The Bone Marrow of Multiple Myeloma Patients Contains B Cell Populations at Different Stages of Differentiation That Are Clonally Related to the Malignant Plasma Cell

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Summary

One of the distinguishing features of multiple myeloma (MM) is the proliferation of a clonal plasma cell population in the bone marrow (BM). It is of particular interest that the tumor plasma cells appear to be restricted to the microenvironment of the BM and are rarely detected in the peripheral system, yet the disease is found widely disseminated throughout the axial skeleton. Furthermore, isolation of MM tumor cell lines has proven to be quite problematic due to their slow growth rate. These observations have instigated the search for earlier cells in the B cell lineage that are clonally related to the plasma cell tumor and that may represent the growth fraction of the tumor. We used allele-specific oligonucleotides (ASO) derived from the third complementarity determining region of the rearranged tumor immunoglobulin heavy chain gene to detect isotypes clonally related to the plasma cell tumor. By reverse transcribing RNA from the BM with a panel of CH primers (μ, δ, α, and γ), followed by ASO–polymerase chain reaction amplification, we demonstrate the existence of preswitch isotype species that are clonally related to the myeloma tumor. Furthermore, we show that separation of the BM cells into CD45$^+$ and CD38$^+$ cell populations results in a lineage-specific expression of the clonally related RNA molecules, with the Cμ and Cδ in the CD45$^+$, and Cγ in the CD38$^+$ population. Interestingly, clonally related Cα transcripts are also derived from the CD45$^+$ fraction. These results confirm the presence of B cell populations clonally related to the plasma cell tumor and are consistent with models that propose the existence of myeloma precursors.

Multiple myeloma is characterized by the clonal expansion of the plasma cell compartment in the bone marrow (BM)[1] and is further distinguished by the production of a monoclonal Ig, as determined by idiotypic determinants and Ig gene rearrangements. A number of reports have suggested that various populations of B cells are clonally related to the plasma cell malignancy (1–14). Antiidiotypic antibodies generated to the myeloma monoclonal protein have been shown to crossreact with B cells displaying different isotypes (1–4). Furthermore, coexpression of early B cell lineage markers and plasma cell markers have been observed on the malignant population (10–14). The implication from these findings is that various phenotypes of a common B lineage may result from an oncogenic process affecting the B cell early in differentiation. Indeed, differentiation from Id$^+$ B cells to plasma cells in culture using cytokines (8), or phorbol ester induction (9), has been reported. However, there is clearly disagreement concerning the evidence that concludes there are early-B progenitors that are transformed and subsequently differentiate to the clonal plasma cell tumor. While cells containing Igμ protein that appear to be idiotypically identical to the myeloma protein have been observed, these may be normal polyclonal B cells bearing crossreactive Ids (15). Moreover, expression of multilineage markers, common in the myeloma plasma cell, has been observed on normal BM plasma cells (16).

If B cells at various stages of differentiation are clonally related, it would be expected that they share common Ig gene rearrangements. Detection of clonal Ig gene rearrangements in mononuclear cells from blood and cultured precursor cells has been inconsistent (5–7, 17–19). In part this may be due to limits in blot hybridization sensitivities.

We (20, 21) and others (22–25) have shown that PCR amplification of the Ig heavy chain gene junctional regions can provide very specific characterization of neoplastic B cell

[1] *Abbreviations used in this paper:* ASO, allele-specific oligonucleotide; BM, bone marrow; MM, multiple myeloma; RT, reverse transcription.

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clones, with a significant increase in sensitivity. The CDR3 sequence is clonally unique due to novel combinations of the V-D-J gene segments, random nucleotide deletions and insertions, and somatic mutations. We have used consensus oligonucleotide primers to amplify CDR3 of rearranged heavy chain alleles from lymphoid tumor samples (20, 21). From the sequence of the amplified product, allele-specific primers can be synthesized and used to detect the tumor-specific allele. Because this approach is specific for the tumor Ig heavy chain allele, it can provide a unique and useful tool to identify clonally related populations of cells. In this study we have generated allele-specific oligonucleotide (ASO) primers from the predominant tumor population in the marrow of patients with IgG or IgA myeloma. RNA was isolated from their marrow, reverse transcribed with μ, δ, γ, and α constant region primers, and the cDNA was amplified using the ASO. Based on the PCR products and their sequence, we confirm the presence of B cells at different stages of development that are clonally related to the malignant population. Sorting of the BM sample into $CD38^+/45^-$ or $CD38^-/45^+$ populations, followed by reverse transcription (RT)-PCR of these populations with γ, α, δ, and μ, demonstrates that the clone-specific transcripts are being generated by different cell compartments. Moreover, sequence comparisons between early stage (μ-δ–expressing) clones and the clonally related, but more differentiated (γ- or α-expressing), clones showed sequence stability (i.e., lack of accumulating somatic mutations) in the junctional region.

