In Multiple Myeloma, Clonotypic B Lymphocytes Are Detectable Among CD19+ Peripheral Blood Cells Expressing CD38, CD56, and Monotypic Ig Light Chain

By P. Leif Bergsagel, Anna Masellis Smith, Agnieszka Szczepak, Michael J. Mant, Andrew R. Belch, and Linda M. Pilarski

Multiple myeloma (MM) is characterized by a plasma cell infiltrate of the bone marrow (BM). However, late-stage monotypic B cells have been detected in the blood. This work analyzes the effects of clinical treatment on late stage CD19+ B cells present in 752 blood samples from 152 MM patients. MM patients have 2 to 8 times as many circulating CD19+ cells as do normal donors. Analysis of the Ig heavy chain (IgH) gene rearrangements using polymerase chain reaction indicates that the CD19+ population includes cells sharing the same clonotypic CDR3 region as is detected in the BM plasma cells, for patients analyzed during chemotherapy or in relapse. They are also monotypic as defined by their cytoplasmic or surface expression of lmg or light chain. The light chain restriction is the same as that of the BM plasma cells. Individual patients observed over 1- to 2-year periods exhibit considerable variation in the number of B cells present in blood; this number does not correlate with the concentration of serum monoclonal Ig. The monoclonal blood CD19+ cells are not eliminated by any of the chemotherapy regimens analyzed and remain at high levels during transient remissions. Patients in the progressive phase of disease or in relapse have significantly higher numbers of B cells than do patients in transient remission or untreated patients. During periods when the quantity of blood B cells approaches normal, phenotypically their quality is highly abnormal, with physical and phenotypic heterogeneity. Most B cells express CD45R0, a high density of CD38, and CD56 characteristic of late-stage B or pre-plasma cells. CD38+ blood B cells had a cyclical presence. We conclude that monoclonal B cells in the blood of myeloma patient populations include drug-resistant reservoirs of clonotypic cells that may underlie relapse.

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Multiple myeloma (MM) is a malignancy of the immune system characterized by accumulations of plasma cells in the bone marrow (BM), usually by a high concentration of monoclonal Ig in serum or urine and lytic bone lesions arising from osteolytic activity of plasma cell-activated osteoclasts. Although many patients respond to chemotherapy, nearly all eventually relapse and become refractory to further treatment. The measure of response to chemotherapy is a reduction in BM plasma cells, loss of the monoclonal Ig peak, and relief from bone pain and other symptoms. However, even though plasma cells are apparently eradicated from the BM by therapy and monoclonal Ig becomes undetectable by conventional assays, for nearly all patients the disease persists in an apparently cryptic compartment. The mean survival postdiagnosis is approximately 3 years.

Although MM is a cancer of the BM, the therapy-induced loss of BM plasma cells does not lead to long-term remissions. It has been suggested that the malignant stem cells in myeloma may be immature cells that colonize hematopoietic microenvironments, including the blood. Accumulating evidence implicates a circulating late-stage CD19+ B cell in myeloma and clonotypic rearrangements as defined by the BM plasma cells in myeloma have been reported among blood lymphocytes. A preliminary report indicates clonotypic sequences among purified CD19+ peripheral blood mononuclear cells (PBMCs). Analysis of CD45 isomorphism on CD19+ cells in the blood and BM of myeloma patients indicates a heterogeneous continuously differentiating B lineage, in contrast to other B-cell malignancies such as B-cell chronic lymphocytic leukemia (B-CLL), lymphoma, or hairy cell leukemia. Monoclonal rearrangements of the Ig heavy chain locus are detectable in blood from a proportion of patients, and mRNA encoding either κ or λ but not both light chains was detectable in myeloma PBMCs. A large proportion of CD19+ cells in myeloma PBMCs have extensive DNA aneuploidy. Unlike plasma cells or BM-localized B cells, and consistent with expectations for an invasive cell type, the monoclonal blood CD19+ cells express adhesion molecules, including CD11b, a2β1, and a6β1 integrin receptors for extracellular matrix, selectins, and CD44.

For nearly all patients with myeloma, circulating CD19+ cells express functional multidrug transporter, p-glycoprotein 170. The number and phenotypic properties of CD19+ cells in the blood of myeloma patients, and their relationship to malignant plasma cells, were analyzed as a function of chemotherapeutic treatment and course of disease. We find clonotypic IgH rearrangements among CD19+ cells expressing cytoplasmic Ig at diagnosis, during therapy and off treatment. Thus, CD19+ B cells persist despite chemotherapy and, for those patients with apparently normal numbers, CD19+ cells in blood are phenotypically abnormal.
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Fig 1. CD19+ B cells in blood persist despite chemotherapy. Each symbol represents a single patient sample. The percentage of CD19+ cells actually detected in PBMCs is presented here, in preference to absolute values in blood, because the percentage is a direct, unmanipulated data point. *P = .07 as compared with untreated patients.

