Tumor-Specific Aneuploidy Not Detected in CD19+ B-Lymphoid Cells From Myeloma Patients in a Multidimensional Flow Cytometric Analysis

By P.A. McSweeney, D.A. Wells, K.E. Shults, R.A. Nash, W.I. Bensinger, C.D. Buckner, and M.R. Loken

Aneuploidy and Ig light chain restriction were used as separate, independent tumor specific markers to study 26 patients with multiple myeloma to determine whether bone marrow B cells, as defined by CD19 expression, are clonally related to myeloma plasma cells. Specimens were characterized using multidimensional flow cytometry to identify the presence of clonality in both the B lymphoid and plasma cell populations using both surface and cytoplasmic staining with antibodies specific for kappa or lambda Ig light chain. In none of the patients with multiple myeloma were CD19+ cells found to be clonally restricted to kappa or lambda. The monoclonal plasma cell populations (MPC) were found to be uniformly negative for CD10, CD19, and CD34, while the CD19+ B lymphoid cells present within the samples expressed normal intensities and relationships of these antigens, which allowed them to serve as internal positive controls. Combined analysis of cell surface antigen expression and DNA content allowed plasma cell populations to be characterized for aneuploidy without interference from normal bone marrow cells. The MPC, detected on the basis of bright CD38 expression (CD38+), demonstrated DNA aneuploidy in 65% of cases (DNA index range of 0.9 to 1.3). These aneuploid DNA distributions had typical cell cycle profiles (including G1, S, and G2 + M) expected of a proliferating population. In all cases, DNA aneuploidy was confined almost entirely to the CD38+, CD19+ malignant plasma cells, while cells expressing CD19 were diploid. These results support the concept that myeloma is a disease process mediated by self-replicating, late compartments of B-cell ontogeny.

© 1996 by The American Society of Hematology.

MATERIALS AND METHODS

Patient samples. Patient's marrow samples were obtained during diagnostic workup for suspected myeloma at Cytometry Associates, or after referral for bone marrow transplantation with an established diagnosis of multiple myeloma at the Fred Hutchinson Cancer Research Center (FHCRC). In several cases, peripheral blood studies were also performed. Consent was obtained in accordance with the policies of the Internal Review Board at the FHCRC. Patients with MPC were identified by demonstrating clonal light chain restriction by exclusive cytoplasmic staining of plasma cells for either kappa or lambda, and the percentage of malignant cells in the samples was determined on that basis.

Surface and cytoplasmic light chain Ig staining. A mononuclear cell preparation was obtained from heparinized peripheral blood and marrow aspirates using ficoll-hypaque (density = 1.077) separation. Cells were washed twice in phosphate-buffered saline (PBS) with 2% fetal calf serum (FCS) and 0.1% sodium azide to remove the presence of light chain Ig containing plasma, and adjusted to a concentration of 2 × 10⁶/mL. Mononuclear cell isolates for cell surface staining were added to monoclonal antibody cocktails conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin-chlorophyll-a (PerCP) consisting of CD45-PerCP (Beckton-Dickinson, San Jose, CA), an irrelevant control mouse IgG monoclonal antibody (MsIgG-FITC), CD19-PE (Dako, Carpinteria, CA), and anti–kappa-FITC or anti–lambda-FITC (both Dako) and other phenotypic analyses, it is possible to ascertain the presence or absence of aneuploidy in distinct cell populations.

This study was designed to establish the relationship between myeloma plasma cells and the CD19+ B lineage cells present in the bone marrow. Multidimensional flow cytometry (MDF) was used to identify and to phenotype monoclonal plasma cell populations (MPC). Plasma cells, mature B cells, and B lymphoblasts (LB) were examined for light chain restriction, as determined by analysis of both cell surface and intracytoplasmic Ig light chain expression. Additional surface antigens were also assessed to aid in delineation of MPC to allow precise phenotyping and clear distinction from other cells that have previously been suggested to be myeloma precursors by single parameter flow cytometric methods. Finally, cell surface antigen expression was combined with propidium iodide (PI) staining to identify aneuploidy as an independent neoplastic marker and to evaluate cell cycle status of phenotypically defined cell populations.

From the Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle; the University of Washington, Seattle; Hematologics, Seattle, WA; and Cytometry Associates, Brentwood, TN.

