Ig V<sub>H</sub> Gene Mutational Patterns Indicate Different Tumor Cell Status in Human Myeloma and Monoclonal Gammopathy of Undetermined Significance

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

Plasma cell tumors display a wide spectrum of clinical progression, ranging from aggressive multiple myeloma to a benign form known as monoclonal gammopathy of undetermined significance (MGUS), which requires no treatment. Because both diseases involve mature Ig-secreting plasma cells, the reason for this variation in malignant behavior is unclear. However, assessment of malignant potential is desirable for choice of treatment protocols. Ig variable (V<sub>H</sub>) gene sequence analysis has previously shown the tumor cell of multiple myeloma to be postfollicular, with mutated homogeneous clonal sequences indicating no continuing exposure to the somatic hypermutation mechanism, and this was confirmed in 7 of 7 patients. Comparison of the V<sub>H</sub> gene sequences in the monoclonal cells in MGUS yielded a different result, with 3 of 7 patients demonstrating mutated heterogeneous sequences consistent with the tumor cells remaining under the influence of the mutator. In 1 of 3 of these patients, an IgM-positive precursor cell was identified that expressed heterogeneous V<sub>H</sub> sequences similar to those of the isotype-switched plasma cell. These results indicate that the clonal cells in MGUS differ from those in myeloma and suggest that the difference may reflect malignant potential.

MULTIPLE MYELOMA is a malignant tumor involving plasma cells that accumulate in the bone marrow (BM). Clinical manifestations are diverse but often include osteolytic lesions and renal impairment. The tumor cells generally secrete a monoclonal Ig paraprotein that can be identified in serum, and free Ig light chains (Bence-Jones proteins) may be present in urine. However, levels of normal Ig tend to be low. Prognosis remains poor despite modern chemotherapy, with a median survival of about 30 months. Recent protocols of intensive chemotherapy, sometimes with an autologous BM transplant, have improved initial response, although long-term survival remains to be assessed.

Although monoclonal gammopathy of undetermined significance (MGUS) also involves monoclonal plasma cells, the clinical manifestations are different. In a study at the Mayo Clinic in 1988, MGUS was distinguished from myeloma by having a lower level of serum paraprotein; little or no monoclonal protein in urine; the absence of lytic lesions, anemia, hypercalcemia, and renal insufficiency; and, most importantly, stability of the level of paraprotein and failure to develop other abnormalities. A further distinction is that patients with MGUS tend to maintain normal levels of polyclonal Ig in serum. With regard to the monoclonal cell populations in the two diseases, patients with myeloma characteristically have greater than 10% plasma cells in the BM, and those cells are more often in division, as judged by the plasma cell labeling index.

The relationship between myeloma and MGUS remains uncertain despite many clinical studies. In fact, the term MGUS was introduced by Kyle and Lust to replace benign monoclonal gammopathy on the grounds that only time will show whether a monoclonal protein is truly benign or the first manifestation of myeloma or another lymphoproliferative disease. The Mayo Clinic observed 241 patients with a known serum paraprotein and found that 36 (15%) developed myeloma within a median time of 9.6 years. Other studies have produced similar figures and have indicated that prediction of patients who may undergo transformation to myeloma is not currently feasible. A particular frustration is in the problem of discriminating between stage I myeloma and MGUS, because the ability to do this is important to both patient and physician, given that prevalence of MGUS is considerable, especially in the older age group. In addition, early prognostic discrimination may influence the entry of patients into protocols involving treatment with cytokines. The limits of current information concerning the monoclonal plasma cell of MGUS also affect nomenclature of the cell of origin. Although a clonal population clearly exists, there are arguments for not referring to it as a tumor. However, in the absence of a suitable alternative, the term tumor will be used to describe the monoclonal cell population of MGUS in this report.

Analysis of rearranged functional Ig variable region genes in B-cell tumors is providing information that relates to the clonal history of the cell of origin. In particular, accumulation of somatic mutations in the V-genes indicates that the cell has been exposed to the hypermutation mechanism and has therefore traversed the germinal center (reviewed by Berke). For myeloma, numerous studies have shown that the tumor cell has undergone extensive somatic hypermutation, but, in contrast to follicular lymphoma, homogeneity of tumor V<sub>H</sub> sequence indicates that it is no longer being exposed to the mutator and can be considered as a postfollicular cell. This report describes a parallel analysis of the tumor cells of MGUS and shows in some patients a heterogeneity of clonal sequences, suggesting a clonal history closer to follicular lymphoma than to myeloma.