MATERIALS AND METHODS

Patients. Patients with MM were observed for periods of up to 2 years, at monthly intervals, after informed consent was obtained. A total of 752 blood samples from 152 patients were analyzed for some or all of the parameters described in the Results. The majority of patients in all groups received a complete phenotypic analysis. A total of 14 patients were analyzed by CDR3 polymerase chain reaction (PCR). Of these, only 8 showed a single major IgH rearrangement among the purified BM plasma cells, consistent with other reports, which allowed identification of a clonotypic rearrangement for comparison with IgH rearrangements in purified blood B cells. Phenotypic information was maintained in a set of linked databases for clinical and research data using dBASEIV (Borland, Scotts Valley, CA). The patients included 50 untreated (at diagnosis), 43 on melphalan/prednisone (M/P), 42 on vincristine/adriamycin/dexamethasone (VAD), 9 on biologic response modifiers interferon (IFN) or IFN plus interleukin-2 (IL-2), and 68 off chemotherapy. Because most patients were observed for prolonged periods of time, a single patient usually appears in several of these groups. Patients on intermittent chemotherapy were studied at least 4 weeks after their latest treatment. The definition of clinical parameters was as described by Durie and Salmon. Seventeen normal, healthy volunteers were analyzed at single time points as controls. Patients were designated as off treatment 2 months after their last cycle of chemotherapy. However, most patients in this category had been off treatment for longer periods of time.

Purification of PBMCs. Venous blood samples were drawn into heparinized vacuumer tubes and the PBMCs were isolated on a Ficoll-Paque (Pharmacia, Dorval, Quebec, Canada) density gradient. Cells harvested from the interface were washed twice in RPMI (GIBCO, Grand Island, NY) and resuspended in phosphate-buffered saline, including 2% fetal calf serum (HyClone Labs, Logan, UT). Methods for depletion of adherent cells were carefully avoided, because the abnormal CD19+ cells in myeloma have adherent properties and are depleted by such procedures.

Antibodies. IgG1 FITC, IgG1 PE, IgG2 FITC, and IgG2PE were from Southern Biotech (Birmingham, AL). Leu15PE (CD11b), Leu17PE (CD38), J55FITC (CD10), and HNK-1 (CD56) were purchased from Becton Dickinson (San Jose, CA). From Coulter (Hialeah, FL), we purchased B4-FITC (CD19) and B1-RD1 (CD20). Monoclonal antibody (MoAb) FMC63 (CD19) was from H. Zola, and UCHL1 (CD45R0) from P. Beverley, and PCA-1 from K. Anderson. For detection of circulating CD19+ B cells in the blood of myeloma patients, either FMC63 or the commercially available B4 MoAb (Coulter) gave comparable results, but these cells were not reliably detected with Leu-12 (Becton Dickinson) in our hands. However, others have reported large numbers of CD19+ cells in PBMCs from myeloma patients using Leu-12. They were not reliably detected with phycoerythrin (PE) conjugates of CD19 MoAbs, probably reflecting steric hindrance from these bulky phycobiliproteins. MoAbs to Ig light chain used in immunohistochemistry were from the American Type Culture Collection (Rockville, MD); those used for immunofluorescence (IF) were F(ab)2 fragments conjugated to fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (Rhodamine). Antibodies. IgG1 PE and goat antihuman IgG-P were from Southern Biotech. Goat antihuman IgG-PE and goat antihuman IgG-P were from Southern Biotech.

Two-color and three-color immunofluorescence staining procedure was used for the study of surface marker expression as previously described. PBMCs were incubated with antibodies to be detected by indirect IF (isotype controls,
Fig 2. Expression of CD19 on myeloma PBMCs and expression of CD20 on the CD19⁺ subset of PBMCs. (A) PBMCs were stained with CD19-FITC and CD20-RD1 (-----) or an isotype-matched IgG2 MoAB (-----). Row 1 is the staining by CD19-FITC and the marker bar (R1) indicates the cells considered to be positive for CD19 and the electronic gate defining CD19⁺ cells. Row 2 is the staining by CD20-RD1 on PBMCs gated for CD19 (R1). Ungated (CD19) and gated (CD20) histograms are from 3 representative patients. Staining by FMC63-FITC and by BCFITC was nearly identical. (B) RT-PCR analysis using CD19 primers to amplify mRNA from freshly isolated and sorted B or T cells from the same patient. PCR was performed on RNA from 10⁶ cells. (C) CD19⁺ PBMCs express Ig. Files were gated for CD19⁺ cells as indicated in the top panel and the expression of Ig plotted as histogram. (-----) Staining with goat antimouse Ig-PE (control). (-----) Staining of CD19⁺ PBMCs with goat antihuman Ig-PE. (-----) Staining of plasma cells with goat antihuman Ig. The values within each peak indicate the proportion of gated CD19⁺ PBMCs or plasma cells with that intensity of staining. Similar results were obtained with PBMCs from 10 other patients; in all 10, the proportion of B cells expressing detectable Ig was 80% or higher.
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UCLH1, or PCA-1), washed, blocked with mouse Ig, and stained with a direct conjugate of B4-FITC or FMC63FITC. A double-direct IF procedure was used for staining of cells with PE-conjugated isotype controls, B1RD1, Leu15PE, or Leu17PE and FMC63FITC. Stained cells were washed twice and fixed in 1% formalin for flow cytometric analysis.