Submitted October 5, 1995; accepted March 11, 1996.

Supported in part by Grants No. CA47748, CA18029, and CA18221 from the National Cancer Institute. P.A.M. is a Fellow of the Leukemia Society of America.

Address reprint requests to P.A. McSweeney, MD, Fred Hutchinson Cancer Research Center, 1124 Columbia St, M318, Seattle, WA 98104.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

incubated for 20 minutes at room temperature (RT). For detection of
cyttoplasmic antigens, surface light chains were blocked using
saturating concentrations of pure unconjugated antibodies directed
at the antigen incubated at RT for 30 minutes. Following the blocking
procedure, excess antibody was removed with a single wash in PBS
containing 2% FCS and 0.1% NaN₃. The cells were stained with
CD38-PE at RT for 20 minutes and permeabilized with PermeaFix
(Ortho Diagnostics, Raritan, NJ) using a 40-minute RT incubation.
The cells were then washed twice in PBS followed by incubation
for 20 minutes at RT with FITC-conjugated polyclonal antibodies
directed at kappa or lambda light chains. Light chain restriction
analyses were performed using a FACSCAN (Becton-Dickinson,
San Jose, CA) or a Cytorion Absolute (Ortho, Raritan, NJ). Lysomiole
files of 30,000 events were analyzed using WinListTM software (Ver-
ity Software House, Topsham, ME). Several different projections of
parameters were taken and analyzed simultaneously so that multidimi-
sional, color-related displays were used to observe relationships
between multiple signals. Multidimensional flow cytometry, as
used in this study, correlates multiparameter data for simultaneous
analysis, creating a multidimensional space for identification of
each cell cluster. 16

Correlated cell surface/DNA analysis. Ficoll-hypaque separated
mononuclear cells were stained with saturating concentrations of
anti-CD19-FITC (Gentrak, Plymouth Meeting, PA), anti-CD38-
FITC (Gentrak) and MsGg-FITC (irrelevant isotype-specific irrele-
vant antibody) and incubated at RT for 30 minutes. Cells were
washed twice in PBS followed by dropwise addition of 1 mL of
70% ethanol to the cell pellet. The cells were lightly vortexed during
the addition of ethanol and then were fixed overnight at 4°C. Excess
ethanol was removed by one wash, 500 μL RNAse added and cells
were incubated at RT for 30 minutes. DNA was then stained by
incubating in 500 μL PI (20 μg/mL) for at least 2 hours at 4°C.

The PI-stained cells were analyzed using an EPICS 752 (Coulter
Electronics, Hialeah, FL) with 100 mW at 488 nm. The ploidy and
S-phase of electronically isolated CD19” and CD38” cells were
determined using a DNA modeling system (Modfit, Verity Software
House). The determination of aneuploidy was based on the collection
of three individual histograms (total cells, CD38+’ and CD19”).
Each histogram was gated on peak versus area signals to ensure
single nuclei events by eliminating doublets. A total of 50,000
events were collected for each histogram using a discriminator level
above 5%.

Other phenotyping studies. Mononuclear cell isolates of peripheral
blood and marrow were stained with CD45-PerCP and combina-
tions of the following directly conjugated antibodies with associated
fluorochromes as follows: (1) CD19-FITC, CD38-PE; (2) CD10-
FITC, CD38-PE; (3) CD20-FITC, CD38-PE; (4) CD28-FFTC
(AMAC, Westbrook, ME), CD38-PE; (5) CD38-FFTC, CD34-PE
(BD), and (6) CD38-FFTC, CD56-PE (Gentrak). Dual FITC and PE
control antibodies used were isotype-specific direct conjugates of
irrelevant antibodies.

RESULTS

Patient characteristics. Demographics on 26 patients with
bone marrow aspirates having either DNA aneuploid or diploid
MPC are included in Table 1. Of these patients, 12 had received
prior therapy, while 14 were presenting de novo. Patient ages
ranged from 30 to 82 years old. Peripheral blood studies were
performed in five patients and included assessment of peripheral
blood DNA aneuploidy in two patients.

Discrimination of monoclonal plasma cells from other
leukocytes. Multidimensional analyses can distinguish
MPC from normal B-lymphoid cells and other leukocytes
by combining multiple cell surface antigens with two light
scatter parameters. Since the complete separation of these
two cell populations using five dimensional analysis is diffi-
cult to depict in two dimensional displays, analysis of a
single patient’s data serves to illustrate the procedure (Figs
1, 2, and 3).