Materials and Methods

Multiple Myeloma (MM) Patient Samples. Approximately 20 × 10^6 frozen BM cells from patients with MM were received from the Mayo Clinic in RPMI containing 10% DMSO and 20% FCS. The samples were washed twice in 1× PBS to remove DMSO and then split in half. DNA was prepared from one half of the sample by standard protocol (26) and then resuspended in 200 μl TE (10 mM Tris, 1 mM EDTA), pH 7.0. Total cellular RNA was prepared from the other half of the sample as described by Chomczynski and Sacchi (27).

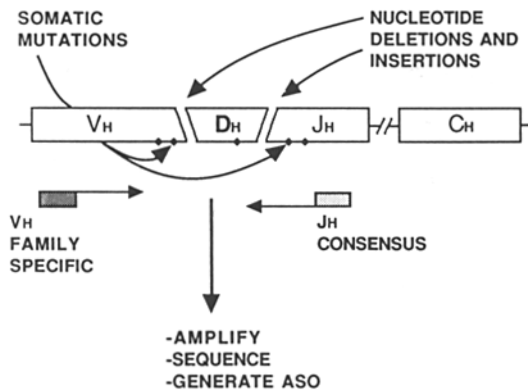
V_H Family–specific Amplification of Rearranged Ig Heavy Chain Genes in Myeloma BM DNA. PCR was performed essentially as described by Saki et al. (28), using a J_H consensus primer (20) or J_4-specific primer, along with primers specific to the six V_H gene families (29). For family-specific PCR of each sample, seven 50-μl reactions were amplified that contained the following: 0.5 μg DNA, 15 pmol of the J_H or J_4 primer, 15 pmol of a V_H family primer, 1.25 U of *Thermus flavus* polymerase (Epicentre Technologies, Madison, WI), 200 μmol/liter of each dNTP, 50 mmol/liter Tris-HCl, pH 8.4, 20 mmol/liter NH_4SO_3, 1.2 mmol/liter $MgCl_2$. The parameters for thermocycling were: 5-min denaturation at 95°C, followed by 35 cycles of: 30-s denaturation at 94°C, 30-s annealing at 60°C, and 45-s extension at 73°C, and finally a 10-min extension at 73°C. Negative controls of normal peripheral blood DNA and no template were run with each set of amplifications. PCR products were separated on a 6% polyacrylamide gel and visualized by UV light after staining with ethidium bromide. Amplification of sample M45 was only successful when amplifying with the J4 oligonucleotide paired with the V_H family oligonucleotides, but not when using the J_H oligonucleotide. Subsequent sequencing of the amplified product showed a deletion encompassing the 5′ region of the J_H gene recognized by the 3′ portion of the J_H consensus oligonucleotide (see Fig. 3), thereby leading to the inability of J_H to amplify this sample.

DNA Sequence of Amplified Products. Amplified products were isolated as previously described (20). The sample was desalted on Ultra-free-MC 100,000 NMWL filters (Millipore, Bedford, MA). DNA sequencing was performed as previously described (20) or using the fmol sequencing kit (Promega Biotec, Madison, WI) as per supplier's protocol with the following alteration. Sequencing reactions containing the sequencing buffer, end-labeled primer, template, and polymerase were subjected to a 5-min denaturation step at 95°C, followed by a snap-cool annealing of the primer to the template by placing in an ice-bath 10 min before distribution into termination mixes. Analysis of the sequencing reactions was performed as previously described (20).

RT of Myeloma BM RNA Using Constant Region Primers. RT of RNA using constant region primers (Table 1) was performed in 20-μl reactions containing: 3–5 μg RNA, 500 μmol/liter of each dNTP, 10 pmol of the constant region primer, 200 U of Moloney murine leukemia virus (MMLV)-RT (GIBCO BRL, Gaithersburg, MD), 20 U RNasin (Promega Biotec), 50 mmol/liter KCl, 20 mmol/liter Tris-HCl, pH 8.4, and 2.5 mmol/liter $MgCl_2$. The RNA and components minus the enzymes were heated to 95°C for 3 min and then placed on an ice-bath for 10 min. Next, the enzymes were added and the reaction was cycled under the following conditions: 10 min at 25°C, 60-min extension of cDNA at 42°C, and a 7-min enzyme inactivation at 95°C.

Allele-specific Amplifications of cDNA. ASOs were designed from sequence information and the primer position was maintained within the CDR3 to obtain the highest specificity (see Fig. 3). First-round amplification of the cDNA was carried out in 50-μl reactions that contained: amplification reaction buffer as described above, but included one-fourth of the RT reaction, 15 pmol of constant region primer (CHA) paired with 15 pmol of a family-specific V_H primer for 30 cycles of: 30-s denaturation at 94°C, 30-s annealing at 62°C, and 45-s extension at 73°C with a final 10-min extension at 73°C. The second-round amplification was carried out in 50-μl reactions and contained: 5 μl of the first-round amplification, 15 pmol of ASO primer, 15 pmol of an internal constant region primer (CHB), and the buffer conditions as described above. The reaction was cycled for 35 cycles under the above conditions with the annealing temperature ranging from 60 to 66°C depending on the melting temperature (T_m) of the ASO. Amplified products were separated on 8% polyacrylamide gel and visualized by UV light after ethidium bromide staining.