Analysis of IF. Samples were analyzed using a FACScan (Becton Dickinson). Red blood cells and dead cells were excluded by electronic gating on forward angle light scatter and files of 10,000 to 20,000 cells were collected. Films were electronically gated for CD19+ cells and the expression of the second MoAb was plotted as a histogram. In all cases, staining with a specific MoAb was compared with its appropriate isotype-specific control, with identical electronic gates for B-cell subsets. To maintain a consistent evaluation of the intensity of staining among different patients, a precise definition was used. In all cases, on a log scale, the intensity of staining was categorized as moderate (between 101 and 102) or high (staining greater than 102).

CD19+ cells were also evaluated for their physical properties as measured by forward (FALS) and side angle scatter (SSC), as previously described.18-20 Cells designated as small were those with SSC less than channel 400, whereas those designated as large had SSC greater than channel 400 on a linear scale. For most patients, FALS was also increased among the large cells.

Sorting. Sorting was on an ELITE flow cytometer (Coulter). PBMCs were stained with FMC63-FITC or CD11b-PE, followed by sorting for staining greater than the isotype-matched control. Sorted B cells were concentrated, counted, and used to prepare lysates for DNA analysis or cytoplasmic Ig for immunohistochemistry. PCA-1+ IgG BM plasma cells were sorted for PCR analysis of CDR3 clonotypic rearrangements.

Ig heavy chain gene analysis. PCR was used to amplify VDJ rearrangements from blood B cells sorted for expression of CD19 and, in some cases, sorted into a CD19 small subset and a CD19 large subset. For those patients tested, the majority (80% to 90%) of CD19+ B cells expressed detectable cytoplasmic Ig. BM plasma cells were purified by sorting for cells which coexpressed the plasma cell marker PCA-1 and a high density of cytoplasmic Ig (c Ig).

IgH fingerprinting. Whole cell lysates of the sorted cell populations were prepared24 and resuspended at a concentration of 1,000 cells/μL. One microliter of the cell lysate was used in a 25-μL PCR reaction in 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 2 mmol/L MgCl2, 200 μmol/L dNTP, 0.025 U/μL Taq polymerase (AmpliTaq; Perkin Elmer, Branchburg, NJ), 0.2 μmol/L FR2a (codons 42-47 TATGAATTCGAGAAGGCGCTGGAGTTG), and 0.2 μmol/L JH1 (codons 114-109 AGGGATCCACTGGAGAGCTGTTTGACC), with cycling 25 times between 94°C for 30 minutes, 52°C for 30 minutes, and 72°C for 30 minutes. From this reaction, 0.5 μL was placed into a second-stage PCR in a 5-μL reaction performed as above, with the oligonucleotides being 1 μmol/L FR2a and 0.1 μmol/L L 32P ATP end-labeled JH2 (codons 110-103 ACGGGATCCGGTACCAGGTNTCCTTGCCCCAG), with cycling for 20 times. Two microliters of this reaction was electrophoresed on a 6% denaturing polyacrylamide urea gel and the gel was exposed to XAR film (Eastman Kodak, Rochester, NY).

Sequence. The CDR3 PCR products were amplified as above and purified from an agarose gel using Promega Magic PCR Preps (Promega, Madison, WI). The nucleotide sequence was determined using end-labeled oligonucleotides FR2 and JH2 with Promega’s fmol PCR sequencing kit (Promega). Allele-specific oligonucleotides ASO were designed based on the nucleotide sequence and synthesized by Genosys Biotechnologies Inc. (The Woodlands, TX). CDR3 PCR and ASO Southern blot. Using the same conditions as above, 1 μL of the cell lysate was amplified with oligos FR2a and JH1 for 30 cycles. From this reaction, 0.1 μL was placed in a second-stage PCR under the same conditions, with oligos FR2b [codons 64-69 ATGGAATTCCAGGC(A/GA/CT)(CTG)TCACCAT] and JH2 for 30 cycles. Ten microliters of this reaction was electrophoresed on a 4% Nusieve GTG agarose gel containing ethidium bromide. The DNA was transferred to a Nylon filter by Southern blotting. The Nylon filters were hybridized to the patient specific end-labeled ASO in 5× SSC, 1% sodium dodecyl sulfate (SDS), and 20% formamide at 42°C for 4 hours, and then washed three times in 5× SSC, 1% SDS at 22°C for 5 minutes and once at 42°C for 5 minutes. The filter was exposed to XAR film for 2 hours at −70°C.