Initial screening of the samples involved collecting the
forward (FSC) versus orthogonal side scatter (SSC) pattern
to eliminate excessive debris (Fig 1A). The SSC versus
CD45 pattern was then generated to determine the distinct
cell populations present within the specimen (Figure 1B).17
 Plasma cells were identified separately on the basis of bright
CD38 staining versus SSC (Fig 1C).10 The plasma cells ex-
pressed the highest levels of CD38 (Red, Fig 1C) while the
lymphoblasts (early B lineage cells) expressed 10-fold less
CD38 (Green, Fig 1C). The CD38++ cells exhibited high
levels of either cytoplasmic kappa or lambda light chain
(Fig 1D-F) with associated clonal light chain restriction,
confirming that they were myeloma plasma cells. In contrast,
the lymphoblasts and lymphocytes identified by CD19 and
CD45, or CD19 versus CD38, expressed lower amounts of
cytoplasmic kappa or lambda, as compared with the MPC
(Fig 2A-C), and did not show clonal restriction. An exception
to these findings was patient no. 11 with plasma cell leukae-
emia in whom MPC in the peripheral blood exhibited only a
fivefold, rather than 10-fold, increase in CD38 expression
as compared with the other CD38 positive cells. The propor-
tion of MPC per nucleated cell ranged from 0.3% to 100%
in bone marrow (Table 1) and 0.2% to 70% in peripheral
blood (Table 2). The use of MDF has previously been shown
to have a sensitivity allowing detection of MPC down to a
level of 0.2%.19

Coexpression of other lineage specific and lineage
associated surface markers on the myeloma cells was also
determined. Once the CD38++ population was defined as clonal
by light chain restriction, it was then possible to use the
high intensity of CD38 expression to identify the malignant
plasma cells for subsequent phenotyping. The CD38++ MPC
from all patients studied were negative for surface expression
of CD19 and CD10 (Table 3). These markers clearly distin-
guish the MPC from the residual B lymphoid cells that
express CD19. In nine of 26 bone marrows, MPC showed weak
surface expression of CD20 (Table 3). Myeloma cells from
15 of 17 patients tested were positive for surface CD56
expression, and eight of 13 patients tested were positive for
surface CD28 expression. All patients evaluated in this study
showed MPC expression of one or the other of these two
antigens. CD45 expression was found to be heterogeneous
on MPC, both within a given patient and between different
patients, with a majority of cells usually showing absent
or reduced expression relative to lymphoid cells (Fig 1B).
Therefore, CD45 expression was not used as a marker to
specifically identify myeloma cells.

Antigen expression of marrow B lymphoid cells in my-
eloma. Immunophenotypic analysis of the remainder of
the B-cell compartment using MDF showed that these cells had
phenotypes of normal B lymphoid cells and did not show
clonal light chain restriction (Figs 2 and 3). The B
lymphoblasts exhibited low levels of CD45 expression and
had decreased SSC as compared to mature B and T lympho-
cytes as previously reported (green v blue, Fig 1B).17 There-
cells expressed CD19. The B lymphoblasts demonstrated gating on the discrete cell population or by backgating using have increased expression of the opposite light chain to that lymphoid cells expressed small amounts of cytoplasmic Ig CDlO with increasing expression of CD20 on the maturing B cells (green, Fig 3B and C, respectively). These immature three-color combinations to isolate cell populations of inter-

Mature B lymphoid cells expressed CD20 without CDlO (green, Fig 2B and C, respectively) and included a small threshold for different cell populations.

Both immature B lymphoblasts and mature B lymphoid cells expressed CD19. The B lymphoblasts demonstrated CD10 with increasing expression of CD20 on the maturing B cells (green, Fig 3B and C, respectively). These immature lymphoid cells expressed small amounts of cytoplasmic Ig (green, Fig 2B and C, respectively) and included a small population of cells with nonclonal surface Ig expression. Mature B lymphoid cells expressed CD20 without CD10 and also showed nonclonal cell surface Ig expression (blue, Fig 3B through E). Monoclonal plasma cells (red, Fig 3A through C) showed considerably more autofluorescence than lymphoblasts and lymphocytes illustrating that different baseline autofluorescence generates different negative thresholds for different cell populations.

