Brief Definitive Report

Evidence for a Bone Marrow B Cell Transcribing Malignant Plasma Cell VDJ Joined to Cμ Sequence in Immunoglobulin (IgG)- and IgA-secreting Multiple Myelomas

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Summary

Multiple myeloma is a B cell malignancy characterized by the expansion of plasma cells producing monoclonal immunoglobulins (Ig). It has been regarded as a tumor arising at the B, pre-B lymphocyte, or even stem cell level. Precursor cells are presumed to proliferate and differentiate giving rise to the plasma cell clonal expansion. Antigenic features and specific Ig gene rearrangement shared by B lymphocytes and myeloma cells have supported this hypothesis. However, the existence of such a precursor is based upon indirect evidence and is still an open question. During differentiation, B cells rearrange variable (V) regions of Ig heavy chain genes, providing a specific marker of clonality. Using an anchor polymerase chain reaction assay, these rearranged regions from five patients with multiple myeloma were cloned and sequenced. The switch of the Ig constant (C) region was used to define the B cell differentiation stage: V regions are linked to Cμ genes in pre-B and B lymphocytes (pre-switch B cells), but to Cγ or Cα in post-switch B lymphocytes and plasma cells (post-switch B cells). Analysis of bone marrow cells at diagnosis revealed the presence of pre-switch B cells bearing plasma cell V regions still joined to the Cμ gene. These cells were not identified in peripheral blood, where tumor post-switch B cells were detected. These pre-switch B cells may be regarded as potential myeloma cell precursors.

Multiple myeloma is a B-cell malignancy characterized by a clonal expansion of plasma cells producing monoclonal Ig. Several lines of evidence suggest B lymphocyte involvement in the pathogenesis of multiple myeloma. First, B lymphocytes, or even pre-B lymphocytes expressing the same idiotype as myeloma protein have been found in bone marrow and peripheral blood (1–3). Second, malignant plasma cells expressing pre-B lymphocyte antigens have been described (4–6). Third, PBMC may show the same IgH gene rearrangement as myeloma cells (7, 8). However, the existence of a myeloma cell precursor is still a subject of controversy (9, 10).

Our molecular approach involved in the use of tumor cell IgH V region as a marker of clonality. The antigen specificity in B lymphocyte clones is derived primarily from the rearrangement of V, D, and J segments of H chain genes. Assembled variable regions (VDJ) contain three CDR that codify for the antigen-binding site. These regions are unique to each B cell clone (11), and can be used to define oligonucleotide primers and probes characteristic of the tumor cell. After antigenic stimulation, B lymphocytes switch the IgH isotype by deleting the Cμ gene, without changing the VDJ-encoded specificity. Hence, pre-B and B lymphocytes (pre-switch B cells) can be discriminated from post-switch B lymphocytes and plasma cells (post-switch B cells) because of their C region usage.

We reasoned that myeloma cell precursor may be located at the pre-switch B cell stage, and be identifiable as a cell in which the IgH transcript is composed of plasma cell VDJ still joined to Cμ gene. The sequence of the plasma cell VDJ was obtained by using an anchor PCR assay (12), whereas the switch region was analyzed by means of a standard PCR protocol (13).

Materials and Methods

Patients and Nucleic Acid Extraction. Four cases of multiple myeloma were evaluated at diagnosis and one at relapse. Two patients had an IgG (MM-20 and MM-23) and three an IgA (MM-15, MM-67, and MM-125) monoclonal protein. Specimens were obtained during standard diagnostic procedures. Bone marrow plasma cells and lymphocytes were separated on a Ficoll-Hypaque density gradient. DNA was obtained by cell lysis, phenol extraction, and ethanol precipitation. RNA was isolated by the guanidinium isothiocyanate and cesium chloride centrifugation method (14).

Synthesis of cDNA and Tailing Reaction. Two syntheses of cDNA were performed: (*a*) total RNA was reverse transcribed using an isotype-specific primer (α or γ) to have the Ig cDNA; and (*b*) total

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RNA was reverse transcribed into total cDNA using an oligo dT-15 primer. 20 μg of total RNA was reverse transcribed with 20 pmol of reverse transcription primer (γ: GACCGATGGGCCCTTGGTGGAAGC [15], or α: GACCTTGGGGCTGGTCGGGGATGC [16]). A 50-μl reaction was carried out in 10 mM dithiothreitol, 1 mM dNTPs (Pharmacia LKB Biotechnology, Uppsala, Sweden), 1× reverse transcriptase buffer (50 mM Tris-HCl, 6 mM $MgCl_2$, 40 mM KCl), final concentration, adding 20 U of ribonuclease inhibitor (RNasin; Promega, Madison, WI), and 400 U Moloney MuLV reverse transcriptase (Superscript, GIBCO BRL, Gaithersburg, MD). The reaction was incubated 1 h at 37°C. The Ig cDNA was then tailed in 200 μM dATP, 1× terminal deoxynucleotidyl transferase (TdT) buffer, final concentration, adding 30 U of TdT enzyme (GIBCO BRL), and incubated 10 min at 37°C.