Immunohistochemistry. Cytopsin were air-dried, fixed in acetone, and stained with Wright’s stain for morphologic evaluation, followed by staining with anti-κ or anti-λ to determine light chain expression. Cells were incubated with primary antibody for 60 minutes at room temperature followed by two washes in phosphate-buffered saline (PBS). Horseradish peroxidase conjugated to goat antimouse Ig was added for a further 15 minutes of incubation at room temperature. Freshly prepared DAB (3,3'-diaminobenzidine-tetrachloride; Sigma, St Louis, MO) in PBS containing 0.008% hydrogen peroxide was added to cells for 4 to 5 minutes and the degree of staining was determined by comparison to isotype-matched controls.

Reverse transcriptase-PCR (RT-PCR) for CD19 mRNA. Based on the sequence of the CD19 gene,23 primers for RT-PCR were designed by and obtained from Dr Tom Tedder (Duke University, Durham, NC). Using Trizol according to the manufacturer’s directions (GIBCO, Burlington, Ontario, Canada), RNA was prepared from populations of sorted B cells and from sorted T cells of the same patient, collected at the same time in a double immunofluorescence sort, to serve as a negative control. After purification, 1 μg of RNA was reverse transcribed using Super-Script reverse transcriptase (GIBCO BRL) and universal primer oligo(dT)12 (Boehringer Mannheim, LaVal, Quebec, Canada) ac-
CD19+ B cells persist in blood despite chemotherapy. Figure 1 shows the distribution of CD19+ cells in individual PBMCs as a function of treatment status. There is a broad range of values in all treatment categories and the mean values are comparable for all groups (Table 1). Although for any given patient an increased percentage of CD19+ cells is always found at some time points throughout the disease progression, the numerical value is not always abnormal, as indicated by the values below 15%, the normal range12 (Fig

Statistical evaluation. Values from patients on chemotherapy or off treatment were compared with those from untreated patients using a two-tailed t-test.

RESULTS

CD19+ B cells persist in blood despite chemotherapy.
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Table 2. Nucleotide Sequence of IgH VDJ Rearrangements Amplified From BM

<table>
<thead>
<tr>
<th>FR3</th>
<th>CDR3</th>
<th>JH</th>
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<tbody>
<tr>
<td>1</td>
<td>TGTACG...GCGGCACTTT..CTCGGCC</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>TGTACGAGA...GCGGCACTTT..CTCGGCC</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TGTACGAGA...GCGGCACTTT..CTCGGCC</td>
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The sequence is given for the patients referred to in Figs 4 and 5. The ASOs were synthesized based on the complement of the regions underscored.

...were synthesized based on the complement of the regions underscored. High proportion of CD19' B cells. Figure 2 (top panel, row 2) shows the CD19 MoAb staining of myeloma PBMCs and the marker bar indicates those cells recorded in Fig 1. These CD19' PBMCs expressed CD19 mRNA, as detected by RT-PCR analysis of sorted CD19' B cells, the absence of CD19 mRNA from T cells sorted from the same PBMCs patient samples (Fig 2, bottom left), and the expression of IgG by the CD19' population (Fig 2, bottom right). PBMCs gated for CD19 expression coexpress CD20, including CD19' population. 

Unrelated BM Unrelated BM PCA1+ CD19+ CD19+ BM BM Unsorted CD19+ Unrelated CD19+

CD19' B cells in blood of nearly all myeloma patients can be subdivided into a set with low SSc (designated "small") and a set with high SSc (designated "large") that is not found in normal donors. The subset of small B cells is phenotypically heterogeneous. In untreated patients, approximately half of the circulating B cells are small B cells and half are large late-stage B cells (Table 1, columns 4 and 5). For patients treated with M/P or with VAD, the number of small B cells was significantly decreased as compared with untreated patients (P ≤ .07). The number of large B cells did not change with treatment. The B cells in patients treated with biologic response modifiers (IFN or IFN/IL-2) or patients off treatment were not significantly different from those in untreated patients.

The number of circulating B cells was compared among patient groups defined by their disease status (Fig 3). A significant difference in the percentage of CD19' B cells in PBMCs was detectable, with the lowest values among those patients in transient remission and the highest in patients with progressive disease or those who have had one or more relapses.

CD19' cells in blood of myeloma patients express clonotypic IgH VDJ rearrangements. Hemi-nested PCR was used to compare IgH rearrangements in sorted populations of blood CD19' cells with sorted PCA-1' plasma cells from the BM. PCR using consensus oligonucleotides to the IgH variable region framework 2 (FR2) and the IgH J segment (JH) genes amplifies rearranged heavy chain genes, but not germline heavy chain genes (because germline FR2(JH) gene sequences are too distant to be amplified). Because of different D gene lengths, and N region diversity, the length of the VDJ rearrangements amplified varies within a range of about 48 nucleotides, changing in increments of three nucleotides (be-
Fig 6. Circulating CD19+ B cells express Ig light chain. Cytospins were made from PBMC B cells of a patient with IgGκ myeloma. Slides were stained with either anti-κ or anti-λ F(ab)2 fragments conjugated to FITC and examined using confocal microscopy. B cells were scanned at 1,400× power at 512 × 512 resolution. The left image is of small B cells with predominantly surface staining and low cytoplasmic staining with anti-κ. The right image is of a large B cell with strong cytoplasmic staining for κ light chain. A variety of gradations of staining patterns between these two extremes were always detectable among the CD19+ blood B cells. No staining was detectable for cells from this patient stained with anti-λ.

