In Multiple Myeloma, Circulating Hyperdiploid B Cells Have Clonotypic Immunoglobulin Heavy Chain Rearrangements and May Mediate Spread of Disease

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ABSTRACT

DNA aneuploidy characterizes a proportion of malignant bone marrow (BM)-localized plasma cells in multiple myeloma (MM). This analysis shows that for most MM patients, circulating clonotypic B cells in MM are also hyperdiploid. Although all normal B cells and some malignant B cells are diploid, hyperdiploidy is likely to be exclusive to those that are malignant. Hyperdiploid MM B cells express CD34 and have clonotypic IgH transcripts, confirming them as part of the malignant clone. For MM, 92% (70/76) of patients had a DNA hyperdiploid subset [5–30% of peripheral blood mononuclear cells (PBMCs)] of CD19+ B cells. All CD19+ PBMCs in MM expressed CD19 and IgH variable diversity joining (VDJ) transcripts, confirming them as B cells. DNA aneuploid cells were undetectable in T or B lymphocytes from normal blood, spleen or thymus, or in blood from patients with B chronic lymphocytic leukemia. In MM, untreated patients had the highest DNA index (1.12). DNA hyperdiploid PBMCs were most frequent among untreated patients and were significantly reduced after chemotherapy. Diploid B cells were significantly more frequent after chemotherapy than at diagnosis. Of the hyperdiploid PBMCs, 81 ± 3% expressed CD34 and CD19. In contrast to circulating CD34+ B cells, CD34− B cells in MM are diploid. In MM, unlike hyperdiploid PBMC B cells, hyperdiploid BM plasma cells lack both CD34 and CD19, suggesting that loss of CD34 correlates with differentiation and BM anchoring. In situ reverse transcription-PCR of the CD34+ (hyperdiploid) and CD34− (diploid) PBMC B-cell subsets was performed using patient-specific primers to amplify clonotypic IgH VDJ transcripts. Confirming previous work, CD34+ hyperdiploid MM PBMCs were clonotypic (86 ± 5%). In contrast, CD34− diploid MM PBMCs had few monoclonal cells (4.8 ± 2%). The lack of hyperdiploidy, together with the relative absence of cells having clonotypic transcripts, suggests these polyclonal CD34− B cells are normal. After culture in colchicine to arrest mitosis, hyperdiploid B cells were reduced and MM B cells accumulated in a diploid G1-M, suggesting that hyperdiploidy in MM may represent a transient S-phase arrest rather than an aneuploid G0 phase. The DNA hyperdiploidy of CD34+ clonotypic B cells suggests these cells may be clinically important constituents of the myeloma clone and that they may play a direct role in the spread of myeloma.

INTRODUCTION

MM3 (1) is a malignancy of the blood and BM characterized by monoclonal B and plasma cells. The IgH rearrangement (IgH VDJ) provides a unique clonal marker to identify cells related to the malignancy. Although the pathology of myeloma results from plasma cells and their products, no correlation is detectable between decrement in serum monoclonal Ig or plasma cell kill after conventional chemotherapy and patient survival (1, 2). A modest increment in survival is found after cytoreduction and autologous transplantation (3). Generative potential within the MM clone may derive from a less differentiated component. A variety of evidence suggests that MM represents a hierarchy of monoclonal B lineage cells in the blood and BM (4–11) that includes late stage B cells and plasma cells (4, 6, 11), pre-B cells (12), and slgM+ preswitch B cells (13–16). B cells expressing the MM idiotype have been detected in peripheral blood (17–22). Circulating B cells with IgH rearrangements characteristic of autologous BM plasma cells have been frequently reported in MM (11, 23–26). Although the number of monoclonal B cells in MM was initially controversial, our recent work, which used single cell and in situ RT-PCR assays, shows that circulating clonotypic B cells are frequent in MM blood (10). Nearly all of the clonotypic MM B cells express the stem cell antigen CD34 (7) and the adhesion receptor CD11b (8). Circulating cells from patients with aggressive myeloma or from granulocyte colony-stimulating factor mobilized blood engraft primary human myeloma to the BM of immunodeficient mice, indicating the presence of blood-borne MM progenitor cells (27).

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3 The abbreviations used are: MM, multiple myeloma; BM, bone marrow; mAb, monoclonal antibody; CV, coefficient of variation; RT-PCR, reverse transcription-PCR; BMC, bone marrow cell; Crbc, chicken RBCs; DAPI, 4′,6-diamidino-2-phenylindole; IgH; immunoglobulin heavy chain; PBMC, peripheral blood mononuclear cell.
Although clearly abnormal, the clinical consequences of circulating clonotypic B cells in MM are as yet unknown. DNA aneuploidy, a property not found in normal B lineage cells, is considered to be evidence of chromosomal abnormalities and thus may be an indicator of malignancy (28). For most myeloma patients, a substantial subset of BM plasma cells are DNA aneuploid (28–38). If the clonotypic B cells circulating in the blood have equivalent abnormalities, DNA aneuploidy may be detectable among circulating MM B cells in those MM patients who have DNA aneuploid BM plasma cells.