Neither clonal surface/cytoplasmic light chain restriction, nor significant skewing towards the MPC specific light chain, were detected in B lymphoid cell populations from specimens of bone marrow (Table 4) and peripheral blood (Table 2). In some instances, mature B lymphocytes were found to have increased expression of the opposite light chain to that found on the MPC. In these cases, the kappa:lambda ratios were within normal limits, and these results probably reflect statistical variation. In addition, the proportion of CD19 + B cells in the majority of bone marrow aspirates (2.1% ± 2.4% of total cells) was reduced as compared with a previously established normal reference value (6.3% ± 4.3%).

Eight patients were tested for CD34 expression and in no case was it detected on MPC (Table 3 and Fig 4). Using backgating analysis on the CD45 and the SSC histogram (as for Fig 1C), it was possible to make a clear distinction between brightly positive CD38 myeloma cells and normal marrow progenitors that expressed both CD38 and CD34. Only the CD38 + , CD34 - cells were clonally restricted with intracytoplasmic expression of either kappa or lambda light chains. The CD34 + progenitor cells in the bone marrow specimens had phenotypes consistent with normal hematopoietic development.

DNA and S-phase analysis in specimens with aneuploidy. DNA aneuploidy was used as an additional tumor specific marker to examine MPC and CD19 + B lymphoid cells. The separate populations of cells were identified by cell surface antigen staining with FITC-conjugated antibodies in combination with PI staining to detect DNA aneuploidy. PI staining has a low coefficient of variation of the G1 peak (below 3%), thereby allowing resolution of small changes in DNA content often found in myeloma including changes in the DNA index (DI) of 0.1 or greater. Electronic gating was

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>DI</th>
<th>S-Phase of Myeloma Cells</th>
<th>Malignant Cells %</th>
<th>CD38 + Aneuploid</th>
<th>CD19 + Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>43/M</td>
<td>1.2</td>
<td>4.3</td>
<td>30</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>2*</td>
<td>61/M</td>
<td>1.1</td>
<td>4.2</td>
<td>2</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>3</td>
<td>69/M</td>
<td>1.3</td>
<td>0.0</td>
<td>27</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>4</td>
<td>75/M</td>
<td>1.2</td>
<td>4.6</td>
<td>25</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>5</td>
<td>58/M</td>
<td>1.2</td>
<td>6.3</td>
<td>27</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>6</td>
<td>67/M</td>
<td>1.2</td>
<td>1.2</td>
<td>25</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>7*</td>
<td>82/F</td>
<td>1.1</td>
<td>2.2</td>
<td>9</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>8*</td>
<td>75/M</td>
<td>1.2</td>
<td>0.0</td>
<td>4</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>9</td>
<td>55/M</td>
<td>1.4</td>
<td>5.4</td>
<td>14</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>10</td>
<td>65/M</td>
<td>1.2</td>
<td>8.1</td>
<td>23</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>11*</td>
<td>47/M</td>
<td>0.9</td>
<td>2.3</td>
<td>100</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>12</td>
<td>30/F</td>
<td>0.9</td>
<td>12.0</td>
<td>53</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>13</td>
<td>73/F</td>
<td>1.1</td>
<td>1.5</td>
<td>11</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>14</td>
<td>74/M</td>
<td>1.1</td>
<td>0.0</td>
<td>29</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>15</td>
<td>53/F</td>
<td>1.1</td>
<td>0.0</td>
<td>14</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>16</td>
<td>77/F</td>
<td>1.1</td>
<td>0.0</td>
<td>6</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>17</td>
<td>61/M</td>
<td>1.1</td>
<td>2.1</td>
<td>39</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>18*</td>
<td>77/F</td>
<td>1.0</td>
<td>1.0</td>
<td>38</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>19</td>
<td>64/M</td>
<td>1.0</td>
<td>1.0</td>
<td>19</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>20</td>
<td>52/M</td>
<td>1.0</td>
<td>0.0</td>
<td>27</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>21*</td>
<td>41/M</td>
<td>1.0</td>
<td>0.0</td>
<td>4</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>22*</td>
<td>59/M</td>
<td>1.0</td>
<td>0.5</td>
<td>2</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>23*</td>
<td>49/M</td>
<td>1.0</td>
<td>0.0</td>
<td>4</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>24*</td>
<td>50/M</td>
<td>1.0</td>
<td>0.7</td>
<td>0.3</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>25*</td>
<td>50/M</td>
<td>1.0</td>
<td>1.0</td>
<td>6</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>26*</td>
<td>51/M</td>
<td>1.0</td>
<td>0.0</td>
<td>3</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