cause the majority of rearrangements preserve an intact open reading frame). When electrophoresed on a high resolution sequencing gel, the rearrangements present in a polyclonal B-cell population thus appear as a ladder of approximately 16 bands, spanning 48 nucleotides. For a monoclonal B-cell population, such as purified BM plasma cells from a patient with myeloma, only a single rearrangement should be amplified. In 8 patients, we amplified a unique rearrangement from their BM plasma cells, and we examined their blood CD19+ cells by Ig fingerprinting.

Figure 4 shows the result of hemi-nested IgH fingerprinting analysis in blood B cells and BM plasma cells for 3 of the 8 patients, chosen to reflect the heterogeneity in the amplification patterns observed. From the blood CD19+ cells of the patient in the left panel, a single rearrangement of exactly the same size (222 bp) as that in the BM was amplified. The rearrangement amplified from the peripheral blood was sequenced and was identical to that in the BM, without evidence of somatic mutation. This is evidence of both clonotypic B cells in the CD19+ population, and a relative absence of other B cells (although all the cells in this population expressed Ig; Fig 2). This does not necessarily mean a complete absence of nonclonotypic B cells, because the hemi-nested PCR may greatly amplify small differences. We have not determined at what level a clonal rearrangement, in the presence of polyclonal rearrangements, will be amplified as a single band without evidence of a polyclonal ladder. For the patient in the middle panel, blood B cells were sorted into the small and large subsets: from the large B cells, only the clonotypic rearrangement (258 bp) was amplified; from the small B cells, a polyclonal ladder of rearrangements was amplified. For the patient on the right panel, many rearrangements were amplified from both the small and large B-cell subsets. Although a rearrangement(s) of the same size as the clonotypic one (252 bp) is evident in both populations, in the presence of so many other rearrangements, one cannot conclude that there are clonotypic cells in these populations. Therefore, the rearrangements amplified from the BM were sequenced and ASOs were synthesized (Table 2).

To confirm the presence of patient-specific clonal rearrangements in the blood CD19+ cells, all of the rearrangements in a sample were amplified using nested CDR3 PCR (Fig 5). The top panel shows an ethidium bromide-stained agarose gel of the nested PCRs from the same patients as in Fig 4, from samples collected at a later time point. This demonstrates that there are IgH rearrangements in all samples. To confirm that the amplified product includes clonotypic sequences, these rearrangements were hybridized to an end-labeled patient-specific ASO under stringent conditions so that only identical rearrangements should hybridize. The lower panel shows that clonotypic rearrangements are detected among the rearrangements in unsorted blood, and in CD19+ cells, but not in the CD19+ cells of an unrelated individual. Because this method analyzes DNA, as opposed to RNA, one need not be concerned that a rare contaminating plasma cell will inordinately influence the result, there being only a single DNA rearrangement per diploid cell. The same results were obtained using ASO-PCR (ie, using the ASO in the PCR reaction with FR2); however, the sensitivity of ASO-PCR is theoretically much greater, and could detect even a very rare cell. By amplifying all of the rearrangements in a sample there is no (or a least very little) selection imposed by the PCR. One can then ask what fraction of the amplified rearrangements are clonal. Although not quantitative, in relative terms a much greater proportion of amplified
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Table 3. Small and Large CD19+ B Cells Are Phenotypically Abnormal in Newly Diagnosed Patients and Those on or off Chemotherapy

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>CD19 Small</th>
<th>CD19 Large</th>
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<tbody>
<tr>
<td></td>
<td>Unit</td>
<td>Tr</td>
</tr>
<tr>
<td>CD20</td>
<td>61 ± 7</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>CD10</td>
<td>33 ± 17</td>
<td>47 ± 12</td>
</tr>
<tr>
<td>CD45R0</td>
<td>61 ± 7</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3 ± 1</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>PCA-1</td>
<td>45 ± 13</td>
<td>50 ± 14</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;+&lt;/sup&gt;</td>
<td>18 ± 3</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>CD56</td>
<td>24 ± 14</td>
<td>21 ± 12</td>
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</table>

PBMC samples were stained with CD19 together with MoAb to the indicated marker in two-color immunofluorescence. Files were gated for CD19<sup>+</sup> cells and the expression of the marker was plotted as a histogram. Cells were considered to be positive only if the staining exceeded that of an identically gated isotype control MoAb. For CD11b and CD38, only the highest density of expression is enumerated here (staining as indicated in the Materials and Methods). For the majority of cells, staining with CD10 and PCA-1 was generally at moderate intensity; staining with CD20, CD45R0, and CD56 was moderate to high as defined in the Materials and Methods. Values in the indicated treatment group or between different types of therapy were not significantly different from each other or from untreated values.