In this work we have assessed the extent of DNA aneuploidy among circulating B cells in 76 patients with MM by using multicolor flow cytometry to define the DNA content of MM PBMCs. DNA aneuploid PBMCs were characterized by their staining profile with fluorescent-tagged mAbs. We show that DNA aneuploid PBMCs coexpress CD19 and CD34, with a nearly complete overlap between the CD34+ and the hyperdiploid subsets. The hyperdiploid CD34+ B cells in MM PBMCs are predominantly monoclonal/clonotypic, as shown by patient-specific in situ RT-PCR to amplify mRNA transcripts encoding the unique IgH VDJ rearrangement of autologous BM plasma cells. In contrast, CD34− B cells in MM PBMCs are predominantly diploid and lack patient-specific IgH VDJ transcripts, as expected for the residual polyclonal set of normal B cells in MM blood.

**MATERIALS AND METHODS**

**Patients and Samples.** One hundred and fourteen peripheral blood and 27 BM samples were obtained from 76 patients with MM at several time points throughout their disease, as well as from 14 patients with B chronic lymphocytic leukemia, after informed consent and approval from the University of Alberta Human Ethics Committee. Blood samples from 12 normal donors were obtained through the Red Cross Blood Transfusion Service. Fragments of two normal spleens from 12 normal donors were obtained through the Red Cross Blood Transfusion Service. Thymus tissue was obtained as part of normal procedures in pediatric cardiac surgery. All blood and BM samples were freshly obtained and were stained within 3–4 h after being drawn. MM samples were obtained from 18 untreated patients at diagnosis, 42 patients on chemotherapy (taken 3 weeks after their last chemotherapy cycle), and 41 patients after chemotherapy (at least 4 months after cessation of their therapy). Some patients appear in all of these categories. PBMCs were purified by centrifugation over Ficoll-Paque (Pharmacia, Dorval, Quebec, Canada) as previously described (11).

**Antibodies.** Leu3-PE (CD4), Leu2-PE (CD8), Leu4-phycocerythrin (PE) (CD3), and Leu17-PE (CD38) were from Becton Dickinson (San Jose, CA). B4-FITC (CD19) was from Coulter (Hialeah, FL), HPCA-1 (Becton Dickinson) or 8G12, from Dr. Peter Lansdorp, were used to detect CD34 in either direct or indirect immunofluorescence as previously described (7). Anti-human κ- or λ-FITC, IgG2a-FITC, IgG1-PE, and goat antimouse immunoglobulin-PE were from Southern Biotech (Birmingham, Alabama). FMC63 (CD19) was directly conjugated to FITC (7, 10, 11). FMC63 cross-blocks with B4 and binds to CD19 transfectants4,5. Identical results were obtained with both B4-FITC and FMC63-FITC. Sorted CD19- B cells had a lymphoblastoid/monocytoid morphology (4, 10, 39) with <1% of plasma cells, as identified by Wright’s stain (not shown), and expressed a level of cytoplasmic immunoglobulin 10-fold lower than that of plasma cells (7, 11), which clearly distinguished them from plasma cells.

**Analysis of DNA Content.** Multiparameter flow cytometry was used to measure DNA content of individual cells defined by their surface phenotype. Briefly, samples were stained for surface phenotype followed by permeabilization of the cell membrane using ethanol, RNase treatment, and staining of DNA with the DNA-binding dye DAPI (40, 41). DAPI binds to AT-rich regions of DNA; it does not bind to RNA. Samples were analyzed with an ELITE flow cytometer (Coulter) using the argon laser to excite FITC and PE at 488 nm and the water-cooled laser to excite DAPI at 353 nm. Files of 50,000–100,000 cells were collected and analyzed using the ELITE software. DAPI was chosen based on its narrow CV of 2–4 for normal lymphocytes, thymocytes, and DNA bead standards. DAPI fluorescence properties allow the use of other fluorochromes to simultaneously characterize the surface phenotype. The patterns of DNA staining obtained using DAPI were identical to those using propidium iodide, 7-amino actinomycin D, or Hoechst 33258. Crbc were added to each sample as an internal standard. PBMCs were stained with CD19-FITC to detect B cells and with a mixture of CD8-PE/CD4-PE to detect autologous T cells within the same aliquot of cells. BM plasma cells were defined as having high scatter, low/no CD19, and an absence of CD4/8. In replicate aliquots, this set was shown to express CD38high. All samples included an aliquot stained with isotype-matched control mAbs (IgG2a-FITC and IgG1-PE). Use of the ELITE gated amplifier eliminates overlap between the emissions of FITC and DAPI. To maximize visual resolution of diploid and hyperdiploid, the peak in the diploid region was placed at channel 300–500 for most samples, as justified in the “Results” section. This required an increase of about 20 V on a 1000-V scale. For those samples where the G2-M region was off scale, a file was collected at a lower voltage to enumerate this subset. These methods allowed a direct comparison of the DNA content of T cells and of B cells from the same patient in the same aliquot of cells and provided an internal standard to define the diploid DNA content for each patient in each aliquot of cells stained. Files of 50,000–100,000 cells were collected and analyzed from the list mode using the ELITE software. The histogram of DAPI staining for T cells was analyzed first to identify the position and CV of the diploid peak in relation to the Crbc standard. Files were then gated for hyperdiploid cells based on a comparison with the position of T cells within the same aliquot of cells.