Abbreviations: DI, DNA index corrected to nearest 0.1 (diploid = 1.0); Pos, positive; Neg, negative.
* Samples from patients who had received prior therapy.
ANEUPLOIDY NOT DETECTED IN MYELOMA B CELLS

Fig 1. MDF analysis of MPC from patient no. 16. (A) Position of lymphoblasts (green, LB), mature lymphocytes (blue), and MPC (red) by forward light scatter and orthogonal light scatter (side scatter) as determined by backgating from (B) and (C). Note the overlapping light scattering properties of these populations and that the orthogonal light scatter of MPC is greater than LB and lymphocytes. (B) Mature lymphocyte population is gated on the basis of CD45 versus orthogonal light scatter. LB and MPC are displayed based on backgating from (C). MPC are predominantly but not uniformly CD45 low. (C) LB and MPC populations are gated on the basis of CD38 versus orthogonal light scatter. In histograms displaying CD38 staining, the voltage gain of the phycoerthrin channel has been reduced to bring the CD38+ myeloma cells on to scale. (D-F) Comparison of expression of cytoplasmic (cKappa, cLambda) light chain Ig on gated plasma cell population showing clonal restriction to lambda. Note that in (F) the majority of cLambda+ MPC are intensely positive, off scale, and consequently not seen in the figure.

Fig 2. Comparison of cytoplasmic kappa and lambda Ig light chain expression of MPC and LB from patient no. 16. Plasma cells (red) were defined as demonstrated in Fig 1. A reference population stained with a negative control antibody (A) is used to compare reactivity of anti-kappa (B) and anti-lambda (C). The plasma cells react strongly with the anti-lambda reagent whereas only a small proportion of LB (green) react with either anti-kappa or anti-lambda.
Fig 3. A nonspecific reference population (A) was reacted with a control antibody with no reactivity for human cells (patient no. 16). The vertical lines can be used as reference positions for lymphocytes and lymphoblasts (LB, grey) as compared with plasma cells (black). The LB react with CD20 and CD10, while a portion of the lymphocytes react with only CD20, but not CD10. Plasma cells do not demonstrate staining with either CD10 or CD20. Surface light chain staining was identified on both the LB and lymphocytes (D and E) with an equal proportion staining with anti-kappa or anti-lambda antibodies. It should be noted that autofluorescence and/or nonspecific staining of plasma cells (A) is significantly higher than seen with the LB or lymphocyte populations, and MPC staining with other antibodies was evaluated using this specific reference population.

Three separate histograms (50,000 events) were collected for each DNA aneuploid specimen; these included all cells, those gated on CD38++ cells, and those gated on CD19+ cells. As shown in Fig 5, the DNA aneuploid population was completely confined to the CD38++ population, while the CD19+ cells were diploid. In all 17 aneuploid specimens, the CD19+ fraction was always diploid, whereas the CD38++ cells were aneuploid. In separate studies using mixing experiments, the use of dual DNA and surface antigen staining allowed detection of DNA aneuploidy as few as 0.5% of aneuploid cells added to a diploid population (data not shown). Therefore, in each case, there were <0.5% aneuploid cells in the CD19+ population of cells.

Monoclonal plasma cells are cycling. The CD38++ DNA aneuploid and diploid MPC were found to have a normal cell cycle profile with G1, S, and G2/M components indicating the presence of a proliferating CD38++, CD19+ compartment in this disease. This was particularly pronounced in the DNA aneuploid cells, but was also identified in the specimens with diploid MPC. The S-phase fraction of the aneuploid and diploid MPC in bone marrow ranged from 0% to 12.0% and in the peripheral blood specimens ranged from 0.2% to 1.8%. Seventeen of 26 bone marrow aspirate specimens and six of six peripheral blood specimens had an S-phase present in the MPC population that was greater than zero, indicating proliferation of the clone.