MATERIALS AND METHODS

Patients. Unselected patients from the Haematology (UK) or Immunology (Germany) Clinics were diagnosed as having multiple

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myeloma (7 patients) or MGUS (7 patients) using clinical and laboratory criteria (Table 1). Among those with myeloma, patients no. 1, 6, and 7 had recently completed a course of chemotherapy for the time of investigation, and the tumor load was reduced, particularly for patient no. 1. Assignment to the myeloma category was performed by the clinician and, for patients no. 4 and 5 with stage I disease, was influenced by the presence of osteolytic lesions. This feature was present in all the patients with myeloma, none of whom had metastatic carcinoma. Six of seven of the group had an identifiable monoclonal Ig in the serum that was of the same Ig class and light chain type as found in the plasma cells in the BM. Patient no. 5 was unusual in having no detectable monoclonal Ig in serum, but IgGa was detectable in plasma cells in the marrow, consistent with nonsecretory myeloma. Four of six evaluable patients in the group had detectable urinary Bence-Jones protein, and all had reduced levels of normal polyclonal Ig in serum.

Patients in the MGUS category were all untreated and had monoclonal Ig in serum at less than 30 g/L, less than 10% plasma cells in BM, less frequent urinary Bence-Jones protein in serum, but IgGa was detectable in plasma cells in the marrow, consistent with nonsecretory myeloma. Four of six evaluable patients in the group had detectable urinary Bence-Jones protein, and all had reduced levels of normal polyclonal Ig in serum.

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**Table 1. Characteristics of MM/MGUS Patients**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Diagnosis/Stage*</th>
<th>Months From Diagnosis</th>
<th>Paraprotein</th>
<th>% Plasma Cells</th>
<th>Polyclonal Ig</th>
<th>Osteolytic Lesions</th>
<th>Bence-Jones Proteinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>MM/II</td>
<td>67</td>
<td>IgGκ</td>
<td>3</td>
<td></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>MM/IIA</td>
<td>0</td>
<td>IgGκ</td>
<td>59</td>
<td>14.0</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>MM/III B</td>
<td>0</td>
<td>IgGκ</td>
<td>79</td>
<td>15.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>86</td>
<td>MM/I</td>
<td>0</td>
<td>IgGκ</td>
<td>18</td>
<td>5.0</td>
<td>–</td>
<td>+ ND</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>MM/I</td>
<td>7</td>
<td>IgGκ</td>
<td>17</td>
<td>8.0</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>74</td>
<td>MM/II</td>
<td>15</td>
<td>IgGκ</td>
<td>43</td>
<td>5.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>78</td>
<td>MM/III A</td>
<td>19</td>
<td>IgGκ</td>
<td>24</td>
<td>11.0</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>63</td>
<td>MGUS</td>
<td>11</td>
<td>IgGκ</td>
<td>18</td>
<td>8.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>MGUS</td>
<td>19</td>
<td>IgGκ</td>
<td>13</td>
<td>0.5</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>41</td>
<td>MGUS</td>
<td>13</td>
<td>IgGκ</td>
<td>3</td>
<td>6.2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>71</td>
<td>MGUS</td>
<td>125</td>
<td>IgGκ</td>
<td>5</td>
<td>8.0</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>78</td>
<td>MGUS</td>
<td>0</td>
<td>IgGκ</td>
<td>8</td>
<td>5.0</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>67</td>
<td>MGUS</td>
<td>3</td>
<td>IgGκ</td>
<td>10</td>
<td>1.3</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>87</td>
<td>MGUS</td>
<td>1</td>
<td>IgGκ</td>
<td>13</td>
<td>7.0</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

* Durie and Salmon.
† Nonsecretory myeloma.

For tumor cells, the V, gene analysis was performed using cDNA. Reverse transcription was performed using an appropriate constant region primer to match the identified tumor-derived Ig. For IgG or IgA, a Cy primer (5'-CACCGTCACCGTGTCGG) or a Ca primer (5'-CTGGGTGCTGCTGAGGCT) was used, respectively, with a first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden).