FACS® Sorting of MM BM Cells into $CD38^+$ and $CD45^+$ Populations. Frozen BM from patients with MM were thawed and diluted into RPMI 1640. These samples were then centrifuged at 1,800 rpm for 5 min, aspirated, and finally resuspended in 4 ml of media. This wash was repeated twice to get rid of DMSO and cellular debris. The cells were resuspended after the final wash and cell counts were performed. 2 × 10^6 cells were aliquoted to each tube for staining with antibody. The antibodies used were FITC-conjugated CD45 and PE-conjugated CD38 (Becton Dickinson & Co., Mountain View, CA). The cells were incubated with the antibodies for 30 min at 4°C then washed twice with RPMI and resuspended in 1 ml of media. Sorts were performed on either a FACStar® or a FacsVantage® flow cytometer (Becton Dickinson & Co.). Cells were collected into tubes containing 1 ml of media, and a total of 1–5 × 10^5 cells were collected into each tube. The cells were sorted into two fractions containing either $CD45^+38^-$ or $CD38^+45^-$. The cells were then frozen in RPMI with 20% FCS and 10% DMSO.



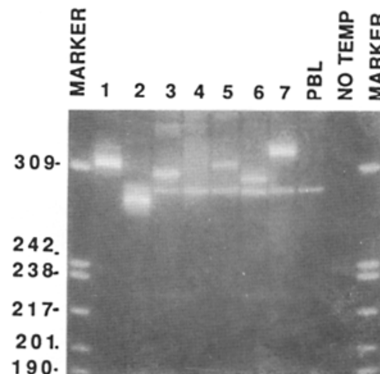


Figure 1. PCR amplification of the rearranged IgH locus using V_H family-specific and J_H consensus oligonucleotide primers. (*A*) Schematic representation of the events that occur during Ig heavy chain gene rearrangement that create a genetically unique sequence that can be used as a marker of the disease. Using family-specific primers to the conserved region of framework 1 and a J_H consensus primer, the rearranged allele(s) can be amplified, sequenced, and an ASO can be generated to the unique sequence. (*B*) Ethidium bromide–stained polyacrylamide gel of PCR products derived from V_H family-specific/J_H consensus amplification of the rearranged Ig heavy chain locus from seven myeloma BM samples. The band migrating at ~290 bp in all lanes is nonspecific.

Preparation of Whole-Cell Lysate for RT-PCR. Sorted cells were washed twice with 1× PBS and then resuspended to give a final concentration of 14.5 × 10^4 cells/μl in 1× PBS. 1 μl of tRNA at 0.5 μg/ml was added and then the cells were lysed at 95°C for 10 min. To this, 2 μl of proteinase K (Boehringer Mannheim Biochemical, Indianapolis, IN) at 10 mg/ml was added and incubated at 55°C for 30 min, followed by enzyme inactivation at 95°C for 10 min. The sample was then centrifuged at full speed for 2 min and the supernatant was transferred to a new tube and stored at −80°C. Approximately 10^5 cells worth of lysate (7 μl) were used in the RT-ASO-PCR amplification (see above). The only modification to the PCR amplification was that the ASO was end labeled with γ-[^{32}P]dATP using T4 polynucleotide kinase as per supplier's protocol (New England Biolabs, Beverly, MA), before amplification. 20 μl of the final PCR product was separated on an 8% polyacrylamide gel, dried, and exposed to x-ray film at room temperature for between 15 min and 5 h.

Results

PCR Amplification of V-D-J Ig Heavy Chain Rearrangements in MM BM Samples. The genetically unique sequence encompassing the V-D-J recombination junction (CDR3) has proven to be a useful tool in detecting and monitoring the presence of B cell malignancies by ASO-PCR (20–24). We isolated DNA and RNA from seven MM BM samples that had been previously characterized to contain a clonal V_H rearrangement, expressed as either an α or γ isotype. Within the marrow of these patients the tumor population constituted >45% of the total cellular content. As diagrammed in Fig. 1 *A*, we used a pool of seven family-specific V_H oligonucleotides (29) to framework 1 paired with a J_H consensus oligonucleotide designed to the 5′ portion of the J_H region to amplify across the CDR3 junction of these MM DNA samples. From six of the samples discrete amplified products derived from the predominant tumor cells could be detected by ethidium bromide staining after electrophoresis through a 6% polyacrylamide gel (Fig. 1 *B*). The sizes of the amplified products range from 280 to 340 bp due to the heterogeneity in D_H gene usage, nucleotide insertions, and deletions. Because of the heterogeneity of the IgH gene rearrangements within the normal peripheral blood DNA sample, only a faint smear was observed. A nonspecific band of ~290 bp was amplified in all of the samples, and was not further characterized. Each of the DNA samples that produced an amplified product by V_H family–specific PCR was then amplified with individual V_H family oligonucleotides paired with the J_H consensus oligonucleotide (or J4 in the case of M45) to determine which V_H family gene was being used (data not shown). The use of a single V_H primer provided additional specificity for subsequent PCR amplifications (see below).