**DISCUSSION**

One hypothesis as to the origins of plasma cell populations found in myeloma is that they develop from cells at earlier stages of B-cell ontogeny, including from within the hematopoietic stem cell compartment. An alternative hypothesis is that the tumor is restricted to self-replicating plasma cells without replenishment from earlier B lymphocyte precursors. If tumor proliferation is occurring in early stages of B-cell ontogeny, evidence of disturbed B-cell maturation should be present. In addition, there should be tumor markers detectable in cells at different stages of B-cell differentiation. However, these markers would not be identified in the earlier B lymphoid cells if tumor replication was restricted to the plasma cell compartment.

This study of myeloma patients used MDF and DNA aneuploidy analysis to explore whether B lymphoid cells, as defined by CD19 expression, share genetic markers with the malignant clone. The combined use of surface staining with CD38 and DNA staining with PI has previously been shown to be an accurate method for identifying monoclonal aneuploid plasma cells in myeloma and evaluating their S-phase. Although DNA staining with bromodeoxyuridine (BRDU) and tritiated thymidine has also previously been used to evaluate myeloma proliferation, the PI method has some advantages in that the malignant cells need not comprise a majority of cells since they can be isolated electronically based on surface phenotype for separate DNA analysis. A dual-staining technique was necessary to specifically study minority populations of MPC (in some cases <5%) for DNA content and S-phase that may not be detectable without surface staining of the myeloma. Similarly, DNA content of the small CD19+ cell populations could only be measured because these populations could be specifically identified by surface marker expression.

Monoclonal aneuploid or diploid plasma cells could be identified based on high CD38 expression and monoclonal light chain expression in the cytoplasm, and these cells were uniformly negative for CD19 expression. Normal nonclonal pre B and mature B lymphoid cells served as an internal positive control for the comparison of CD19 and CD38 expression. It has previously been shown that a high percentage (97% ± 2%) of the aneuploid cells segregate with the CD38++ phenotype. While very small populations of aneuploid cells expressing a lower amount of CD38 were detected in some patients, these cells were not detected in the CD19+ population, and other phenotypic characteristics of these cells remain to be determined. The absence both of detect-
Table 2. Analysis of Peripheral Blood MPC and CD19− B Cells From Five Myeloma Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Malignant Cells %</th>
<th>DI</th>
<th>S-Phase of Myeloma Cells</th>
<th>DI</th>
<th>CD38- CD19-</th>
<th>% Total Cells</th>
<th>% Total CD19 Kappa</th>
<th>% Total CD19 Lambda</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.8</td>
<td>1.2</td>
<td>1.0</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>0.9</td>
<td>40</td>
</tr>
<tr>
<td>11*</td>
<td>16</td>
<td>0.9</td>
<td>1.4</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>3.3</td>
<td>17</td>
</tr>
<tr>
<td>11*</td>
<td>70</td>
<td>0.9</td>
<td>1.8</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>0.9</td>
<td>21</td>
</tr>
<tr>
<td>21</td>
<td>0.2</td>
<td>0.9</td>
<td>0.6</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>6.0</td>
<td>16</td>
</tr>
<tr>
<td>22</td>
<td>2.0</td>
<td>1.0</td>
<td>0.2</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>1.1</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Studied twice at intervals of 6 weeks.

able aneuploidy and of light chain restriction or skewing of the CD19+ cells suggests that these CD19+ B lymphoid cells were not genetically identical with the MPC clone. Further, we did not find disturbed or aberrant patterns of expression of normal B-cell maturation markers in the marrow as can be detected in acute lymphoblastic leukemia and non-Hodgkin's lymphoma. In several cases, S-phase analysis showed that significant proliferation was occurring in a CD38+/CD19− cell population. This suggests that self-replication of MPC occurs and could account entirely for tumor growth without the need for myeloma progenitors from earlier in B-cell ontogeny.

With the techniques used in this study, it is possible to detect 0.5% aneuploid B lymphoid cells among normal B cells. Furthermore, since the mean percent of CD19+ B cells in the marrow was 2.3%, then the percent of aneuploid CD19+ B cells that could have been present without detection among the total cell population in the specimens was approximately 0.01% (0.5 × 0.023). This calculation is in keeping with the observation that aneuploid CD19+ cells were not detected in list mode files of at least 50,000 cells. Overall, these findings are consistent with those made by some other investigators who found no evidence of an expanded B-cell compartment in myeloma patients. The presence of rare CD19+ aneuploid cells in the patients studied cannot be absolutely excluded. However, if these cells are present, they represent a very small subpopulation of the malignant clone.