rearrangements are clonal for patients no. 1 and 2 and, by comparison, for patient no. 3, for whom only a minority of the products in the band hybridize to the patient-specific ASO probe. Patient no. 3 has had a prolonged plateau phase perhaps related to the relative paucity of clonotypic B cells and the expression of the marker was plotted as a histogram. Cells were considered to be positive only if the staining exceeded that of an identically gated isotype control MoAb. For CD11b and CD38, only the highest density of expression is enumerated here (staining as indicated in the Materials and Methods). For the majority of cells, staining with CD10 and PCA-1 was generally at moderate intensity; staining with CD20, CD45R0, and CD56 was moderate to high as defined in the Materials and Methods. Values in the indicated treatment group or between different types of therapy were not significantly different from each other or from untreated values.

In a total of 5 of the 8 patients, a single clonotypic rearrangement was amplified from the blood B cells or large/small B-cell subsets on IgH fingerprinting, indicating clonotypic involvement. In all 8 patients clonotypic rearrangements were detected in the blood B cells or B-cell subsets using one or more technique of IgH fingerprinting, nested CDR3-PCR probed with an ASO, and ASO-PCR. B cells taken from the same patient over a 2- to 4-month period consistently exhibited the same clonotypic band. Purified T cells from the patient taken at the same time and B cells from an unrelated individual did not contain the clonotypic rearrangement.

CD19<sup>+</sup> cells in blood of myeloma patients have restricted light chain expression. To confirm Ig gene expression, cytoplasmic Ig light chain was evaluated in either sorted B cells or in total PBMCs. Cytospins were stained with either anti-κ or anti-λ, followed by microscopic examination. Samples were also evaluated using confocal microscopy of sorted B cells stained with fluorescent anti-light chain. Figure 6 shows representative examples of the small B cells with predominantly surface staining (Fig 6A) and large B cells with strong cytoplasmic staining (Fig 6B). Parallel staining with the opposite anti-light chain reagent gave no fluorescent staining. In 13 of 13 patients analyzed, sorted CD19<sup>+</sup> B cells were positive for either κ or λ but not both, and the light chain expressed was the same as that for the monoclonal serum Ig or urine protein. The light chain type was the same for blood and BM B cells. Three patients were analyzed at two to three time points with consistent light chain restriction. For sorted B cells from all patients analyzed, the majority of B cells expressed κlg and the intensity of staining with anti-light chain was heterogeneous, ranging from barely detectable to relatively intense (comparable to the fluorescent staining shown in Fig 6) but less than that of BM plasma cells, consistent with our phenotypic characterization of this population as heterogeneous and comprising a range of B-cell differentiation stages. Finally, the patient PBMCs analyzed in other studies for monoclonal Ig rearrangements, expression of light chain mRNA, or expression of Ig were also part of this study, providing confirmation of Ig monoclonality for a total of approximately 60 patients.

CD19<sup>+</sup> PBMCs are phenotypically abnormal, expressing CD38, CD56, and other antigens not detected on normal B cells. A significant proportion of CD19<sup>+</sup> PBMCs expressed both CD38 and CD56 (Table 3). Figure 7 shows representative histograms for the staining of CD38 during and after chemotherapy (Fig 7A), and of CD56 (Fig 7B), on CD19<sup>+</sup> PBMCs in myeloma. Among subsets of CD19<sup>+</sup> cells gated for granularity (SSC; Table 3), for the majority of patients, the small B-cell subset expressed CD45R0 and PCA-1, unlike normal B cells, but had only minor expression of CD56 (moderate intensity) or expression of a high intensity of CD38. The large B cells coexpressed CD19 and CD20, as expected for B cells. As previously shown with a smaller cohort, the majority of large B cells were CD10<sup>+</sup> CD45R0<sup>+</sup> CD11b<sup>+</sup> PCA-1<sup>+</sup>, and are here shown to express a moderate intensity of CD56 and nearly half express a high intensity of CD38 (Fig 7 and Table 3). Overall, although clearly heterogeneous and including multiple B-cell differentiation stages, no significant differences in phenotype of MM blood B cells were found between different treatment groups. Because normal PBMC B cells do not express CD10/PCA-1, CD38<sup>+</sup>, CD11b<sup>+</sup>, CD56, or CD45R0, Table 3 shows that numerically normal CD19<sup>+</sup> populations in blood in all cases include phenotypically abnormal cells. The majority of small B cells, the subset most comparable to normal B cells in its physical properties (FALS/SSC), are qualitatively different
from and considerably more heterogeneous than normal B cells.

