Circulating Clonal Lymphocytes in Myeloma Constitute a Minor Subpopulation of B Cells

By Bengzeng J. Chen and Joshua Epstein

The mononuclear cells in the blood of myeloma patients have been reported to contain a high proportion of phenotypically abnormal myeloma B lymphocytes. These cells have been proposed to constitute the drug-resistant proliferative myeloma cell compartment. To determine the extent of B lymphocyte involvement, the proportion of clonotypic cells among the CD19-expressing cells from myeloma patients was estimated by quantitative polymerase chain reaction analysis of the third complementarity determining region (CDR3). The results indicate that the B lymphocytes constitute, on average, 6% of blood mononuclear cells, and that only a minor fraction of these are clonally related to the myeloma cells. While the small number of circulating clonal cells may be incompatible with their proposed role as a reservoir of proliferating myeloma progenitors, the majority of the B cells appear not to be clonally related to the myeloma cells.

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MATERIALS AND METHODS

Patient samples. Heparinized bone marrow aspirates and blood were obtained from patients with plasma cell myeloma during routine visits to the clinic. Patient-signed informed consents, in keeping with institutional policy, are on record. Mononuclear cells were prepared by Ficoll-Hypaque density centrifugation (specific gravity 1.077). Patient characteristics and disease status are given in Table 1.

B-cell and mature plasma cell purification. Blood mononuclear cells were reacted with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to CD19 (FMC63; a gift from Dr H. Zola) at a saturating concentration. CD19-expressing cells and CD19-negative cells were sorted on a FACStar Plus cell sorter (Becton Dickinson, Mountain View, CA). CD3-expressing (Leu4; Becton Dickinson) lymphocytes were also sorted and studied as negative controls. Because the goal of this study was to determine the frequency of clonotypic myeloma progenitor cells among the CD19-expressing cells, patients with plasma cell leukemia or samples with detectable CD38<sup>dim</sup>CD45<sup>dim</sup> plasma cells were excluded from this study.

Myeloma plasma cells were purified from the bone marrow aspirates on the basis of CD38 and CD45 expression and light scatter properties, as previously described.

Consensus amplification of CDR3. Genomic DNA was extracted from 1 x 10<sup>6</sup> to 10 x 10<sup>6</sup> bone marrow cells (greater than 10% plasma cells) and from 0.2 x 10<sup>6</sup> to 2 x 10<sup>6</sup> purified plasma cells by lysis in 1 mL 1× saline sodium citrate (SSC) containing 50 mmol/L EDTA and 1% sodium dodecyl sulfate (SDS). In some cases, total RNA was also isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (TRI reagent; Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized from up to 2 μg RNA in a 30-μL reaction using avian myeloblastosis virus (A-MLV) reverse transcriptase (Promega, Madison, WI) according to the supplier-recommended protocol.

Amplification of the CDR3 region using consensus primers was performed using a modification of a previously reported protocol. Briefly, each DNA sample was amplified in seven reactions. Each reaction contained 0.5 μg DNA, 20 pmol of β2m primer and one of six V<sub>λ</sub> family-specific primers, 1.25 U of Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT), 5 μL of 10× buffer (670 mmol/L Tris-HCl, 77 mmol/L MgCl<sub>2</sub>, 160 mmol/L [NH<sub>4</sub>]SO<sub>4</sub>), 2.5 μL of 5% dimethyl sulfoxide (DMSO), and 2.0 mmol/L of each deoxynucleotide triphosphate (dNTP). Samples were amplified for 35 cycles of PCR as follows: 12 seconds of denaturation at 92°C, annealing for 15 seconds at 62°C, and extension for 25 seconds at 74°C. After 35 cycles, a final extension step of 10 minutes at 74°C was applied.

Amplification conditions were modified for reverse transcription (RT)-PCR: 50-μL reactions contained 20 pmol/L β2m and FR3A consensus primers, 10 μL cDNA, 5 μL 10× PCR buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.3), 1.25 U Taq DNA polymerase,
1.5 mmol/L MgCl₂, and 1.0 mmol/L of each dNTP. The tubes were cycled through 35 amplification cycles, each consisting of 30 seconds of denaturation at 92°C, 1 minute of annealing at 62°C, 1.5 minutes of extension at 74°C, and a final extension step of 10 minutes at 74°C.