The PCR-amplified products were sequenced and the V-D-J boundaries were established based on previously published sequences (30, 31) (see Fig. 3). As previously shown (20, 21), the variety of nucleotide deletions and insertions between the coding junctions, accompanied with the diverse use of D_H and J_H gene segments, generates a very unique sequence and can provide a genetic marker of the clonal malignancy. ASOs were designed to each of the CDR3 with the 5′-3′ orientation toward the J_H and C_H segments (see Fig. 3). DNA ASO-PCR amplifications were performed on the six MM samples as previously described (20, 21) to assess the specificity of the ASO. All of the samples produced a single, amplified product of the expected size and none of the ASOs were found to crossamplify any BM MM DNA for which it was not specific or normal peripheral blood DNA (data not shown).

Detection of Clonally Related but Isotypically Distinct RNA Species by ASO RT-PCR. Although myeloma is characterized as a clonal proliferation of a plasma cell, there is continued debate over the presence of earlier lineage cells that are clonally related to the malignancy, and that may represent clonal precursors. The MM samples characterized in this study expressed either Cγ or Cα heavy chain Ig, as determined by isotype-specific antibodies (not shown). We rea-

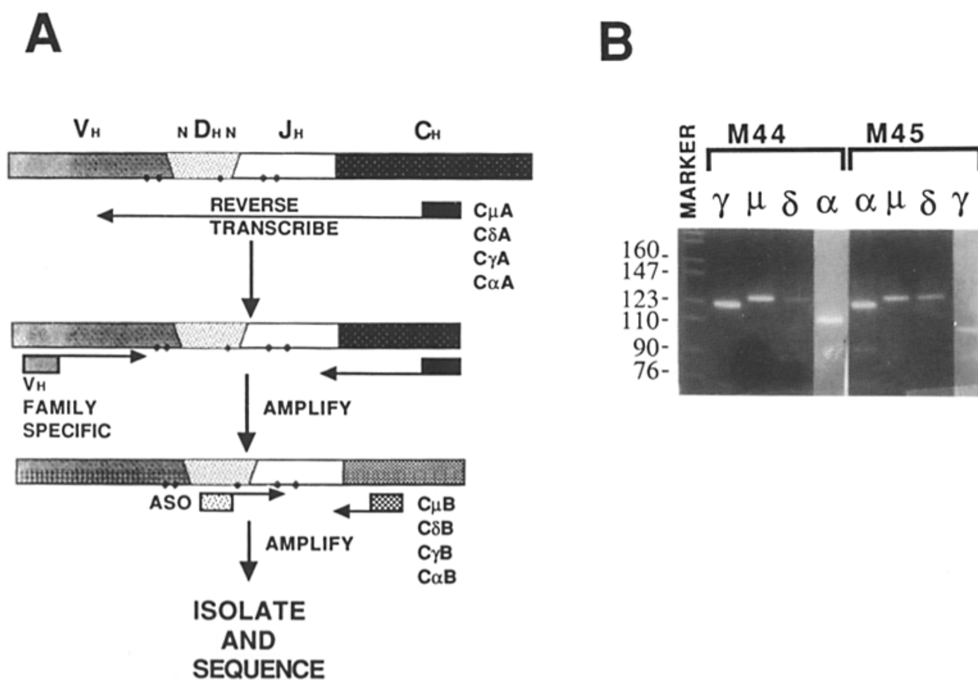


Figure 2. Allele-specific amplification of CHμ, δ, γ, and α reverse-transcribed tumor RNA. (*A*) Depiction of an RNA molecule from the rearranged heavy chain Ig gene. The experimental method used to detect RNA transcripts bearing different constant region isotypes that are clonally related to the myeloma is shown. (*B*) Ethidium bromide-stained polyacrylamide gel of two representative patient samples showing the existence of different isotype RNA molecules that are clonally related to the tumor. The first lane in each patient sample set denotes the isotype of the predominant tumor population.

soned that if B cells earlier in development were present, they may express preswitch isotypes (Cμ and Cδ) and show clonal identity with the plasma cell by virtue of the V-D-J junctional region. The tumor-specific ASO was used to examine this possibility. The approach taken is shown in Fig. 2 *A*. RNA from the patient marrow was reverse transcribed with one of several CHA primers specific for α, γ, μ, or δ (see Table 1). Oligonucleotides Cγ and Cα (both CHA and CHB) are consensus primers and recognize the four gene isoforms and two gene isoforms, respectively. Addition of the V_H family primer that corresponded to the plasma cell tumor and PCR cycling with one of the CHA primers resulted in selective amplification of a subset of RNA species (i.e., isotype and V_H gene family restricted). A second round of PCR amplification was performed using the tumor-specific ASO paired with an internal C_H region primer (CHB in Fig. 2 *A* and Table 1). This amplification selectively amplified only cDNAs generated from the first amplification that contained the CDR3 identical to the plasma cell tumor. A representative example of the RT-ASO-PCR products obtained from two of the patient samples is shown in Fig. 2 *B*. The first lane in each set shows the product of ASO paired with the C_H expressed by the plasma cell tumor (M44-Cγ; M45-Cα). The remaining lanes show products obtained by pairing the ASO with other isotype primers. Detection of the predominant RNA species derived from the plasma cell tumor re-