PCR Conditions. Three reactions were performed. The anchor-PCR assay (Fig. 1) included PCR-1 and -2, whereas PCR-3 was a standard amplification. For PCR-1, 20% of dA-tailed Ig cDNA was amplified in 10% DMSO, 200 μM dNTPs, 1× Taq buffer (50 mM KCl, 10 mM Tris-HCl pH, 8, 1 mM $MgCl_2$, 1% [wt/vol] gelatin) final concentration, adding 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus Corp., Norwalk, CT), 10 pmol of RE-TTTT (GGTGGATCGATGAATTCTTTTTTTTTTTTTTTT), 20 pmol of RE (GGTGGATCGATGAATTCTT), and 20 pmol of JH-RE2 (GGATGGTACCAAGCTTTGAGGAGACGGTGACCA) primers in a final volume of 50 μl. Using a DNA thermal cycler (Perkin Elmer Cetus Corp.) the tailed cDNA was amplified by linking two files together. File A: 94°C for 1 min; 50°C for 3 min; 72°C for 40 min; 1 cycle. File B: 94°C for 1 min; 56°C for 2 min; 72°C for 1 min; 40 cycles, followed by a 7-min final extension at 72°C. The 5′ primers (RE-TTTT, RE) contained ClaI and EcoRI restriction enzyme sites, whereas the 3′ primers (JH-RE2, RE2) had KpnI and HindIII sites. The PCR product was electrophoresed on 1.5% low melting point agarose and the 500-bp band cut out under shortwave UV light. For PCR-2, the slice of low melting point agarose was melted at 68°C and 5–10% reamplified in the conditions described above, except for 2 mM $MgCl_2$ concentration in the 1× Taq buffer and 20 pmol of RE and RE2 primer (GGATGGTACCAAGCTTTGA), instead of using RE-TTTT and JH-RE2. File C was used for this PCR: 94°C for 1 min; 54°C for 30 s; 72°C for 30 s; 32 cycles, followed by a 7-min final extension at 72°C. For PCR-3, the amplification was used to detect the presence of clonal pre-switch B cells in bone marrow and peripheral blood RNAs. 5 out of 50 μl of total cDNA was amplified in the same PCR-2 conditions, except for the 1.5 mM $MgCl_2$ concentration in the 1× Taq buffer. The 5′ primers were derived from CDRII, and were specific for each patient (Table 1), whereas the 3′ primer was from the Cμ first exon (μ-1: GTTGGGGCGGATGCACT [17]). File C was used at 61–64°C annealing temperature for 48–55 cycles.

Oligonucleotide Synthesis. Oligonucleotides were chemically synthesized with a DNA synthesizer (model 391 PCR-MATE EP; Applied Biosystems, Inc., Foster City, CA) on a 0.2-μmol scale, according to the manufacturer's instructions.

Cloning and Sequencing. The PCR product was digested with EcoRI and HindIII enzymes according to the manufacturer's instructions at 37°C for 3 h. Digested DNA was electrophoresed through a 1.5% low melting point agarose gel, the 500-bp band cut out, phenol/chloroform-extracted and precipitated in 2 vol of ethanol, and then cloned in a Bluescript SK® vector (Stratagene, San Diego, CA) according to standard procedures (14). Restriction enzyme analysis was carried out on plasmid DNAs prepared by the alkaline lysis method (14). Miniprep plasmid DNAs were sequenced with the Sequenase version 2.0 kit (United States Biochemicals Corp., Cleveland, OH) according to the manufacturer's recommendations. Sequence analysis was performed using the PC-GENE programs (IntelliGenetics, Inc., Mountain View, CA).

Southern Blot and Direct Sequencing Analysis. 20% of the PCR product was analyzed by agarose gel electrophoresis. The amplified DNA was blotted overnight onto nylon membranes (Stratagene), and hybridized to CDRIII probes end-labeled with γ[^{32}P]ATP (14).