Amplification and sequencing of V, genes. For analysis of the V, genes of tumor cells, the first-to-one-to-one-third of a sample of cDNA was amplified by PCR using a mixture of 5' oligonucleotide primers specific for each of the V, leader sequences of V,1-6 families (V,1: 5'-CTCACCATTGGACCTGACCTGAG; V,2: 5'-ATGGAATACATCTTTTGGTCACGTC; V,3: 5'-CCATGGAGTTTGGGCTGACG; V,4: 5'-ACATGAAACAYCTGTGGTTClTCC; V,5: 5'-ATGGGTCAACCCGACATCCTCC; V,6: 5'-ATGGCTGTTCCTTCTTCTACCTCC; together with 3' primers specific for the constant region (see above). In all cases, polymerase chain reaction (PCR) conditions were as described. Gel-purified products of predicted size were blunt-end ligated into pGEM-T-A vector (Promega, Madison, WI) and used to transform JM109 competent cells (Promega). Clones found to contain an appropriate size by restriction analysis of plasmid DNA were sequenced by the dideoxy chain termination method, with alignment being made current EMBL/GenBank and V-Base sequence directories, using MacVector 4.0 sequence analysis software (International Biotechnologies, Inc, New Haven, CT). At least two independent PCR amplifications were performed from each sample.

Investigation of tumor-related V,-Cp transcripts. For patient no. 13, precursor V,-Cp transcripts with the tumor-related clonal signature in CDR3 were sought using a three-step seminested PCR approach. Total RNA (5 µg) was reverse transcribed using an outer Cα primer (5'-GACGGATCCATCTGAGGAC). In step 1, one-fourth of the cDNA (18 µL) was amplified using the 5'-V, leader primer together with an inner 3'-Ca2 primer (5'-CGAGGGGAAAGGG). The product of predicted size was cloned and randomly selected colonies were sequenced to confirm the presence of Cα. In step 2, 1/25 of the PCR product of step 1 was amplified with a 5'-CDR3-specific primer (5'-GGATATTAYTATGATMG) together with the 3'-Ca2 primer. Amplification conditions were modified to include an annealing temperature of 42°C for 1 minute. In step 3, to obtain a full V, sequence, 1/25 of step 1 product was

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amplified with the 5'-V\textsubscript{H}3 leader primer together with a 3'-CDR3-specific primer (5'-GYAGTTACCACCCACKACATACA) and an annealing temperature of 52°C for 1 minute. PCR products of predicted size were cloned and sequenced.

**RESULTS**

\( V_\text{H} \) \textit{sequences from tumor cells of patients with myeloma.} Preparations of cDNA obtained from BM MNC populations of 7 patients with myeloma were amplified with mixed 5'-\( V_\text{H} \) leader-specific primers together with a 3'-constant region primer chosen from the Ig class of the tumor-derived paraprotein (Table 1). The PCR products were cloned and sequenced; in all cases, a predominant \( V_\text{H} \) sequence with an identical CDR3 clonal signature was evident and was confirmed by repeated PCR. Remaining clones contained individually distinct \( V_\text{H} \) sequences, presumably derived from normal plasma cells or B cells. Deduced amino acid sequences of the predominant clones are shown in Fig 1; nucleotide sequences have been submitted to the EMBL database (accession nos. X88806-12).

Sequence analysis of the tumor-related clones (Fig 1 and Table 2) indicated that 6 of 7 were derived from the \( V_\text{H}3 \) family and 1 of 7 from the \( V_\text{H}1 \) family. For all sequences, homology with germline sequence was quite low, suggesting that a significant degree of somatic mutation has occurred. Deviations from germline sequence were also evident in JH, again consistent with somatic mutation events. However, although the \( V_\text{H} \) repertoire now includes a large content of allelic variation, assignment of each nucleotide change to a corresponding germline gene in the individual patient and this has not been performed. In Table 2, each nucleotide change may be interpreted as a replacement mutation (R) or silent mutation. However, these ratios did not exceed 2:5:1 for the remaining 5 patients. This inconsistency in distribution of replacement mutations is similar to that reported for other cases of myeloma\textsuperscript{[14]} and leaves open the role of antigen selection.

**\( V_\text{H} \) \textit{sequences from tumor cells of patients with MGUS.}** A similar approach was used to identify the tumor-related sequences from patients with MGUS. Repeated sequences were again identified after PCR and cloning, although the
number of sequences derived from contaminating B cells tended to be slightly higher (Table 3). Deduced amino acid sequences are shown in Fig 2 with nucleotide sequences again available from the EMBL data base (accession nos. X88813-15, X88824-26, and X88842). Sequence analysis showed that, as for the cases of myeloma, the most common VH family used was again VH4 (4/7), with the remaining 3 from the VH1, VH2, and VH4 families. Deviations from corresponding germline sequences in the database suggested a high degree of somatic mutation, with the percentage of homologies being similar to those found for the myeloma sequences. Somatic events were confirmed by nucleotide changes in the relatively nonpolymorphic VH4 gene (DP-67) used by patient no. 14 and by changes in JH sequences (Fig 2 and Table 3). Analysis of the distribution of somatic mutations indicated an increased R:S ratio in the CDRs for some patients but not all, giving a heterogeneous picture similar to that seen in myeloma. The sequence derived from VH4 did not show clustering of replacement mutations in the CDRs (R:S = 5:3), and the question of the role of antigen selection in myeloma or MGUS remains to be decided.