Table 1. *Oligonucleotides Used in PCR and RT*

Oligo	Sequence	Domain + position	
J4	5′-GTGAGGACTCACCTGAGGAG-3′	J4	
CμA	5′-GGGTTGCCGAAGAAGCCGCCGCGGGGTGGG-3′	CH2	255
CμB	5′-GACGGAATTCTCACAGGAGAC-3′	CH1	125
CδA	5′-CCCAGTTATCAAGCATGCCAGGAC-3′	CH1	139
CδB	5′-TGGGTGTCTGCACCCTGATAT-3′	CH1	125
CγA	5′-TC$_{T/C}$GAATTCAGGG$_{T/C}$GCCAGGGGGAGAC-3′	CH1	121
CγB	5′-GGGGAAGACCGATGGGCCCT-3′	CH1	117
CαA	5′-GGGATTCGTGTAGTGCTTCACGTG-3′	CH1	209
CαB	5′-GAGGCTCAGCGGGAAGACCTT-3′	CH1	120

* The amino acid number given designates the position of the 3′ end of the oligo. All sequences and position assignments are as previously reported (30, 42).

quired only a single round of amplification. Thus, the intensity of the plasma cell RNA amplification product appears comparable to the other isotypes detected in the figure, but in fact actually represents the dominant species. Significantly, in all cases we demonstrated the existence of RNA species clonally related to the plasma cell tumor, but associated with different isotypes. Based on the sequence of the original tumor CDR3, and the known location of each of the primers, we could precisely predict the sizes of the expected related PCR products. In each case, the size met the prediction. Our results demonstrate the presence of preswitch transcripts (μ and δ) that amplified with the tumor-specific ASO. Interestingly, other clonally related isotypes could also be detected (α in γ tumor samples and γ in α tumor samples). This result is not unexpected in light of previous reports demonstrating idiotypic identity between myeloma cells and other mature isotypes (1). Similar results were obtained from the RNA isolated from all six patients.

Sequence Analyses of the Clonally Related RNAs Show Identity in the CDR3 and Little Accumulation of Additional Somatic Mutations after Switching. To confirm the clonal relationship of each of the PCR products obtained from the myeloma samples, all of the products were sequenced (Fig. 3). The first line shown for each sample represents the sequence corresponding to the predominant plasma cell malignancy. In each case the sequence of the other isotypes amplified with the tumor-specific ASO showed almost complete sequence identity in the CDR3, through the D and J gene segments, thus confirming the clonal relationship. Differences in sequence from published germline sequences were noted in repeated sequence analyses, and presumably represent somatic mutations. These mutations were identical for all the isotypes within a single patient sample. Rare additional mutations were observed, two in the Cα transcript of M42, and one in the Cγ transcript of M44. This demonstrates a significant sequence stability after switch recombination, and suggests somatic mutations do not accumulate during switch, in the absence of antigen selection (see Discussion).

Distinct B Cell Populations Express the Transcripts Clonally Related to the Malignant Plasma Cell. The antigenic CD cell