Table 3. Analysis of Markers Expressed by Marrow MPC Populations

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>MPC %</th>
<th>cKappa</th>
<th>cLambda</th>
<th>CD38+ CD19-</th>
<th>HLA-DR</th>
<th>CD38- CD19-</th>
<th>CD38+ CD19-</th>
<th>CD38- CD19-</th>
<th>CD38+ CD19-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>13</td>
<td>11</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>16</td>
<td>6</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>17</td>
<td>39</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>18</td>
<td>38</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>20</td>
<td>27</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>21</td>
<td>4</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>22</td>
<td>2</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>23</td>
<td>4</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>24</td>
<td>0.3</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>25</td>
<td>6</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>26</td>
<td>3</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>NT</td>
<td>Weak</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Abbreviations: NT, not tested; MPC, monoclonal plasma cells.
ANEUPLOIDY NOT DETECTED IN MYELOMA B CELLS

Flow cytometric identification of myeloma cells and their phenotypic characteristics depends on accurately identifying and electronically isolating the MPC clone. A multidiimensional approach is necessary to delineate the relevant cell populations, as no single marker distinguishes plasma cells from all other marrow leukocytes. It was only after confirming clonal light chain restriction of the CD38+ population that intensity of CD38 expression was used to define the plasma cells for subsequent phenotypic analysis. An important additional factor is that the background nonspecific fluorescence is dramatically different between plasma cells and other lymphoid cells as illustrated in Figs 2 and 3. In this example, plasma cells could erroneously be attributed the expression of CD20 and CD10 if they were not delineated as a separate population for analysis, and if different control references backgrounds were not set for the separate populations.

Using an MDF approach to phenotyping, we did not detect expression of CD34, CD10, or CD19 on MPC. Because some heterogeneity of surface markers between tumors from different patients does exist, these markers may be expressed on the MPC in a minor subset of patients. The absence of CD34 expression on MPC is consistent with polymerase chain reaction (PCR) studies that did not detect myeloma IgH rearrangements in highly purified CD34 positive cell populations obtained from bone marrow of myeloma patients. CD34 expression was also not detected on mature peripheral blood B lymphocytes (data not shown) as previously has been reported. The absence of CD10 expression on MPC from 24 patients suggests that CD10+ myeloma is an uncommon phenotype, in contrast to the findings of others. However, the possibility that CD10 is expressed on rare myeloma cells is not excluded by our data. Even if it were demonstrated that rare tumor cells from all myeloma patients express a marker usually expressed earlier in B-cell ontogeny, it would remain another matter to prove that these cells have a role as the so-called myeloma progenitor cells.

We have confirmed that CD56 and CD28 are frequently expressed on myeloma cells and clonal myeloma B-cell populations (including up to 80%) in the peripheral blood. One conceivable explanation for differences between our findings and some previous reports is that phenotypic differences exist between peripheral blood and marrow B cells. In five patients reported here, there was no discordance noted between marrow and peripheral blood MPC or B lymphoid immunophenotype. Furthermore, as marrow samples usually contain an admixture of peripheral blood MPC or B lymphoid immunophenotype. Further, as marrow samples usually contain an admixture of peripheral blood, expanded aneuploid populations of CD19+ cells would have been detected if present. In addition, treatment effects do not explain the variance between other studies and our observations because prior therapy had no effect on our results (14 of 26 patients had received no prior therapy).

A recent approach to identifying myeloma precursor cells has involved PCR studies to detect myeloma IgH rearrangements in cell populations obtained by cell sorting. This has led to reports of myeloma IgH rearrangements being detected in CD10 positive and CD19+ B-cell populations. Caution is required in interpreting these PCR-based studies. Because of the extreme sensitivity of PCR, sorting purities need to be absolute to exclude plasma cells from the sorted population. Plasma cells could inadvertently be sorted into the CD10+ or CD19+ cell populations as a result of the increased autofluorescence of these cells unless specific measures using electronic gating are taken to exclude the MPC. Furthermore, although IgH rearrangement sequences are highly reliable clonal markers of B cells, they are not per se markers of