Hot start was used for all amplifications. PCR products were separated on a 2% low melting agarose gel and visualized with ethidium bromide.

Direct sequencing of PCR products. Amplification products generated by PCR were approximately 300 bp long. These were extracted from the 2% low melting agarose gel in ddH₂O at 37°C overnight and were purified using microconcentrators. Amplification products were separated on 6% polyacrylamide gels, stained with silver using microconcentrators (Microcon-30; Amicon, Beverly, MA). Purified PCR products were directly sequenced using fmo DNA Sequencing System (Promega) and J₅ primer that was end-labeled with γ²⁵P-deoxyadenosine triphosphate (dATP) using T4 polynucleotide kinase (Promega) as recommended by the supplier. Allele-specific oligonucleotides (ASOs) were designed from the sequence information for each patient (Table 2) and were used to identify and estimate the frequency of clonotypic cells in the sorted cell populations.

Estimation of the frequency of clonotypic cells. Each patient's ASO was end-labeled with γ²⁵P-dATP as above and was used together with the specific V₅ family-specific primer to amplify DNA from the different purified cell preparations. Standard curves were generated by amplification of serial 10-fold dilutions of DNA obtained from the patients' bone marrow or purified myeloma plasma cells with DNA extracted from blood mononuclear cells of a healthy donor. The range of dilutions was from 10⁻¹ to 10⁻⁷ tumor cells (10% to 0.001%). Conditions for the PCR were as described above.

Amplification products were separated on 6% polyacrylamide gels, and the gels were washed for 15 minutes in 1X Tris-Borate EDTA buffer and were purified by Wizard PCR Preps (Promega). Amplification products (100 bp long) were extracted from 8% polyacrylamide gels and visualized with silver.

Table 1. Patient Characteristics

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<tr>
<th>Patient No.*</th>
<th>Sex</th>
<th>Salmon-Durke Stage</th>
<th>Disease Status</th>
<th>Isotype</th>
<th>Serum M Protein (g/dL)</th>
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<td>Newly diagnosed</td>
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Abbreviations: ABMT, autologous bone marrow transplantation; VAD, vincristine, adriamycin, dexamethasone.

* Patient numbers are consistent throughout this study.

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Table 2. ASOs

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Sequences of the CDR3-PCR amplification products used for design of the ASOs used as primers for each patient are listed. ASOs are represented by bold, underscored segments.
Fig 1. Proportion of CD19-expressing lymphocytes among blood mononuclear cells of myeloma patients. Results are presented as mean ± SD for a population of 90 patients (n = 90) and for the nine patients studied by ASO-PCR (study).

Discussion

Suggestions that circulating B cells in myeloma contain the myeloma stem cell compartment and, thus, are responsible for disease dissemination and for relapse were based primarily on phenotypic data. Recent reports that circulating B cells in myeloma patients contain myeloma clonotypic cells, as determined by CDR3 analysis of sorted CD19 cells and by the presence of mutated ras oncogene, lend support to this notion. However, careful quantitative analysis demonstrates that only a very small proportion of the circulating B lymphocytes in myeloma patients are clonally related to the myeloma plasma cells. The small proportion of clonotypic circulating B cells is not incompatible with the notion that these cells represent the proliferative myeloma stem cell compartment. In fact, these cells were shown to differentiate in vitro into monotypic plasma cells when induced with interleukin-3 (IL-3) plus IL-6 or after coculture with bone marrow stromal cells. However, the small size of the clonotypic cell population implies that the reported abnormalities in the phenotype of B cells from myeloma patients, in fact, represent phenotypic changes in the normal B cells, probably in response to the malignant process, rather than an aberrant myeloma cell phenotype.