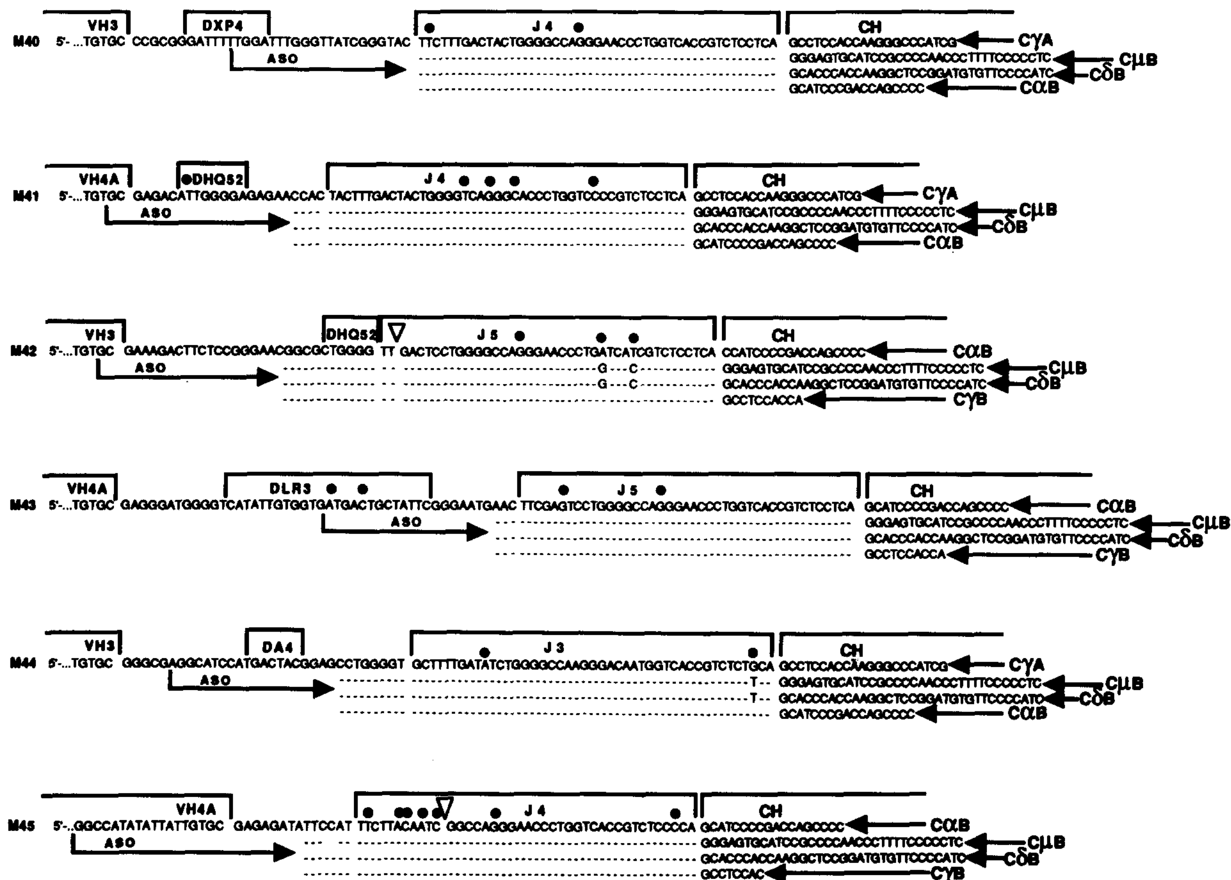


Figure 3. DNA sequence of the products isolated from myeloma BM RNA that was reverse transcribed with the isotype-specific CH primers and amplified using the tumor-specific ASO. Sequence analyses of the RNA transcripts are shown. The initial sequence shown for each patient sample denotes the predominant tumor isotype. Gene boundaries are shown as boxed areas above the sequence with the gene segments identified. The ASO is underlined and the arrowhead designates the direction of priming. The dashed lines represent sequence identity among the RNA transcripts and base changes are indicated. The isotype primers being used to generate the sequence are given on the right and the sequence 5′ of the primer are shown. The open triangles indicate deletions and the filled circles represent somatic mutations based on published sequence information (30, 31).