**Identification of Hyperdiploid B Cells.** B cells were characterized as hyperdiploid only if their DNA content exceeded by at least 1 SD that of the T-cell CV in the same aliquot of cells. In many patients, this represented a discrete peak; for

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4 H. Zola, personal communication.
5 L. M. Pilarski, unpublished data.
some, there was an extended shoulder to the main diploid peak; and for others, the entire peak was shifted relative to the position of diploid T cells. Representative examples are shown in the results or in previously published work (4, 40). The DNA index was defined as the mean channel DAPI staining of a B-cell subset divided by the peak channel of the autologous T cells. In all cases, the values recorded in the results report only those B cells having a DNA content that was at least 1 SD greater than the autologous T cell CV. Using this method, the DNA content of MM B cells (1.03) always exceeded the DNA content of B cells from normal donors (1.01–1.02), as compared to autologous T cells in the same sample. For all patient PBMC samples, at least two and usually three to six replicate stainings were done. For all MM patients, the DNA index for MM B cells was identical in each of several replicates. The analysis performed here underestimates the extent of B-cell hyperdiploidy because it excludes cells in the region where T- and B-cell peaks overlap. Cells falling in the hypodiploid region were also excluded from this analysis because of the difficulty in distinguishing them from early apoptotic cells. Thus, the results presented here are likely to represent an underestimate of the total extent of DNA aneuploidy in MM, but a relatively accurate quantitation of B cells with the greatest hyperdiploid DNA content.

**In Situ RT-PCR.** PBMCs from MM patients were stained in double direct immunofluorescence with mAbs CD19-FITC, with CD3-PE, or with CD34-PE and fixed in 10% formalin/PBS overnight. Using the ELITE Autoclone (Coulter), total CD19⁺ and CD3⁺ PBMC or CD34⁺19⁺ and CD34⁺19⁺ B cells were sorted onto slides and processed for *in situ* RT-PCR as previously described (7, 10). Primers to the CDR2 and the CDR3 regions of the rearranged IgH VDJ from individual BM plasma cells were designed and used for *in situ* RT-PCR as previously described (7, 8, 10). The optimal primer sequences in CDR2 and CDR3 were chosen based on computerized analysis. The IgH VDJ sequence used for patient-specific amplification of PBMC subsets was confirmed to be expressed by >80% of autologous BM plasma cells using *in situ* RT-PCR. For *in situ* RT-PCR, stained PBMCs or BMCs were fixed for 18 h in 10% formalin/PBS before sorting directly onto slides using the ELITE flow cytometer with an Autoclone cell deposition unit (Coulter). Rapid processing before the fixation step was essential to preserving mRNA (particularly for B cells that have fewer IgH mRNA transcripts than do plasma cells). All blood specimens were processed within 3–4 h after being drawn. Briefly, cells were permeabilized using pepsin (Boehringer Mannheim, Laval, Quebec, Canada) and then digested overnight with DNaseI (Boehringer Mannheim) before reverse transcription and PCR using patient-specific primers and digoxigenin labeled UTP (Boehringer Mannheim) as an indicator, as described in detail previously (7). After 25 cycles, slides were incubated with anti-DIG Fab conjugated with alkaline phosphatase (Boehringer Mannheim) followed by incubation with the chromogen nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Boehringer Mannheim). After air drying, slides were mounted and examined microscopically. Only those slides with acceptable positive
Cell Sorting and Confocal Microscopy. For sorting, PBMCs were stained, fixed with 0.1% formaldehyde, and sorted using the ELITE flow cytometer; analysis of aliquots of the sorted PBMCs indicated that CD19⁺ B cells were >80% cytoplasmic immunoglobulin⁺ (7, 11). Sorted subsets were 95–98% pure as determined by flow cytometric reanalysis of the sorted population.

Ethanol-fixed PBMCs stained with DAPI were sorted for the hyperdiploid subset. Cytospins of sorted hyperdiploid cells were stained with anti-k or anti-l F(ab)₂ fragments conjugated to FITC (Southern Biotech, Birmingham, Alabama) and viewed using a laser scanning confocal microscope (Leica, Toronto, Ontario, Canada) with excitation at 488 nm.

**Fig. 2** Hyperdiploid PBMCs in MM express CD19. For this patient, 24% of PBMCs were hyperdiploid with a DNA index of 1.31. Files were gated for the hyperdiploid subset. Marker bar: staining above that by an isotype-matched control mAb. The expression of CD19 is comparable before or after fixation and DNA staining and is also comparable to that of normal B cells. The intensity of staining was comparable for either FMC63-FITC or B4-FITC.

Table 1 DNA aneuploidy among MM PBMC B cells is greatest in untreated patients (Percent of CD19⁺ PBMCs or BMCs, and DNA index)⁺

<table>
<thead>
<tr>
<th>Treatment (no. of samples)</th>
<th>% diploid</th>
<th>% hyperdiploid</th>
<th>Ratio of diploid:hyperdiploid</th>
<th>DNA index of hyperdiploid PBMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt PBMCs (18)</td>
<td>32 ± 6</td>
<td>44 ± 8</td>
<td>2.2 ± 0.6</td>
<td>1.12 ± 0.05</td>
</tr>
<tr>
<td>Tr PBMC (42)</td>
<td>41 ± 4</td>
<td>46 ± 4</td>
<td>1.8 ± 0.4</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>Off Tr PBMCs (41)</td>
<td>55 ± 5³</td>
<td>31 ± 4⁴</td>
<td>3.8 ± 0.6⁵</td>
<td>1.07 ± 0.02</td>
</tr>
<tr>
<td>BMCs untreated (11)</td>
<td>43 ± 6</td>
<td>36 ± 8</td>
<td>2.1 ± 0.6</td>
<td>1.10 ± 0.03</td>
</tr>
<tr>
<td>BMCs during/after treatment (16)</td>
<td>42 ± 6</td>
<td>41 ± 7</td>
<td>2.8 ± 0.7</td>
<td>1.16 ± 0.06</td>
</tr>
</tbody>
</table>

⁺ PBMCs and BMCs were stained and analyzed as indicated in Materials and Methods. Only those samples having a hyperdiploid subset were included in this table. This comprises 101/114 MM PBMCs and 19/27 MM BMC samples analyzed. Values are the mean ± SE.

