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.L. 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 cells (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) of 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.

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incubated for 20 minutes at room temperature (RT). For detection of cytoplasmic 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). Listmode files of 30,000 events were analyzed using WinListTM software (Verity Software House, Topsham, ME). Several different projections of parameters were taken and analyzed simultaneously so that multidimensional, color-related displays were used to observe relationships between multiple signals.16,17 Multidimensional flow cytometry, as used in this study, correlates multiparameter data for simultaneous analysis, creating a multidimensional space for identification of each cell cluster.17

**Correlated cell surface/DNA analysis.** Ficoll-hypaque separated mononuclear cells were stained with saturating concentrations of anti-CD19-FITC (Gentak, Plymouth Meeting, PA), anti-CD38-FITC (Gentak) and MsIgG-FITC (irrelevant isotype-specific irrelevant 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.18 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 combinations 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-FITC (AMAC, Westbrook, ME), CD38-PE; (5) CD38-FTTC, CD34-PE (BD), and (6) CD38-FTTC, CD56-PE (Gentra). 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.10 Since the complete separation of these two cell populations using five dimensional analysis is difficult to depict in two dimensional displays, analysis of a single patient’s data serves to illustrate the procedure (Figs 1A, 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 expressed 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 leukemia 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 proportion 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 distinguish 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 myeloma.** 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 lymphocytes 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 population of cells with nonclonal surface Ig expression. 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 monoclonal plasma cells (red, Fig 3A 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 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

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**Table 1. Demographics, Marrow Plasma Cell Content, and DNA Studies of Bone Marrow From Patients With MPC**

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<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>DI</th>
<th>S-Phase of Myeloma Cells</th>
<th>Malignant Cells %</th>
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<th>CD19+ Aneuploidy</th>
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<td>Neg</td>
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</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.

Control FITC

CD20 FITC

CD10 FITC

Surface Kappa FITC

Surface Lambda FITC

Fig 4.

Forward Light Scatter

CD45 PerCP

CD38 FITC

Side Scatter

Progenitors

IgG-FITC

CD38 FITC

Lymph

Myeloid

MPC

Progenitors
used to exclude doublets. DNA aneuploidy was found in 65% of patient bone marrow samples (17 of 26, Table 1). In peripheral blood specimens, DNA aneuploidy was detected in two of five different patient samples (Table 2). The range of the DI was 0.90 to 1.40 with a mean of 1.1 in bone marrow (diploid = 1.0).

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 in 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 GI, 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

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<th>Patient No.</th>
<th>Malignant Cells %</th>
<th>DI</th>
<th>S-Phase of Myeloma Cells</th>
<th>cKappa</th>
<th>cLambda</th>
<th>CD38+ CD19*</th>
<th>% Total Cells</th>
<th>CD19 Kappa</th>
<th>% Total CD19 Lambda</th>
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* 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 x 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.

Results of molecular studies also have suggested that tumor replication is occurring late in B-cell ontogeny.

Table 3. Analysis of Markers Expressed by Marrow MPC Populations

<table>
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<th>Patient No.</th>
<th>MPC %</th>
<th>cKappa</th>
<th>cLambda</th>
<th>CD38+ CD19*</th>
<th>CD38+ HLA-DR*</th>
<th>CD39+ CD20+</th>
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Abbreviations: NT, not tested; MPC, monoclonal plasma cells.
ANEUPLOIDY NOT DETECTED IN MYELOMA B CELLS

The expression of CD20 and CD10 if they were not delineated
important additional factor is that the background nonspecific
the plasma cells for subsequent phenotypic analysis. An im-
lation that intensity of CD38 expression was used to define
eloma patients did not identify B-cell clones with the tumor-
and electronically isolating the MPC clone.\textsuperscript{10,12} CD34 expression on MPC is consistent with polymerase
chain reaction (PCR) studies that did not detect myeloma
rearrangements in highly purified CD34 positive cell
populations obtained from bone marrow of myeloma pa-
CD34 expression was also not detected on mature
peripheral blood B lymphocytes (data not shown) as pre-
viously has been reported.\textsuperscript{5,6} The absence of CD10 expres-
on MPc from 24 patients suggests that CD10\textsuperscript{+} myeloma
is an uncommon phenotype, in contrast to the findings of others.\textsuperscript{7} 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.\textsuperscript{6,9-11} CD56 and/or CD28 was
most likely derived from plasma cells.\textsuperscript{25-27} A study of Ep-
stein-Barr virus (EBV)-transformed B-cell clones from myel-
amia patients did not identify B-cell clones with the tumor-
specific IgH rearrangement.\textsuperscript{24} Recent studies of myeloma
IgH rearrangements\textsuperscript{26-32} have shown that VDJ sequences have
undergone extensive somatic hypermutation and re-
main stable with disease progression,\textsuperscript{30} suggesting that my-
eloma self-renewal occurs in cells late in B-cell ontogeny.\textsuperscript{13}

Flow cytometric identification of myeloma cells and their
phenotypic characteristics depends on accurately identifying
and electronically isolating the MPC clone.\textsuperscript{10,12} A multidimen-
sional 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\textsuperscript{+} pop-
ulation that intensity of CD38 expression was used to define
the plasma cells for subsequent phenotypic analysis. An im-
portant 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
reference backgrounds were not set for the separate popula-
tions.

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 pa-
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.

In contrast to our observations, some investigators have
reported detection of marrow myeloma B-cell precursors\textsuperscript{1,4}
and clonal myeloma B-cell populations (including up to
80%) in the peripheral blood.\textsuperscript{8,11} One conceivable explanation
for differences between our findings and some previous re-
ports 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 periph-
eral blood MPC or B lymphoid immunophenotype. Further,
as marrow samples usually contain an admixture of periph-
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as marrow samples usually contain an admixture of periph-
eral blood MPC or B lymphoid immunophenotype. Further,
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 premelomatous 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