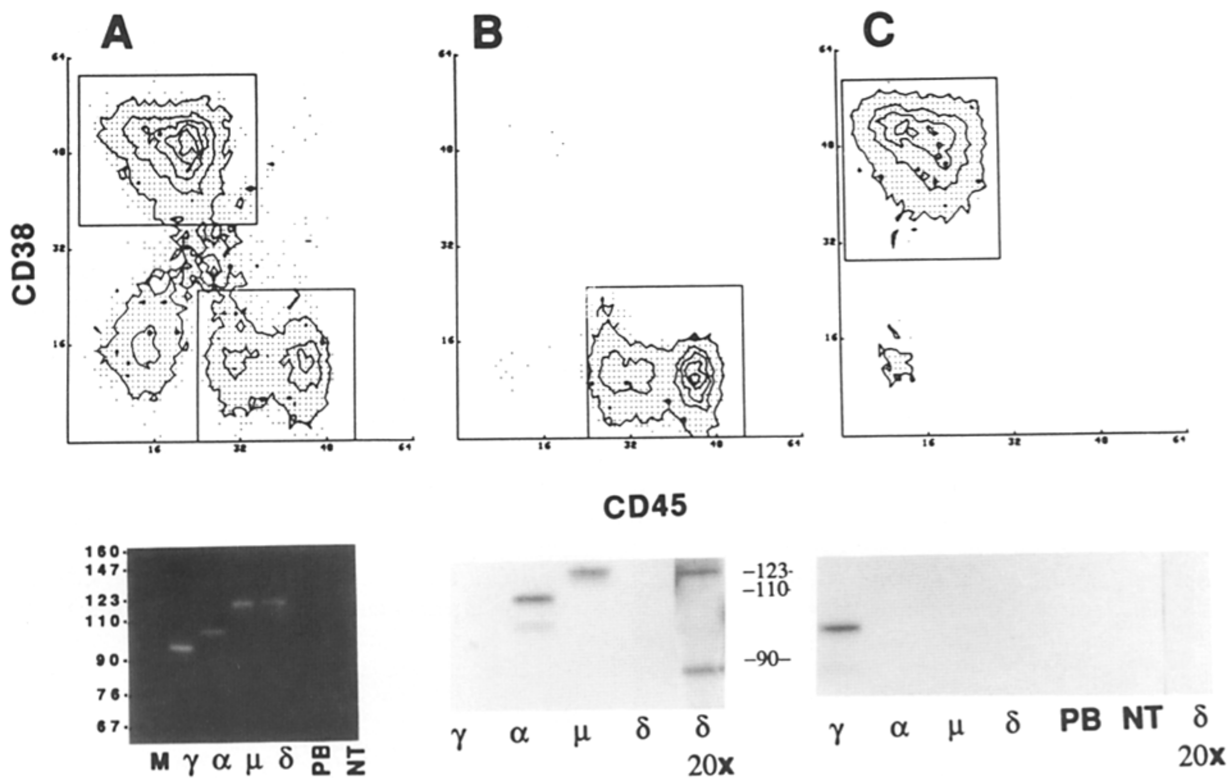


Figure 4. RT-ASO-PCR identification of clonally related but isotype distinct RNA molecules in CD45/38 cell-sorted populations. (*A*) Cell populations in the presort separation of MM BM sample M41 as detected by CD45 and CD38 antibodies. Shown below the plot is an ethidium bromide–stained polyacrylamide gel of the products detected by ASO-RT-PCR using the isotype-specific primers for RT. The δ-amplified product could only be observed after reamplification of 5 μl of the original amplification. (*B*) CD45 sort of M41 BM sample along with the autoradiogram showing the isotypic clonally related RNA molecules detected within this population by RT-ASO-PCR. To detect the δ transcript the film was exposed 20 times (*C*). Results from the CD38 sort of M41 BM sample along with the autoradiogram of the isotype-specific RNA detected within this sorted population by RT-ASO-PCR.

surface markers are useful in separating populations of lymphoid cells at different developmental stages. Expression of two different isoforms of CD45 characterize normal B cell development. Expression of CD45RA occurs at all stages of pre-B and B cell development, whereas CD45RO occurs late after B cell activation. End-stage plasma cells lose all CD45 expression (32). In contrast, CD38 is expressed in early pre-B and immature B cell stages, is lost during subsequent B cell differentiation, and then is expressed at high levels in end stage plasma cells (33, 34). The majority of MM tumor cells are $CD38^+/CD45^-$. Thus, separation of cells based on the expression of these two markers can yield distinct B cell populations.

Using FACS® analysis, the Cγ-expressing tumor M41 was found to express high levels of CD38 on its surface (Fig. 4). A two-color FACS® analysis using a PE-conjugated antibody against CD38 and a FITC-conjugated antibody that recognizes both isoforms of CD45 demonstrates a clear separation of $CD38^+/CD45^-$ and $CD38^-/CD45^+$ cell populations (Fig. 4 *A*). As seen before, RT-ASO-PCR analysis of the total, unsorted cell population shows the presence of all the clonally related but isotypically distinct RNA species (Fig. 4 *A*, *bottom*). The M41 marrow sample was then FACS®-sorted into the two distinct populations, and each population was analyzed for the presence of the clonally related transcripts (Fig. 4, *B* and *C*). Yields of cells from the FACS® sort were between 5×10^5 and 1×10^6. To reduce the loss of RNA from these samples, PCR assays were performed on whole cell lysates (see Materials and Methods). Each PCR amplification was performed on lysate from $\sim 10^5$ cells. In addition, sensitivity of detection was increased by end-labeling the 5′ end of the ASO primer with ^{32}P before the amplification. The PCR amplifications were carried out as described above, and the products were visualized by autoradiography after gel electrophoresis. The results shown in Fig. 4 *B* demonstrate that the Cμ, Cδ, and Cα transcripts that amplify with the ASO are present in the $CD38^-/CD45^+$–sorted population, while the tumor-derived Cγ transcript is not. In contrast, the $CD38^+/CD45^-$–sorted cells uniquely express the Cγ tumor transcript, but none of the other isotypes. The 20× exposed film does show a very faint band corresponding to an amplified Cγ transcript in the $CD38^-/CD45^+$–sorted population, although amplified μ, δ, and α transcripts are not observed in the $CD38^+/CD45^-$–sorted population (data not shown). The observation of the Cγ transcript in the $CD45^+$ population may have resulted from contaminating $CD38^+$ cells in the $CD45^+$ pool or it may represent a transition cell phenotype ($CD38^+/CD45^+$).

Nevertheless, these results clearly show that the transcripts clonally related to the tumor population, but isotypically distinct, are derived from a population of cells separate from the terminally differentiated plasma cell tumor.

Discussion

A number of reports have suggested the existence of earlier B lineage cells that are clonally related to the plasma cell tumor population in myeloma patients (1–14). Some reports have demonstrated the presence of B cells in the peripheral blood of MM patients that share the same IgH gene rearrangements (5–7). However, there has been no direct evidence that these cells represent clonally related preswitch populations, distinct from the plasma cell. Earlier B lineage cells have been shown to be present that express Ig idiotypically related to the plasma cell tumor; however, antiidiotypic antibodies have been shown to crossreact with normal peripheral blood cells. Finally, it has recently been reported that in the PBL of MM patients there exists a monoclonal population of B lymphocytes that express late-stage B cell differentiation CD markers (32), but there was no evidence that these populations were clonally related to the plasma cell tumor in the marrow. In this report we have demonstrated the presence of Igμ and Igδ transcripts that are clearly clonally related to the terminally differentiated plasma cell tumor (expressing Igγ or Igα). This clonal relationship was confirmed by application of a tumor-specific RT-ASO-PCR technique, and verified by sequence analysis of the different isotype-associated transcripts in the unique CDR3 of the IgH locus. Our results are in agreement with a previous abstract report (35) in which μ transcripts were found to be amplified with primers specific to the terminally differentiated myeloma population.