³ The ratio of diploid:hyperdiploid CD19⁺ PBMCs was first calculated individually for each patient sample and then these ratios were analyzed statistically to obtain mean values ± SE. The individual ratios are obscured in calculating the overall means of columns 1 and 2, explaining the apparent discrepancy between columns 1 and 2 and the value of column 3.

°C Unt, untreated; Off Tr, off treatment; Tr, treated, on intermittent chemotherapy.

⁴ P = 0.007 as compared to untreated diploid values.

⁵ P < 0.02 as compared to patients on treated statistical analysis using the Mann-Whitney rank sum test and a Kruskal-Wallis one way ANOVA on ranks using SigmaStat 2 (SPSS Inc, San Rafael, CA).

⁶ P = 0.09 as compared to untreated patients.

and negative control spots were read and counted. Slides were scored visually. After developing, counting of positive cells on each slide was done by a person blinded to the identification of the slide. For all patients and all in situ RT-PCR runs, the specificity of the patient-specific amplification was confirmed by testing the primers using RNA isolated from PBMC B cells of healthy donors. For some runs, specificity was also confirmed using CD38hi cIg1 BM plasma cells of healthy donors and unrelated myeloma B and plasma cells as negative controls, as indicated in the "Results" section.

The intensity of staining was comparable for either FMC63-FITC or B4-FITC.
RESULTS

Staining of DNA with DAPI Yields a Narrow CV for Human Thymocytes and Normal Donor PBMCs. To validate and optimize the analysis of the DNA content used here, a number of standards were stained with DAPI, a DNA-staining dye that does not bind to RNA, to determine the CVs for normal human lymphocytes (Fig. 1). For DNA bead standards, the CV was 3.6 when the peak was electronically positioned at the lower end of the fluorescence scale. Despite the visual appearance of a wider peak when positioned during data acquisition at higher channels of the DAPI fluorescence scale, the actual CV became narrower with a CV of 2.9 when the peak was positioned at channel 800. Normal human thymocytes had a CV of 2.14, as did PBMCs from normal donors (range for individual samples, 2.86–2.95; Fig. 1). Because the visual resolution of the hyperdiploid DNA content and the CV were optimized when the main peak was positioned at higher channels, most samples were electronically positioned between channels 300–500 (scale of 0–1023) to detect deviation from diploid as well as 4N cells.

Normal PBMC or spleen cells were stained with antibodies detecting T (CD4, CD8) or B (CD19) cells and with DAPI to determine the DNA content. Electronic gating for T cells gave a DAPI histogram with a narrow CV (2.21–4.07) and a diploid DNA content. B cells from 12 normal PBMCs and 2 normal spleens were analyzed, and for all donors, the DNA index of B cells was 1.01–1.02 as compared to T cells in the same aliquot of PBMCs. This is consistent with other estimates of DNA content for T and B cells from normal donors (42). For B chronic lymphocytic leukemia, PBMCs had a narrow CV (1.72–4.05 for the 14 patients) and a DNA content of 1.0.

B cells in PBMCs of MM Patients Include a Hyperdiploid Subset. A total of 114 PBMC samples from 76 MM patients were analyzed to determine the extent of DNA hyperdiploidy among circulating cells in MM. Of all MM PBMC samples analyzed, 101/114 (89%) of PBMC samples from 70/76 MM patients (92%) have detectable DNA hyperdiploidy. For patients assayed more than once, a consistent DNA index was seen for sequential samples. Table 1 details the number of diploid and hyperdiploid B cells in the set of PBMC samples exhibiting hyperdiploidy (101 samples) and of BMCs. In those
Clonotypic Myeloma B Cells are DNA Aneuploid

Table 2 Circulating CD19+ PBMCs express mRNA for CD19 and IgH VDJ: in situ RT-PCR

<table>
<thead>
<tr>
<th>Sorted subset of MM PBMCs</th>
<th>Primer</th>
<th>% of subset</th>
<th>No. × 10^9/liter blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19+ B cells</td>
<td>Histone</td>
<td>89 ± 3</td>
<td></td>
</tr>
<tr>
<td>CD3+ T cells</td>
<td>Histone</td>
<td>88 ± 6</td>
<td></td>
</tr>
<tr>
<td>CD19+ B cells</td>
<td>CD19</td>
<td>86 ± 2</td>
<td>0.53 ± 0.18</td>
</tr>
<tr>
<td>CD3+ T cells</td>
<td>CD19</td>
<td>0.8 ± 1</td>
<td></td>
</tr>
<tr>
<td>CD19+ B cells</td>
<td>IgH VDJ</td>
<td>89 ± 3</td>
<td>0.51 ± 0.15</td>
</tr>
<tr>
<td>CD3+ T cells</td>
<td>IgH VDJ</td>
<td>0.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>CD19+ B cells</td>
<td>CD34b</td>
<td>65 ± 7</td>
<td></td>
</tr>
<tr>
<td>Normal donor B cells (3)</td>
<td></td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>CD19+ B cells</td>
<td></td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>IgH VDJ</td>
<td></td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>CD34b</td>
<td></td>
<td>0.8 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