Temporal changes in CD19+ PBMCs occur during chemotherapy and over the course of disease but do not correlate with mIg levels. Individual patients were observed over time to evaluate the number of blood B cells together with clinical parameters. The patterns for 2 representative patients are shown in Fig 8. In all patients the number of B cells varied over time, as measured by the percentage of PBMCs or by the number in blood. For most patients, the number of B cells was relatively high at diagnosis and tended to increase or remain stable throughout chemotherapy, with periods when the level increased substantially (as shown in Fig 8). These increases did not correlate with increased monoclonal Ig, but would not be expected to do so. mIg is a measure of plasma cell tumor burden, and would not be expected to correlate with the numbers of PBMC B cells that have not yet acquired the capacity for a high rate of Ig secretion. For patient no. 1, the number of B cells decreased transiently after cytoreductive therapy and transplantation, but recurred within a few months posttransplant. The analysis of patient no. 2 began 2 years postdiagnosis after relapse and treatment with M/P. For this patient, the number of B cells appeared to increase with the cessation of therapy and with relapse, but decreased with therapy. The B cells from both patients were shown to be monotypic by analysis of light chain mRNA and/or of cytoplasmic Ig. A cyclical pattern of CD19+ B-cell levels was seen in 30 of 40 patients analyzed at multiple time points, including the period when chemotherapy terminated. Six of 40 patients maintained a high expression of CD19+ B cells throughout, and 4 of 40 had decreased CD19+ levels after initiation of therapy that have not yet recurred to high levels.

Cyclical presence of CD38hi B cells in blood is consistently detectable for most patients. A high density of CD38 (intensity of staining greater than 10^2, Fig 7A) appears only late in the differentiation towards plasma cells and is not seen on normal B cells in adults. The percentage of CD38hi B cells was plotted over time for individual patients at the indicated month postdiagnosis (Fig 9). The percent of CD19+CD38hi B cells increased with the initiation of chemotherapy (month 1) and continued to increase for several months. Levels became depressed for a brief interval posttreatment (months 5 and 6) and then increased again (months 8 and
CLONOTYPIC B CELLS IN MYELOMA

40 0.2

The number of CD19+ cells in blood varies with treatment and as the disease progresses, but remains high when Ig levels are reduced. Each plot represents the results for 1 individual patient over the indicated time period. Patient no. 1 was diagnosed as MGUS in 1989 and was diagnosed as stage 2A myeloma in November 1991 at age 82. M/P was initiated in April 1989 for 6 cycles. He relapsed in May 1990 and again in December 1992, and responded to reinstitution of M/P at both relapses.

Fig 8. The number of CD19+ cells in blood varies with treatment and as the disease progresses, but remains high when Ig levels are reduced. Each plot represents the results for 1 individual patient over the indicated time period. Patient no. 1 was diagnosed as MGUS in 1989 and was diagnosed as stage 2A myeloma in November 1991 at age 82. M/P was initiated in April 1989 for 6 cycles. He relapsed in May 1990 and again in December 1992, and responded to reinstitution of M/P at both relapses.

DISCUSSION

For all patients having a monoclonal band among their BM plasma cells, this study demonstrates the persistent presence of monoclonal CD19+ B cells in the blood that share clonotypic VDJ Ig heavy chain rearrangements with the BM plasma cells, as measured by hemi-nested IgH fingerprint analysis, nested CDR3-PCR probed with an ASO, and a specific oligomer PCR, and sequence analysis. As expected, and providing a negative control for the PCR reaction, clonotypic IgH rearrangements cannot be amplified from purified T cells derived from the same PBMCs as the CD19+ B cells. The rearranged Ig genes are functionally expressed in CD19+ B cells as evidenced by the presence of low to moderate amounts of monotypic cytoplasmic Ig light chain. As defined by physical parameters, CD19+ PBMCs include a small and a large subset, both of which are predominantly late-stage CD19+20+ B cells, previously shown to coexpress CD24.10,12 Many of the large B cells coexpress CD56 and a high density of CD38. Although distinguished from plasma cells by the extent of Ig expression, by phenotype, and by morphology,10,13,15,19,20,28 these circulating CD19+ populations include cells within the malignant lineage as defined by the presence of clonotypic VDJ rearrangements with a sequence identical to that of the BM plasma cells, although there is as yet no direct evidence to indicate that they are themselves malignant.

Clonotypic rearrangements were detected among the VDJ rearrangements amplified from the purified B cells, or B-cell subsets in all 8 informative patients, confirming the presence of clonotypic cells and, in some samples, a relative absence of nonclonotypic B cells. The best way of quantitating the frequency of clonotypic B cells relative to all B cells is by probing a nested CDR3 PCR, which amplifies all rearrangements, with an ASO. These data suggest that in some patients the clonotypic cells represent a majority of all B cells, and in others a minority. Further study is required to determine which B-cell subsets are predominantly clonal and how this correlates with a patient's clinical course.