In previous reports we have estimated the percentages of tumor-related cells in both the marrow (20) and peripheral blood (21) using a DNA-based PCR assay. In this report we have used an RNA-based PCR assay. We can conclude that the preswitch transcripts comprise a very small percentage of the total tumor-related RNA, based on the fact that an additional 30–40 cycles of PCR were required to detect the preswitch isotypes. However, because this extensive amplification is well beyond the linear range of PCR, accurate estimates of relative transcript levels are not possible. Moreover, the relative levels of related RNA transcripts are certainly dependent on rates of transcription, which vary significantly among B cell and plasma cell populations. Thus, a quantitative comparison of related, but isotypic distinct, RNA transcripts would have no bearing on actual cell numbers.

An additional observation we made was that there was a lack of significant accumulation of somatic mutations, when comparing the μ and δ sequences with the switched plasma cell γ or α sequences. From the six patients analyzed we noted only three nucleotide differences in the CDR3s sequenced. The most commonly accepted mechanism for isotype switch is by the switch recombination of V-D-J to a downstream heavy chain constant region. Switched heavy chain alleles frequently accrue a significant amount of somatic mutations during the normal immune response due to continued antigenic stimulation and selection. The lack of accumulating somatic mutations from the preswitch to the postswitch isotype observed in our study suggests that the clonal proliferation and differentiation of the tumor occurs in the absence of antigen stimulation and selection. A similar observation was noted in a cell line that was programmed to switch in vitro without antigenic stimulation (36).

Due to the sequence identity observed within the clonally related transcripts and the recent observation of an IgG paraproteinaemia that simultaneously expressed cytoplasmic IgM and IgG (37), we wanted to rule out the possibility that the expression of clonally related isotypes we were observing was derived from a single cell population. It was possible that the distinct isotype transcripts we observed were the result of differential splicing of large transcripts, as has been observed in some mouse B cells (38), or *trans*-splicing (39) from a single population of cells. By sorting the cells based on cell surface expression of CD38 and CD45, we demonstrated that the clonal μ and δ transcripts were being made in a cell compartment distinct from the plasma cell tumor. The expression of CD38 is of a discontinuous nature; it is found early in B cell development, lost on mature and activated B cells, and reexpressed on almost all myeloma plasma cells (34). CD45 expression also occurs early in B cell development, through activated B cells (RA subgroup) and preplasma cells (RO subgroup), but is lost on plasma cells (32). Our results from sorted populations of one patient show that the clonally related transcripts were derived from distinct populations. Because the cells expressing the clonally related μ and δ transcripts were found in the CD38$^-$/CD45$^+$ population, this suggests these transcripts were derived from a mature B cell phenotype. If a myeloma precursor population is present, it would seem likely that the cell would have already progressed through heavy and light chain rearrangements before the transforming event, otherwise clonal rearrangements with identical CDR3s would be required to obtain the clonal plasma cell population found in the marrow. However, because the plasma cell tumor is generally expressing a predominant switched isotype, a precursor model would require that μ-expressing cells undergo a dominant switch recombination to produce the clonal isotype observed. Indeed, programmed switching of murine B cells has been documented (40).

Although the plasma cell tumors in these patients show a clonal isotype, we did find evidence for clonally related cells that express different switch isotypes, likely from other switch recombinations (i.e., we could detect clonally related α expression in predominantly γ myelomas, and γ expression in α myelomas). This would suggest that even if switch is programmed, other minor switch recombinations do occur. Previous reports showing programmed switches have also noted other minor switch isotypes (40). Interestingly, Vesole et al. (41) have found that in a significant percentage of relapsing myeloma patients who had received autologous bone marrow transplants, relapses expressed different isotypes. They suggest that their therapeutic strategies may provide a selective advantage to new populations of clonally related

cells. Our observations would serve to explain these results. It there is a preswitched progenitor population, clonal outgrowths of new isotypes may be possible The clonal relationship of initial and relapse cell populations could be addressed by the RT-ASO-PCR approach we described in this report.

Our analysis was confined to BM populations derived from banked samples of newly diagnosed myeloma patients. We did not have access to peripheral blood samples; however, this should be an important future consideration in light of studies that demonstrate monoclonal populations of late-stage B cells in PBL of MM patients (32). As described in a previous report (21), we found by ASO-PCR analysis of PBL DNA that in some MM patients there was a significant number of cells clonally related to the plasma cell tumor, despite the fact there were no detectable clonal plasma cells in the PBL. Finally, Bergui et al. (8) have reported an IL-3- and IL-6-dependent proliferation and differentiation of malignant precursors from PBL of MM patients to plasma cells in vitro. All of these observations lend support to the presence of precursor populations, and strongly suggest the peripheral blood should be carefully examined.

Additional studies will be required to further characterize the preswitch population of cells we have identified, and their potential to influence the course of the disease. The clonally related μ cell population cannot be assumed to be transformed solely on the evidence that it is related to the plasma cell tumor. Subsequent studies should address this issue and help elucidate the true malignant stage in multiple myeloma. In addition, new protocols in the treatment of myeloma include the use of purged autologous marrow or marrow reconstitution with peripheral blood stem cells. Our results highlight the need to identify and characterize clonally related cells whose presence may influence the outcome of these therapeutic approaches.

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