PBMCs were sorted for the indicated markers and analyzed using in situ RT-PCR. The number of CD19+ B cells in MM PBMCs ranged from 6 to 36% as previously reported (8, 10, 11, 40). CD3+ T cells represented 15–45% of MM PBMCs. Mean values ± SD are calculated from data for three to eight different patients. The absolute number of cells per liter of blood was calculated as: (WBC count × 10^9/liter) × (% lymphocytes) × % of PBMCs expressing CD19 or IgH VDJ.

Data taken from reference (7).

instances when simultaneous blood and BM samples were available, for many patients, the DNA index was comparable for blood B cells and BM plasma cells. However, for some patients, the DNA index of blood B cells was less than or greater than that of the matched BM plasma cells. Overall, for those samples exhibiting hyperdiploidy, the number of hyperdiploid B cells was greatest among untreated patients and significantly lower in patients off treatment. Fig. 2 shows a representative DNA distribution in PBMCs and the expression of CD19 on the hyperdiploid subset. For this patient, 24% of PBMCs were hyperdiploid with a DNA index of 1.31, and the concentration of hyperdiploid cells in the circulation was 0.31 × 10^9/liter of blood. Nearly all MM PBMCs in the G2-M region of the DAPI histogram were CD19+ cells.

To evaluate the extent of DNA hyperdiploidy, the DAPI profile of CD19+ PBMCs was compared to that of T cells in the same sample (Fig. 3). For all 114 analyzed, the full CV of the T-cell DAPI peak was within the range of that seen for T cells from normal donors, providing an internal control for B cells in the same aliquot of PBMCs. As shown for three representative MM patients in Fig. 3, B cells were markedly hyperdiploid as compared to autologous T cells. For patient 1, 40% of B cells were hyperdiploid (DNA index of 1.12), for patient 2, 50% of B cells were hyperdiploid (DNA index = 1.08), and for patient 3, 35% of B cells were hyperdiploid (DNA index of 1.09 to 1.46 as compared to T cells). The same patterns and degree of B-cell hyperdiploidy were obtained in three to six replicate aliquots of each sample. For a given patient over time, the DNA index remained relatively constant, although the proportion of hyperdiploid B cells varied. For the 70/76 MM patients (92%) having hyperdiploid PBMCs, nearly all hyperdiploid cells were CD19+ (see below).

CD19+ MM Blood B Cells Express mRNA for CD19 and IgH VDJ. B cells are unequivocally identified by their rearranged IgH genes and their expression of IgH mRNA and usually of immunoglobulin protein. To confirm that the CD19+ cells identified here were bona fide B cells, in situ RT-PCR was used to detect CD19 and IgH VDJ transcripts in sorted CD19+ MM PBMCs. Sorted CD19+ and CD3+ PBMCs were analyzed using in situ RT-PCR. Specificity of the priming was defined as a detection of an amplified product in B cells, but also as a lack of amplified product from T cells. Positive cells were counted microscopically as those with an accumulation of colored substrate (Table 2). To confirm that sorted cells had intact mRNA at the time they were placed on the slide, an aliquot of both T and B cells was amplified using primers to histone. Histone transcripts were detected in 89% of cells (Table 2).

Expression IgH and CD19 mRNA was considered as definitive identification of a B cell. These assays will detect any B cell with rearranged immunoglobulin transcripts, including both monoclonal and polyclonal populations. IgH transcripts were detected in 89% of sorted CD19+ PBMCs (Table 2). Because this was also the number of cells having intact mRNA (Table 2, line 1), by extrapolation, essentially all sorted CD19+ cells had IgH mRNA. Sorted autologous T cells (purity of 96–98%) for each patient had <1% IgH or CD19+–positive cells, confirming the specificity of the assay. On average, 86% of CD19+ MM PBMCs expressed CD19 mRNA, confirming that the CD19 mAb staining detected bona fide CD19+ B cells. Confirming our previous report (11), MM B cells are present at an average number of 0.5 × 10^9/liter of blood.

To confirm that hyperdiploid MM blood B cells expressed immunoglobulin, PBMCs were sorted based on DNA content and
then stained with antilight chain antibodies. The images of Fig. 4 show two hyperdiploid B cells with cytoplasmic $k$. Identically processed images of anti-$\lambda$-stained cells revealed no detectable cytoplasmic light chain, indicating monotypic immunoglobulin expression of the paraprotein type by hyperdiploid populations, which is consistent with previous observations (11).

Postchemotherapy, the Frequency of Diploid B Cells in PBMC Is Increased, and Hyperdiploid B Cells Are Reduced. The proportion of diploid and hyperdiploid CD19$^+$ MM PBMCs was compared in patients at diagnosis, during intermittent chemotherapy, and after therapy had been discontinued. DNA abnormality was measured as: (a) the extent of hyperdiploidy (DNA index), and (b) the proportion of lymphocytes that are hyperdiploid (Table 1). The extent of DNA hyperdiploidy was highest for untreated patients who had a mean DNA index of 1.12. There was a trend toward a decreased extent of hyperdiploidy during or after chemotherapy with a DNA index of 1.06–1.07 for patients on intermittent chemotherapy or off treatment.