The nucleotide sequence of the junction between the VDJ and Cμ genes was determined by direct sequencing of amplified DNA. 5 μl of bone marrow total cDNA was amplified using CDRII and μ-5 (GGGAATTCAAGGAAGTCCTGTGCGAG [17]) primers. μ-5 is located 42 bp from μ-1 in the 3′ direction. Amplification conditions were as described above. The CDRIII oligonucleotides (case MM-15 and MM-25) were also used as sequencing primers after end-labeling with γ-[^{32}P]ATP. Sequencing reactions were performed as previously described (18). Reaction products were run on a 6% acrylamide/urea gel, fixed in 10% acetic acid, and then exposed at −70°C for varying periods of time.

Results

The anchor-PCR assay is outlined in Fig. 1 (19). Briefly, Ig cDNA was synthesized from bone marrow total RNA using primers specific for plasma cell C region, and then a dATP tail was added by TdT enzyme to the 3′ end. The dA-tailed Ig cDNA was amplified twice, using two sets of primers (JH-RE2, RE-TTTT, and RE, RE2). Finally, the amplified VDJ were cloned and sequenced. For each patient 10–20 clones were sequenced. The first sequencing gel analysis was per-

Table 1. *Tumor-specific Primers and Probes*

Patients	CDRII 5′ primer	Distance	CDRIII probe
		bp	
MM-15	TTTCAGACCGAAGTCCGAGT	138	AGACTGAGGGCCAGTTGTTAC
MM-20	CTACATCAGTGGTACTGGTAATTC	126	GGTCTCTCCCCATACTGACTA
MM-23	CCTTTAGCAGATATGATCTC	124	TTGTTTGGTGCGCATATCGG
MM-67	ATAAGTGGAAGTGGCATTC	130	GGACGGGGAACTTGGATA
MM-125	CGTGATGGGAGTAGTACAAG	136	GGACATAACAGTGGCTGG

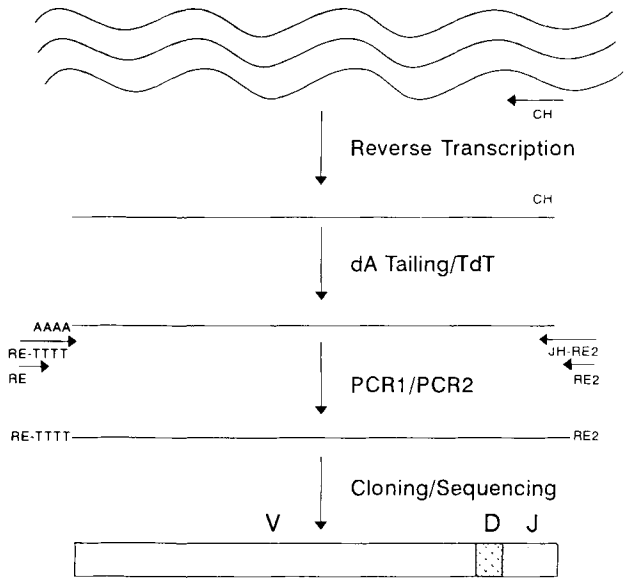

Figure 1. Schematic representation of the anchor PCR assay. Bone marrow total RNA was reverse transcribed into Ig cDNA using an isotype-specific primer (*CH*), and then dA-tailed by TdT enzyme for anchor PCR. The tailed cDNA was amplified twice (PCR1/PCR2) by using two sets of primers (JH-RE2 with RE-TTTT, and RE and RE2). The amplified VDJs were then cloned and sequenced. After sequencing analysis, the CDR were identified and tumor-specific primers and probes were generated.