Fig 9. Cyclical expression of high-density CD38 over the course of disease. The expression of CD38 on large B cells was plotted as a function of time postdiagnosis (patients no. 1 and 4) or postautologous transplant (patient no. 3). Patient no. 1: initial time point, MGUS; month 6, end of VAD; month 11, pre-autologous BM transplant sample (ABMT). The results are as for Fig 8. Patient no. 2: initial time point, post-ABMT; month 6, post-autologous BM transplant; month 14, death. Patient no. 4: initial time point, last; month 6, end of VAD; month 8, leukapheresis sample and autologous transplantation. The kinetics of disease for this patient have been presented elsewhere.6 Breaks inserted in lines indicate periods longer than 1 month. Patient no. 1: break, 2 years including a transition from a diagnosis of MGUS to that of myeloma. Patient no. 2: break, 10 months. Patient no. 1 is also shown in Fig 8. Patient no. 1, (III): patient no. 2, (+); patient no. 4 (*).
Although mean values indicated little change in the circulating B-cell component of myeloma, the values in individual patients exhibited a wide spread, with normal numbers in some patients. To determine if quantitative normality indicated qualitative normality, physical and phenotypic properties of the B cells were compared. By both measures, the B cells in myeloma blood from untreated and treated patients were highly abnormal, expressing a variety of markers not found on normal resting B cells, including CD38 and CD56. Large CD19\(^+\) \(20-38\) \(56\) B cells are not found in normal blood. Treatment had no detectable effect on the large B-cell subset. Significant decreases in the number of small B cells were detectable in patients treated with M/P or VAD. Because small B cells frequently have heterogeneous VDJ Ig rearrangements, this is being evaluated more closely to determine the effects of chemotherapy on the clonotypic subset of small B cells. Extensive DNA aneuploidy comparable to that of BM plasma cells (Pilarski et al, manuscript in preparation) among the small B cells suggests that a large proportion are likely to be within the malignant lineage after chemotherapy.\(^{15,16}\) The number of small B cells returned to the untreated values once therapy was discontinued. The extensive phenotypic abnormality confirms that, if any normal polyclonal B cells remain in myeloma blood,\(^{26-38}\) they have been altered by the disease process.

The CD19\(^+\) subsets described here can plausibly be interpreted to represent sequential stages in the malignant B lineage leading to end-stage plasma cells.\(^{10,16,20}\) The temporal analysis of CD19\(^+\) BMPCs in myeloma suggests that chemotherapy does impinge on the circulating CD19\(^+\) population, although it does not eradicate clonotypic B cells. Even in patients with normal numbers, the CD19\(^+\) BMPCs were phenotypically abnormal, expressing a high density of CD38 and a moderate to high density of CD56. Both of these markers have been used to characterize BM-localized plasma cells,\(^{35,36}\) and their appearance on B cells before the acquisition of plasma cell morphology appears to be a late event in B-cell development.\(^{10,25,35}\) Among the blood B cells, the majority of blood B cells with DNA hyperdiploidy were CD38\(^+\) (Pilarski et al\(^{16}\) and manuscript in preparation).

The lineage relationship between BM plasma cells and the clonotypic B cells detected among the CD19\(^+\) BMPCs is unknown. Because myeloma is likely to originate from malignant transformation within a chronically stimulated antigen responsive B-cell clone(s), perhaps exemplified in monoclonal gammopathies of undetermined significance (MGUS), they could include activated members of the original antigen-responsive clone coexisting with its malignant relatives. Second, they could be migratory progeny of a BM-localized or extramedullary stem cell, consistent with their motile phenotype and repertoire of receptors associated with migratory behavior. Finally, they may include the generative stem cell that perpetuates myeloma. Given the heterogeneity of the CD19\(^+\) BMPCs and the distribution of clonotypic cells among several CD19\(^+\) subsets\(^{26}\) (Bergsagel et al, manuscript in preparation), all of the above interpretations are possible and should not be viewed as mutually exclusive. The results presented here support the notion that the malignant clone in myeloma is heterogeneous, involving multiple differentiation stages with different circulating B-lineage subsets in ascendency at different times and stages of disease. The possibility also exists that similar CD19\(^+\) subsets in patients with MGUS\(^{12}\) may reflect dormant myeloma, which, when released from the mechanisms maintaining dormancy, results in the diagnosis of frank myeloma. Overall, the presence of clonotypic cells among CD19\(^+\) populations in blood, at times when the BM plasma cells have been rendered undetectable by chemotherapy, is consistent with properties expected for a drug-resistant reservoir of malignant disease.\(^{15,26}\) If drug-resistant circulating CD19\(^+\) cells are responsible for perpetuating myeloma despite killing of the BM-localized plasma cells, new modes of therapy may be required to eradicate the malignant clone.

ACKNOWLEDGMENT

This work would not have been possible without the skilled technical assistance of Darlene Paine, Eva Pruski, Dorota Rutkowski, and Kimberly Howland. Joanne Hewitt assisted in collection of clinical information and data entry. Dr Ben Ruether provided some of the patient samples.

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In multiple myeloma, clonotypic B lymphocytes are detectable among CD19+ peripheral blood cells expressing CD38, CD56, and monotypic Ig light chain [published erratum appears in Blood 1995 Jun 1;85(11):3365]

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