The hyperdiploid cells comprised 5–90% of B cells in MM PBMCs. On average, patients off treatment had significantly higher proportions of diploid blood B cells than did untreated patients ($P = 0.007$; Table 1). The lowest proportion of diploid B cells were found in PBMCs from untreated patients (column 1). Patients off treatment had proportionately fewer hyperdiploid B cells in PBMCs than did those undergoing intermittent chemotherapy ($P = 0.03$).

The ratio of diploid to hyperdiploid PBMC B cells, as calculated from the individual patient values, provides a measure of the balance between these subsets of CD19$^+$ MM PBMCs (Table 1, column 3). A ratio $>1.0$ indicates a larger diploid set. This ratio was significantly greater in patients off treatment as compared to patients on intermittent chemotherapy ($P < 0.02$) and approached significance as compared to untreated patients ($P = 0.09$).

Hyperdiploid B Cells Express CD34. Of circulating B cells in MM, 65% express CD34 protein and transcript (Ref. 7; summarized in Table 2). To determine the extent to which CD34$^+$ B cells were DNA aneuploid, MM PBMCs were stained for CD19, CD34, and DNA in multiparameter immunofluorescence. Fig. 5, top panel shows the presence of a large CD34$^+$ set (62% of B cells) and a smaller CD34$^-$ set (38% of B cells), as previously reported (7), and the distinct increase in DNA staining among CD34$^+$ PBMCs. To more precisely analyze this, files were gated for CD34$^+$19$^+$ or CD34$^-$19$^+$ MM PBMCs, and the DNA content compared to that of autologous T cells.
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To determine the proportion of total hyperdiploid MM PBMCs that were CD34+ B cells, files were gated for the hyperdiploid subset, and the expression of CD34 and CD19 was plotted (Fig. 6; Table 3). In contrast, when BM plasma cells were analyzed, 81 ± 3% of hyperdiploid plasma cells were CD34+ CD19− B cells (Fig. 6; Table 3). In time lacked both CD34 and CD19 expression (CD34+ 19−; Table 4).

Hyperdiploid CD34+ 19+ MM PBMC Express Clonotypic IgH VDJ Rearrangements, but Diploid CD34− B Cells Appear Polyclonal. To determine the expression of patient-specific (clonotypic) IgH VDJ transcripts by individual B cells and to obtain a quantitative measure of the proportion of aneuploid cells having clonotypic IgH rearrangements, we took advantage of the fact that for MM patients with hyperdiploid PBMCs, hyperdiploid and CD34− subsets of MM PBMCs are almost completely overlapping. Subsets of CD34+ and 34− B cells were sorted from the blood of 4 MM patients for whom the clonotypic IgH VDJ rearrangement had been identified. The IgH VDJ sequence identified for each patient was confirmed as clonotypic by its expression in >80% of BM plasma cells (10).

Table 4 shows that nearly all CD34+ MM PBMCs express clonotypic IgH VDJ transcripts (86 ± 5%), extending our previously published work (7). In contrast, the population of diploid CD34− B cells in MM PBMCs contains few clonotypic cells (4.8 ± 2%), indicating it is a polyclonal subset. The lack of CD34 expression and diploid DNA content, both properties of normal B cells, are consistent with the identification of this B-cell subset as predominantly normal with little MM involvement.

Treatment with Colchicine Results in a Loss of Hyperdiploid B Cells and an Accumulation of Cells in the Diploid G2−M Region. In 1978, Hulin et al. (43) described a population of cells in MM BM, which were apparently arrested in the S phase and unable to incorporate thymidine in vitro. Haraldsdottir et al. (44) have shown with in vivo labeling studies that hyperdiploid MM cells resolve to diploid. These observations, coupled with the absence of a defined hyperdiploid G2−M peak in MM patients, raised the possibility that hyperdiploid cells may include those arrested in a diploid S phase. Our preliminary data indicated that hyperdiploid cells were frequently reduced in number after culture (not shown). To determine whether this reflected an in vitro release of a putative S-phase arrest, MM PBMCs were cultured with colchicine for 3 days in the absence of any deliberate stimuli. Because colchicine inhibits mitosis, any cycling cells will accumulate in G2−M.

Table 3 CD34+ PBMC B cells are hyperdiploid PBMCs and CD34− PBMC B cells are diploid, but CD34+ 19− plasma cells in BM are hyperdiploid

<table>
<thead>
<tr>
<th>A. Properties of phenotypically defined subsets (gating for phenotype):</th>
<th>CD34+ 19+</th>
<th>CD34+ 19−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Of total B cells</td>
<td>77 ± 6%</td>
<td>23 ± 6%</td>
</tr>
<tr>
<td>DNA index</td>
<td>1.07 ± 0.003b</td>
<td>1.01 ± 0.004</td>
</tr>
<tr>
<td>Of aggregate cells in G2−M (4N)</td>
<td>74%</td>
<td>26%</td>
</tr>
</tbody>
</table>

b 10 MM patients were analyzed for CD34, CD19, and DNA content. For the bottom half of the table, files were gated for hyperdiploid PBMCs or BMCs, and the expression of CD34 and CD19 displayed as a dot plot (as shown in Fig. 6). Of the MM PBMCs, 8/10 had a detectable hyperdiploid subset, whereas for two patients, PBMC B cells were diploid. Of the BMCs, 5/5 had a detectable hyperdiploid subset of plasma cells, which is comparable to the hyperdiploidy of B cells in blood samples taken at the same point in time.

