applies to the G8 CRI which is more represented in normal B-cells and polyclonal IgM than G6 CRI. [40, 41] As for the Lc1 CRI, this idiotope is thought to be encoded by more than one member of the V_H1 gene family, yet to be identified. Our findings regarding the V_H1-CRI suggest that selection of the genes coding for these CRI is differentially regulated in $CD5^+$ B-cell malignancies such as B-CLL, where these genes are overexpressed[15] as compared with MM.

Contribution of somatic mutation within our MM samples could be envisaged by the lack of expression of the V_H6-associated CRI recognized by the MAb JE6. The V_H6 family contains a single functional gene (V_H6-1) located adjacent to the D gene cluster.[29] Germline encoded, but not somatically mutated products of this gene are identified by the MAb JE6.[18] This allows the use of this antibody to serologically differentiate between germline encoded and somatically mutated V_H6 gene products. Since two (4.6%) of our MM paraproteins were from the V_H6 family and none were found to express this idiotope, this could imply involvement of somatic mutation in these samples. Interestingly, the only V_H6 gene so far reported to be rearranged in MM, was found to be extensively mutated with a maximum of 89% homology to the germline counterpart.[28]

Finally, the pattern of expression of the V_H3-associated CRI identified by MAb D12 may also imply, to a lesser extent, the influence of somatic mutation. Three of our samples (1 IgG and 2 IgA) (7%) expressed the D12 CRI (*Table 4*). However, this idiotope is expressed on 14–18% of both normal B-cells and polyclonal immunoglobulin,[40,41] suggesting involvement of multiple members of the V_H3 gene family in its expression. At least 5 distinct gene segments from the V_H3 family have been demonstrated to express the D12 CRI. Interestingly, the idiotope was recently mapped to the external face of the heavy chain involving FR1, FR3 and CDR2. A single amino acid mutation at position 57 could result in complete loss of the idiotope.[42]

In summary, our findings suggest non-random distribution of some V_H gene families and V_H-associated CRI in Iranian MM patients, which could partly be attributed to either negative selection or somatic mutation.

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