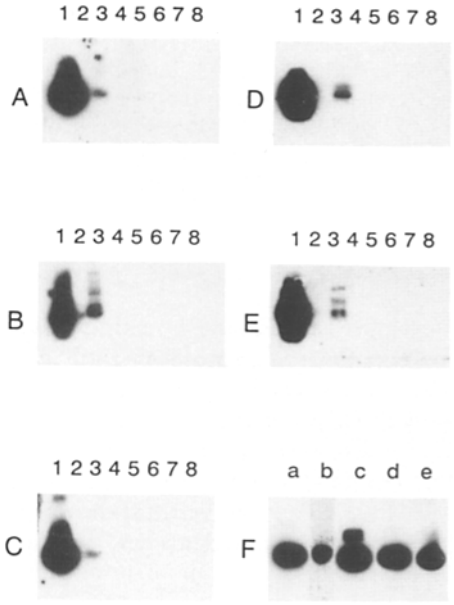

Figure 2. PCR-based detection of pre-switch B cells. Detection of clonal pre- and post-switch B cells in bone marrow and peripheral blood total cDNA of five myeloma patients. The amplifications were performed using CDRII 5′ primer and different C region 3′ primers. Amplified DNAs were then hybridized to CDRIII probes. (*A*) MM-15 patient; (*B*) MM-20 patient; (*C*) MM-23 patient; (*D*) MM-125; and (*E*) MM-67. Lane *1*, bone marrow total cDNA amplified with γ or α primers; lane *2*, empty; lane *3*, bone marrow total cDNA amplified with μ-1 primer; lane *4*, peripheral blood total cDNA amplified with μ-1 primer; lane *5*, empty lane; lanes *6* and *7*, polyclonal cDNAs; and lane *8*, no DNA control. (*F*) lane *a*, MM-15 peripheral blood total cDNA amplified with α primer; lane *b*, MM-20 peripheral blood total cDNA amplified with γ primer; lane *c*, MM-23 peripheral blood total cDNA amplified with γ primer; lane *d*, MM-125 peripheral blood total cDNA amplified with α primer; and lane *e*, MM-67 peripheral blood total cDNA amplified with α primer.

formed to examine the 3′ part of the VDJ to assess how many clones were identical, and this number corresponded to the percentage of tumor cell infiltration in the specimen. A second analysis then gave the complete sequence of the three CDRs. The assay specificity was tested for each patient, generating clone-specific primers and probes from the CDR sequences. No amplification products were detectable when several polyclonal DNAs were used as templates (19). However, further confirmation of myeloma specificity of the cloned VDJ sequence was achieved by using the clone insert, containing the supposed tumor VDJ, as a probe. Patient genomic DNA, was digested with HindIII restriction enzyme, and hybridized under stringent conditions with this probe. Southern blot analysis showed a rearranged band, confirming that the cloned VDJ really represented the tumor VDJ, and excluding any nonspecific bias during the amplification (data not shown).

Bone marrow total RNAs from each patient were reverse transcribed in total cDNA, and analyzed by using tumor-specific oligonucleotides to detect pre-switch B cells having the same plasma cell VDJ. Total cDNAs were tested for the presence of tumor CDRs attached to the first exon of Cμ gene. A PCR amplification was carried out using CDRII and μ-1 primers. A second PCR was performed with γ or α 3′ primer to reveal post-switch B cells in the same cDNA. Then, the amplified DNAs were hybridized to a CDRIII probe. All cases revealed an intense band corresponding to the post-switch B cells, amplified by the second PCR, and one corresponding to pre-switch B cells bearing the Cμ gene (Fig. 2, *A–E*). To control the assay specificity, total cDNAs from two polyclonal LN were always used as a negative controls and no bands were detectable even after a long period of exposure. To further confirm Southern blot results, the bone marrow cDNA of MM-15 and MM-125 cases were