**Analysis of intraclonal sequence heterogeneity.** The tumor-derived VH sequences from each patient could be identified from the CDR3 clonal signature, and, by comparing the mutational patterns of the individual clones at the nucleotide level, it was possible to investigate intraclonal sequence variation. For the cases of myeloma, all the clones obtained from each patient were sequenced (Table 2) and all had identical sequences with no nucleotide differences, consistent with an absence of intraclonal variation. In contrast, for the cases of MGUS, there was evidence for intraclonal variation among tumor-derived VH sequences from 3 of 7 patients, indicative of ongoing mutational events in the neoplastic cell. This intraclonal heterogeneity is illustrated by the nucleotide sequences of the VH genes of the three patients' tumor cells shown in Figs 3, 4, and 5. The heterogeneous mutations appear to vary in incidence among the 3 patients and to be scattered throughout the VH sequences. However, replacement mutations occur in CDR3 in all cases. For patients no. 10 and 13, many of the nucleotide changes were identified in more than one sequence and indicate that several clonal members were undergoing further mutational events. For patient no. 14 and for some of the changes in patients no. 10 and 13, nucleotide changes occurred in only one sequence, and there is a theoretical possibility that such differences could have arisen due to Taq polymerase error. However, application of the

**Table 3. Analysis of VH Genes From Cases of MGUS**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Ig Class</th>
<th>VH Family</th>
<th>GL Donor</th>
<th>% Homology</th>
<th>RS Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FWR</td>
</tr>
<tr>
<td>8</td>
<td>IgA</td>
<td>VH3</td>
<td>DP-49</td>
<td>91.5</td>
<td>3/8</td>
</tr>
<tr>
<td>9</td>
<td>IgG</td>
<td>VH1</td>
<td>V1-24P</td>
<td>93.9</td>
<td>6/5</td>
</tr>
<tr>
<td>10</td>
<td>IgG</td>
<td>VH2</td>
<td>S12-4</td>
<td>95.3</td>
<td>5/2</td>
</tr>
<tr>
<td>11</td>
<td>IgG</td>
<td>VH3</td>
<td>DP-53</td>
<td>90.5</td>
<td>9/7</td>
</tr>
<tr>
<td>12</td>
<td>IgG</td>
<td>VH3</td>
<td>VH3-8</td>
<td>93.2</td>
<td>8/5</td>
</tr>
<tr>
<td>13</td>
<td>IgG</td>
<td>VH3</td>
<td>DP-68</td>
<td>94.6</td>
<td>6/4</td>
</tr>
<tr>
<td>14</td>
<td>IgG</td>
<td>VH4</td>
<td>DP-67</td>
<td>93.9</td>
<td>6/4</td>
</tr>
</tbody>
</table>

**Fig 2.** Deduced amino acid sequences of the VH regions of the tumor-related clones from patients with MGUS. Comparisons are made with the closest germline VH genes. Upper case letters, replacement mutations; lower case letters, silent mutations. Replacement mutations in the JH regions are underlined.
Fig 3. Intraclonal heterogeneity of the nucleotide sequences of the Vδ gene used by IgG-positive tumor cells from patient no. 10 with MGUS. 10G1 through 10G9 represent individual cloned sequences that are compared with the closest germline gene, S12-4. Silent or replacement mutations are indicated below the sequences, in lower case or starred upper case letters, respectively.

sequences from patient no. 13 were more consistent with existence of a major clone (13G1) that has accumulated different individual single mutations or double mutations (Figs 4 and 6). The pattern of intraclonal variation may be explained by assuming the existence of a parent clone (13G0) that acquired an additional mutation to become 13G1, but that also diverged to accumulate different mutations to generate the minor clone 13G2, together with additional mutated progeny (Fig 6). Because of the low
percentage of tumor sequences obtained from patient no. 14, only four clones were available for investigation, but heterogeneity was detected, with all the changes generating replacement amino acids.