Table 4 In MM blood, CD34+ B cells express clonotypic IgH VDJ mRNA, but CD34− B cells are polyclonal

<table>
<thead>
<tr>
<th>Sorted PBMC subset (Expression of patient-specific IgH VDJ)</th>
<th>CD34+ 19+</th>
<th>CD34+ 19−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients no.</td>
<td>74%</td>
<td>0.9%</td>
</tr>
<tr>
<td>Patient 3</td>
<td>a Nov 96</td>
<td>71%</td>
</tr>
<tr>
<td>Patient 4</td>
<td>b Feb 97</td>
<td>96%</td>
</tr>
<tr>
<td>Patient 6</td>
<td>93%</td>
<td>&lt;0.3%</td>
</tr>
<tr>
<td>Patient 18</td>
<td>96%</td>
<td>10%</td>
</tr>
<tr>
<td>Other MM patients (7)b</td>
<td>69%</td>
<td>7%</td>
</tr>
<tr>
<td>Total mean (12)</td>
<td>86 ± 5%</td>
<td>4.8 ± 2%</td>
</tr>
<tr>
<td>Normal B cells (39)</td>
<td>&lt;0.3%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Normal plasma cells (19)</td>
<td>&lt;0.3%</td>
<td></td>
</tr>
<tr>
<td>Unrelated MM</td>
<td>&lt;0.3%</td>
<td></td>
</tr>
<tr>
<td>Plasma cells (16)</td>
<td>&lt;0.3%</td>
<td></td>
</tr>
</tbody>
</table>

b Normal B cells were sorted from the PBMC of 10 different normal donors. Normal plasma cells (CD38+ Ig+ BMCs) were sorted from four different normal BM samples. IgH VDJ clonotypic sequences are from (10).

Includes data from Szczepke et al. (7) plus three additional patient samples. The four patients shown in lines 1–5 and the additional three in line 6 are different from those analyzed by (7).
for PBMCs from five patients, at day 3, there was a 4.6-fold decrease in the number of hyperdiploid B cells coupled with a 3.5-fold increase in the number of B cells in G2-M. Although a formal relationship has not been proven, this does indicate that MM B cells lose hyperdiploidy and accumulate in G2-M when subjected to colchicine, which is consistent with the idea that hyperdiploid B cells may be in a diploid S-phase arrest.

**DISCUSSION**

This study demonstrates that DNA hyperdiploid B cells in the blood of patients with MM are CD34\(^+\) cells expressing clonotypic IgH transcripts, which confirms their clonal relationship to autologous BM plasma cells in MM. DNA aneuploidy is likely to indicate abnormalities characteristic of malignant cells. Although not all MM patients have hyperdiploid PBMCs, for the 89% of patients who do, the presence of both DNA hyperdiploidy and clonotypic IgH transcripts in CD34\(^+\) B cells provides an experimental basis for supposing that they may have malignant status. The CD19\(^+\) PBMCs detected here are definitively identified as B cells based on their expression of IgH VDJ and CD19 transcripts. Their expression of CD34 has been confirmed at the mRNA level in individual B cells (7). Extending our previous work (7), in situ RT-PCR amplification of patient-specific IgH VDJ rearrangements in the mRNA of individual CD34\(^+\) B cells indicated that 86 ± 5% are clonotypic. As shown here, unlike CD34\(^+\) B cells, CD34\(^-\) B cells in MM PBMCs are diploid, and most lack detectable clonotypic IgH mRNA, indicating that they are polyclonal, a pattern identical to that of B cells from normal donors.

Circulating DNA hyperdiploid MM PBMCs have DNA indices significantly higher than diploid, although for some patients, MM B cells are diploid. We have also detected diploid BM plasma cells in some MM patients (not shown). Thus, diploidy does not necessarily indicate a nonmalignant cell. In this study, DNA hyperdiploidy was absent from normal B cells but consistently present in a subset of MM B cells from most patients. The DNA indices (1.04–1.30) for MM B cells are comparable to those reported by others for BM plasma cells (29, 30, 32, 34-37). The excess DNA detected is within the range expected based on the complex chromosomal abnormalities described in MM (36, 38, 45-47). The majority of the cells described here are lymphoblastoid cells with an intensity of

**Table 5** Loss of hyperdiploid B cells and accumulation of B cells in G2/M after treatment of MM PBMCs with colchicine

<table>
<thead>
<tr>
<th>Gated population</th>
<th>Treatment (% with DNA content) for cells harvested at day 3 (mean ± SE)</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperdiploid B cells</td>
<td>Untreated 3.4 ± 0.4</td>
<td>Colchicine 13.8 ± 1.2</td>
</tr>
<tr>
<td>B cells in G2/M</td>
<td>Untreated 3.8 ± 1.2</td>
<td>Colchicine 13.8 ± 1.2</td>
</tr>
</tbody>
</table>