Clonotypic cells were detected only among the CD19-expressing cells in seven of the eight patients studied, indicating the absence of mature, CD19+ clonal plasma cells at the detection level of the assay used (0.005% to 0.001%, depending on the number of sorted cells obtained). In one patient, the CD19− cells contained 0.07% clonotypic cells, suggesting the presence of a few myeloma plasma cells, as compared with 0.34% clonotypic cells among the CD19+ cells. Sorted CD3+ cells were always negative. The observed absence of circulating myeloma plasma cells appears to be in conflict with recent reports regarding the prognostic significance of the number and labeling index of circulating myeloma plasma cells. These reports relied on monotypic cIg light chain content for the identification of myeloma plasma cells. The maturation levels of these cells, in terms of CD19 expression, was not tested, and their clonality was not demonstrated. The circulating monotypic cIg-containing cells could be early, CD19-expressing cells. Alternatively, these cells could represent nonclonal cells, induced to an increased rate of proliferation by the myeloma process. This is also compatible with the reported abnormal phenotypic characteristics of B cells in myeloma, which, as our results indicate, reflect myeloma-associated changes in normal B cells.

Dilution curves of bone marrow cells were identical to those of purified plasma cells. This confirms that the parameters used for myeloma cell identification and sorting—namely, expression patterns of CD38, CD45, and light scatter properties—allowed recognition of most myeloma cells in the bone marrow aspirates. The similarities of the dilution


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patient, which was diluted with normal DNA to contain the equivalent of from 10% to 0.001% myeloma plasma cells. A standard curve was constructed (Fig 2B) from the intensities of these bands as measured on a PhosphorImager, and the proportion of clonotypic cells in the CD19+ population was calculated from the regression equation. Sorted CD19+ cells were simultaneously analyzed to detect the presence of circulating CD19− plasma cells. DNA from T cells was used as a negative control. The results of similar analyses for the nine patients are summarized in Table 3. The proportion of clonotypic cells among CD 19-expressing cells ranged from 0.04% in patient 7 to 5% in patient 9, with a mean of 1.33% and a standard deviation of 1.6. Only in one patient were clonotypic cells detected in the CD19− cells, suggesting the presence of 0.07% plasma cells.

In addition to plasma cells, bone marrow aspirates of myeloma patients could contain significant amounts of clonal, preplasmacytic B cells that are not recognized by CD38/CD45 flow cytometry. This could result in an underestimation of the number of circulating clonotypic cells. To account for this possibility, the quantitative analysis of DNA extracted from bone marrow cells was compared with dilutions of DNA from purified plasma cells from the same patient. Results from one such experiment are presented in Fig 3 and demonstrate that total bone marrow cells and purified myeloma plasma cells are equally suitable for the quantitative estimation.

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Fig 2. Estimation of circulating clonotypic cells. Serial 10-fold dilutions of bone marrow DNA from patient 8, containing the equivalent of 10% to 0.001% myeloma plasma cells, were used to establish a dilution curve. DNA from sorted CD19-expressing cells (CD19+), CD19-negative cells (CD19-), and CD3-expressing cells (T) were simultaneously analyzed. The proportion of circulating clonotypic cells in each fraction was determined from the regression curve, as detailed in Materials and Methods. (A) PCR results. (B) Dilution curve.

Fig 3. ASO-PCR analysis of unselected bone marrow (BM) and purified plasma cells (PC). Serial 10-fold dilutions of DNA from unselected bone marrow cells and from plasma cells purified on the basis of CD38/CD45 expression from patient 1 were analyzed simultaneously. (A) PCR products. (B) Dilution curves: solid circles, unseparated bone marrow; open circles, purified plasma cells.
curves also indicate the absence of a large, clonotypic, preplasmacytic cell compartment in the marrow aspirates.

Collectively, the findings presented reveal the presence of only a small compartment of circulating, clonotypic B cells that are phenotypically distinct from the mature myeloma plasma cells. The role of these cells in the myeloma disease process needs to be established.

ACKNOWLEDGMENT

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REFERENCES

Circulating clonal lymphocytes in myeloma constitute a minor subpopulation of B cells [see comments]

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