**V₉** mutational pattern of an IgM-positive tumor precursor cell. In 1 of the cases of MGUS (patient no. 13) that had shown intraclonal **V₉** sequence variation in the IgG-positive tumor cell, it was possible to detect a preswitched IgM-positive cell that belonged to the tumor cell clone. Identification of the precursor was made by using a 5' CDR3 primer together with a 3' Cu primer (step 2 of the seminested PCR in the Materials and Methods). Sequencing of 10 clones of the product showed a single sequence homologous to 13G1 (Fig 7), which did not contain the additional mutations seen in the CDR3/β₉ regions of the IgG clones 13G7 or 13G10 (Fig 4). To obtain the full **V₉**Cu sequence, the third step of the seminested PCR was performed, and the sequences are shown in Fig 8. Clonal relationship with the isotype-
switched tumor cells was indicated both by the CDR3 sequence and by the match to the DP-58 germline gene. There were also common deviations from germline in the VH sequences of both the IgG clones and the IgM clones (Figs 4 and 8).

These data allowed a parallel analysis of intraclonal heterogeneity of the VH sequences of the IgM-positive tumor cells (Fig 8). Overall, the degree of heterogeneity in the IgM-positive cells appeared less than in the IgG-positive cells. None of the IgM-derived sequences was identical to the isotype-switched sequences; however, the predominant IgM sequence (13M1) closely resembled that of the predominant IgG clone 13G1, with only a single nucleotide difference in codon 69 (Figs 4 and 8). The other IgM clones were similar to 13M1 but had other scattered single mutations, one of which (G to A in codon 11 of 13M4) was also present in
an IgG clone (13G11). Other mutations present in the IgG clones related to the divergent 13G2 group (Fig 6) were not seen in IgM, perhaps indicating that at least some of the additional mutations occurred after isotype-switching.

**DISCUSSION**

During maturation of a B lymphocyte, Ig V<sub>H</sub> genes undergo a process of recombination to generate a VH-DH-JH-constant region unit. Heterogeneity of usage of the individual genetic components, together with gain and loss of nucleotides at the joints, leads to a unique amino acid sequence particularly in CDR3. In a normal B cell, this process produces a wide range of available sequences for antigen recognition, with the unique CDR3 present at the center of the antigen-binding site. In neoplastic B cells, the CDR3 presents a useful clonal marker for detection and tracking of the tumor clone. When a normal B cell enters the germinal center, the V<sub>H</sub> genes are exposed to the somatic hypermutation mechanism, thereby generating nucleotide substitutions throughout the sequence. Antigen selection can then lead to a concentration of replacement amino acids in the CDR sequences, which are known contact points for antigen.

Because neoplastic B cells are considered to be frozen at a point in differentiation, analysis of V<sub>H</sub> gene sequences can show aspects of the clonal history of the tumor cell. In the case of chronic lymphocytic leukemia, the V<sub>H</sub> genes appear
to have a low number of somatic mutations, indicating that they may not have been in contact with the mutator that is likely to be localized in the germinal center. In contrast, cases of follicular lymphoma (FL) tend to harbor more mutations, as would be expected from their residence in the germinal center. Interestingly, tumor cells from patients with FL often display heterogeneous clonal sequences, with the same clonal signature in CDR3, but with additional mutations occurring in V_H. This picture is consistent with continuing exposure of the tumor cells to the mutator subsequent to neoplastic transformation. A similar phenomenon has also been observed in cases of endemic Burkitt’s lymphoma.

Although the role of antigen in driving tumor cell growth remains unknown, it is likely that the neoplastic event releases tumor cells from the apoptotic death that awaits normal unselected B cells.

Several groups have analyzed V_H gene profiles in cases of multiple myeloma, and a consistent pattern has emerged. It appears that the tumor cells are extensively somatically mutated and that the sequences are homogeneous. These findings suggest that the tumor cell has traversed the germinal center, but that it is no longer exposed to the mutator. Absence of intraclonal variation in myeloma has been observed at different stages of disease from presentation to plateau, indicating that it is a feature of the tumor cell population rather than a consequence of outgrowth of a member of the clone. It could be concluded from this that the neoplastic event has occurred at a postfollicular stage, but this conclusion is questioned by the finding of IgM-positive B cells in the BM of identical twin. This picture is consistent with continuing exposure of the tumor cells to the mutator subsequent to neoplastic transformation. A similar phenomenon has also been observed in cases of endemic Burkitt’s lymphoma.

Whether this type of analysis is useful for distinguishing between early stage myeloma and MGUS is not yet evident; investigation of larger numbers of patients and a longer period of observation will be required. Heterogeneity was observed only in 3 of 7 patients, and, although that can be compared with a lack of heterogeneity in more than 60 patients with myeloma, it is not diagnostic for MGUS. More patients will need to be investigated to see if heterogeneous delineates a subset of patients and if chromosomal changes correlate with the V_H gene profiles. Clearly, availability of V_H gene tags is providing new tools for old questions.

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