Table 4. Analysis of Surface Light Chain Expression of B Lymphocytes From Bone Marrow of Myeloma Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>clone LC</th>
<th>% Total Cells</th>
<th>% CD19+</th>
<th>% CD19+ k(Lambda)</th>
<th>% CD19+ K:Lambda</th>
<th>CD19 K:L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kappa</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>Kappa</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>Kappa</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>Lambda</td>
<td>1.3</td>
<td>53</td>
<td>29</td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Lambda</td>
<td>1.2</td>
<td>54</td>
<td>29</td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Kappa</td>
<td>2.0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>Kappa</td>
<td>2.0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>Kappa</td>
<td>2.0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>Kappa</td>
<td>1.5</td>
<td>51</td>
<td>49</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Kappa</td>
<td>3.0</td>
<td>28</td>
<td>40</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Lambda</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>12</td>
<td>Lambda</td>
<td>1.6</td>
<td>57</td>
<td>43</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Kappa</td>
<td>2.3</td>
<td>31</td>
<td>43</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Lambda</td>
<td>0.5</td>
<td>39</td>
<td>30</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Kappa</td>
<td>2.0</td>
<td>24</td>
<td>24</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Lambda</td>
<td>1.4</td>
<td>31</td>
<td>31</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Kappa</td>
<td>6.0</td>
<td>65</td>
<td>35</td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Kappa</td>
<td>4.0</td>
<td>24</td>
<td>24</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Kappa</td>
<td>0.9</td>
<td>24</td>
<td>19</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Lambda</td>
<td>0.5</td>
<td>30</td>
<td>27</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Kappa</td>
<td>0.5</td>
<td>29</td>
<td>17</td>
<td>1.71</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Kappa</td>
<td>0.3</td>
<td>15</td>
<td>18</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Kappa</td>
<td>0.4</td>
<td>21</td>
<td>21</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Kappa</td>
<td>2.4</td>
<td>27</td>
<td>26</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Kappa</td>
<td>2.2</td>
<td>11</td>
<td>5</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Lambda</td>
<td>0.9</td>
<td>19</td>
<td>20</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

Mean 2.3 ± 2.5

Abbreviations: LC, light chain; K:L, kappa:lambda; NT, not tested.
malignancy. None of the PCR-based studies have yet shown that another independent marker of malignancy is also found in the same B cells that have the IgH clonotypic marker. The detection of clonal myeloma IgH rearrangements in CD19+ cells also could suggest that a small clone of premalignant or premyelomatous B cells persists after one cell transforms into the malignant clone (as manifested by the presence of aneuploidy in some cases), and that this population does not actively contribute to myeloma clonal expansion. Definitive evidence as to the genetic and functional relationships between myeloma plasma cells and other B lineage cells, therefore, requires testing of highly purified B-cell populations free of contaminating plasma cells. Only then can the presence or absence of tumor-specific genetic changes associated with malignant transformation be assessed with PCR or equivalent techniques.

Current treatments for myeloma remain unsatisfactory and even with intensified therapy in the setting of bone marrow transplantation, relapses remain a major impediment to the success of both autografting and allografting. New strategies for treating myeloma could involve specific targeting of the malignant phenotype with monoclonal antibodies. Based on our data, the specific targeting of conventional B-cell phenotypes would appear to be insufficient for either treatment of myeloma or purging of stem cell grafts because the CD19+ MPC appear to be a self-replicating population. Directing attention to antigens such as CD56, CD28, and CD38 may prove more effective in this regard. In an autografting setting where autografts are usually contaminated with some level of tumor, CD34 selection of peripheral blood stem cells might be followed by active purging of tumor cells with antibodies against CD28 and/or CD56 to generate a tumor free graft. Targeted radiotherapy, perhaps using monoclonal antibodies against CD38,
could potentially be employed to intensify stem cell transplant regimens without significant increases in transplant toxicity and thereby lead to improved results.

REFERENCES


is the common phenotype of myeloma cells. Ann Hematol 64:132, 1992
Tumor-specific aneuploidy not detected in CD19+ B-lymphoid cells from myeloma patients in a multidimensional flow cytometric analysis

PA McSweeney, DA Wells, KE Shults, RA Nash, WI Bensinger, CD Buckner and MR Loken