Hyperdiploid B cells were defined as indicated in Materials and Methods. Cells in G2/M (4N) had a DNA content twice that of the diploid peak. There was no peak of cells with a DNA content greater than 4N. The means represent culture of PBMCs from each of five randomly selected MM patients. Because insufficient cells were available to analyze PBMCs at both day 0 and after tissue culture, only the day 3 analysis was performed.
CD20 about 10-fold lower than that of normal B cells (11). As such, they would have been excluded by the criteria for defining B cells in a recent analysis showing a lack of chromosomal abnormalities in CD20<sup>hi</sup> small lymphocytes from MM PBMCs (48). CD20<sup>hi</sup> expression defines the predominantly polyclonal CD34<sup>−</sup> subset of B cells in MM blood shown here (49), a subset of circulating B cells that is diploid, as expected based on the work of Zandecki et al. (48). Nearly all CD34<sup>+</sup> B cells in MM PBMCs have clonotypic IgH VDJ rearrangements (this work and Ref. 7). Their extensive hyperdiploidy provides presumptive evidence for their involvement in the malignant process and suggests that they may play a significant role in the disease.

In the blood, hyperdiploidy occurs preferentially among circulating CD34<sup>−</sup> MM B cells. We have previously speculated that the expression of CD34 on B cells in MM may enhance their migratory properties and facilitate the dissemination of myeloma to distant skeletal sites as the disease progresses (7, 50, 51). In initial work to establish a sequential relationship between circulating clonotypic MM cells and BM disease, we have shown that blood from patients with minimal disease includes MM progenitor cells able to engraft primary human myeloma to the marrow of nonobese diabetic severe combined immunodeficient mice (27). If the B cells in blood and the plasma cells in BM are sequentially related, our work implies that terminal differentiation within the myeloma hierarchy is accompanied by loss of CD34. Consistent with our previous work (7) and with the work of others (28, 52, 53), we find that in the BM, hyperdiploid plasma cells are CD34<sup>−</sup>19<sup>−</sup>, which is in direct contrast to the predominant expression of CD34 and CD19 on hyperdiploid cells found in the peripheral blood.

A significant finding in all 101 PBMCs and in 19 BM samples with a hyperdiploid subset was the absence of a G<sub>2</sub>-M peak corresponding to the hyperdiploid DNA content. However, in BMcs and PBMCs from MM, a clearly defined G<sub>2</sub>-M peak corresponding to diploid was always apparent. In absolute numbers, the diploid G<sub>2</sub>-M cells in the circulation are predominantly CD34<sup>−</sup> clonotypic B cells, but a small subset of CD34<sup>+</sup> polyclonal B cells is also detectable. Among BMcs however, nearly all cells in the diploid G<sub>2</sub>-M region of the DNA histogram are CD34<sup>−</sup>19<sup>−</sup> plasma cells. The absence of an aneuploid G<sub>2</sub>-M population in the samples analyzed here raises the possibility that hyperdiploid PBMCs and BMs may represent transiently noncycling populations arrested in the diploid S phase. Hulin et al. (43) have described a population of MM BM cells that have an S-phase DNA content but do not incorporate thymidine and postulated that these were arrested in the S phase. Haraldsdottir et al. (44) have shown that in vivo in hyperdiploid myeloma, cycling BM plasma cells return to the diploid, not the hyperdiploid compartment. These observations and our evidence that the majority of G<sub>2</sub>-M cells in MM PBMCs are CD34<sup>−</sup> B cells, which we show to be both hyperdiploid and clonotypic, are consistent with the idea that CD34<sup>−</sup> hyperdiploid B cells in the blood may resolve to a diploid G<sub>2</sub>-M and ultimately to a diploid G<sub>0</sub> DNA content. Alternatively, hyperdiploid B and plasma cells may be very slowly cycling cells in the aneuploid G<sub>0</sub>-phase. To begin to distinguish between these possibilities, MM PBMCs were subjected to mitotic arrest in vitro using colchicine. Under these conditions, hyperdiploid B cells were lost, and B cells in G<sub>2</sub>-M accumulated; these results support the in vivo results of Haraldsdottir et al. (44) and are consistent with a resolution of hyperdiploid B cells to diploid G<sub>2</sub>-M in vitro.

Ultimately, a clinically valid interpretation of these findings requires an understanding of the biological mechanism(s) giving rise to hyperdiploid cells. However, as a clinical indicator, DNA aneuploidy among PBMCs may provide an easily accessible window to detect malignant traffic throughout the body. Because we show here that most circulating clonotypic MM B cells are both CD34<sup>+</sup> and hyperdiploid, the analysis of hyperdiploidy provides a surrogate marker for clonality that may facilitate monitoring the effects of therapy. Overall, this work suggests that the DNA content of circulating clonotypic B cells should be considered in evaluating the effects of treatment and progression of the malignancy.

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clonalotypic IgH VDJ rearrangements with autologous bone marrow plasma cells in multiple myeloma, as measured by single cell and in situ RT-PCR. Blood, 92: 2844–2855, 1998.


In Multiple Myeloma, Circulating Hyperdiploid B Cells Have Clonotypic Immunoglobulin Heavy Chain Rearrangements and May Mediate Spread of Disease

Linda M. Pilarski, Nadia V. Giannakopoulos, Agnieszka J. Szczeppek, et al.


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