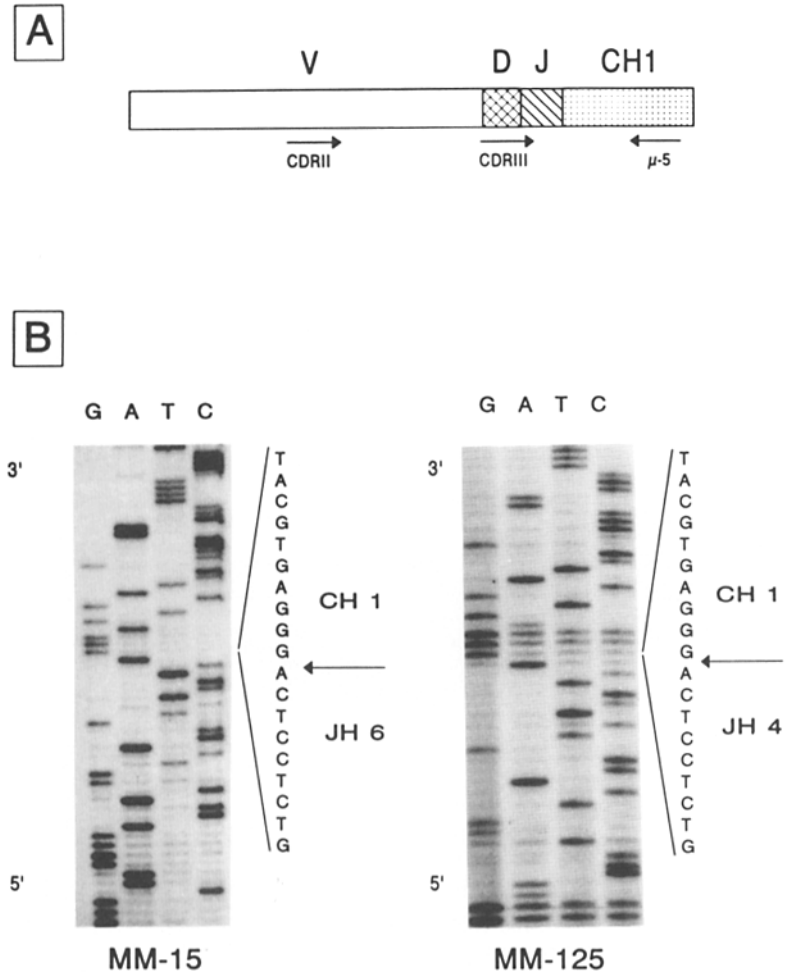

Figure 3. Demonstration by direct sequencing of the junction of the plasma cell VDJ with the Cμ sequence. (*A*) Schematic representation of primers used in the amplification (*CDRII*, *μ-5*) and sequencing (*CDRIII*) of the VDJ-Cμ junction. (*B*) After the amplification of bone marrow total cDNA (case MM-15, and MM-125), direct sequencing analysis showed the joining of the 3′ end of plasma cell VDJ (*JH6* and *JH4*) to the first exon (*CH1*) of Cμ gene. (*Arrows*) Junction point between JH6, JH4, and CH1. G, A, T, C (dideoxynucleoside triphosphates) represent the order of loading of the sequencing gel.

reamplified using CDRII and μ-5 primers. The use of μ-5 allowed the amplification of a longer fragment of Cμ first exon. PCR products were then sequenced and the junction of tumor VDJ to the Cμ gene was definitively demonstrated (Fig. 3). 80 bp of the Cμ gene was read and showed a germline configuration.

Our analysis was also extended to the peripheral blood total cDNA samples. The amplifications performed with CDRII and μ-1 primers did not detect the presence of clonal pre-switch B cells. Conversely, PCR reactions performed with CDRII and α or γ 3′ primers revealed the presence of tumor post-switch B cells (Fig. 2 *F*).

Discussion

The involvement of pre-B and/or B lymphocytes in multiple myeloma has been mainly deduced from the observation that they share some of the plasma cell antigenic features (1–5, 20) and that myeloma-specific IgH gene rearrangements take place in PBMC (7, 8). However, some technical aspects have prevented such involvement from obtaining universal acceptance. For instance, myeloma protein may nonspecifically bind to Fc receptors of PBL, which may then erroneously be recognized as neoplastic by anti-idiotypic antibodies (21). In addition, the specificity of these antibodies has been shown to be limited, since they react with more than one myeloma protein and also recognize several normal B cell clones (22, 23). Finally, the presence in the peripheral blood of myeloma-specific IgH gene rearrangements has been ascribed to plasma cells (8, 9). For these reasons, therefore, the B lymphocyte origin for multiple myeloma has thus been disputed, and it has been regarded as a tumor originating at plasma cell level.

We devised an experimental strategy based upon the molecular analysis of IgH genes. The CDRs provided a specific marker of clonality, whereas the presence of Cμ gene allowed the distinction between pre- and post-switch B cells (24). The hypothesis that a malignant plasma cell derives from a pre-switch B cell implies the existence of a B cell in which the tumor VDJ is still joined to Cμ gene. We showed that the VDJ sequences identified by our assay represent the malignant plasma cell VDJ, and then we looked for a clonal pre-switch B cell population. Analysis of bone marrow RNAs demonstrated the existence of such a population. Conversely, these pre-switch B cells were not found in the peripheral blood, where tumor post-switch B cells were detected. It should

be pointed out that pre-switch B cells in the bloodstream could be too few to be detected by our assay.

An IgH transcript in which the VDJ is still attached to Cμ sequences can be mainly attributed to pre-B and/or B lymphocytes, even though memory B lymphocytes might be included. It has been reported that memory B lymphocytes may coexpress Cμ and Cγ or Cα genes (25, 26), i.e., they can synthesize Ig molecules with different isotype linked to the same idiotype. The switch mechanism postulated for this finding is the alternative splicing of a large Ig RNA transcript, and not the switch recombination characterized by Cμ gene deletion (for a review see reference 27).

The bone marrow B cells carrying the tumor VDJ, and still expressing Cμ genes (pre-switch B cells), may be regarded as plasma cell precursors. It should be pointed out, however, that our data do not provide a direct demonstration of malignancy, and do not exclude the existence of even more immature precursors. The presence of peripheral blood cells in which the tumor VDJ sequences are linked to Cα or Cγ genes (post-switch B cells) leads us to speculate that these cells may be responsible for the dissemination of the disease throughout the axial skeleton.

The contamination of tumor cells in peripheral blood is lower than bone marrow, and the apparent absence of pre-switch B cells in the peripheral blood indicates that it could be an alternative source of normal stem cells for autograft procedures.

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