Peripheral blood stem cells (PBSCs) mobilized with high-dose chemotherapy and hematopoietic growth factors are now widely used to support myeloablative therapy of multiple myeloma and effect complete remissions in up to 50% of patients with apparent extension of event-free and overall survival. Because tumor cells are present not only in bone marrow, but also in virtually all PBSC harvests, it is conceivable that autografted myeloma cells contribute to relapse after autotransplants. In this study, the kinetics of mobilization of normal hematopoietic stem cells were compared with those of myeloma cells present in PBSC harvests of 12 patients after high-dose cyclophosphamide and granulocyte-macrophage colony-stimulating factor administration. CD34+ and CD34+Lin-Thy+ stem cell contents were measured by multiparameter flow cytometry, and myeloma cells were quantitated by immunostaining for the relevant light chain and by a quantitative polymerase chain reaction for the myeloma-specific CDRIII sequence. Results indicated marked heterogeneity in the percentages of mobilized stem cells among different patients (0.1% to 22.2% for CD34+ cells and 0.1% to 7.5% for CD34+Lin-Thy+ cells, respectively). The highest proportions of hematopoietic progenitor cells were observed early during apheresis, with 9 of 12 patients mobilizing adequate amounts of CD34+ cells for 2 autotransplants (≥4 × 10^6/kg) within the first 2 days, whereas peak levels (percent and absolute numbers) of myeloma cells were present on days 5 and 6 (0.5% to 22.0%). During the last days of collection, mobilized tumor cells exhibited more frequently high labeling index values (1% to 10%; median, 4.4%) and an immature phenotype (CD19+). The differential mobilization observed between normal hematopoietic stem cells and myeloma cells can be exploited to reduce tumor cell contamination in PBSC harvests.

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Materials and Methods

Mobilization and procurement of PBSCs. Patients with recently diagnosed (N = 5) or previously treated myeloma (N = 7) received 6 g/m^2 of cyclophosphamide over 12 hours, GM-CSF was initiated at a dose of 250 μg/m^2 subcutaneously within 24 hours after the completion of cyclophosphamide treatment and continued daily until the completion of the PBSC collections. A Quinton double lumen catheter was inserted in the subclavian vein for stem cell apheresis. Apheresis procedures were performed on a Cobe Spectra Blood Cell Processor (Lakewood, CO) and PBSCs were collected once the leukocytes count exceeded 0.5 × 10^9/L (untransfused). Collections continued until at least 6 × 10^6/kg mononuclear cells/kg were obtained (usually within 4 to 6 days) or for a maximum of 14 days. However, there was no set target for CD34+ or for CD34+Lin-Thy+ cells. All patients signed an informed consent form approved by the local Institutional Review Board.

Flow sorting of plasma cells and generation of the CDRIII primer for ASO-PCR. Ficoll-Hypaque–separated BM mononuclear cells (BMMCs) were stained for the CD38 and CD45 antigens (CD45-fluorescein isothiocyanate [FITC]; CD38-phycocerythrin [PE]; Becton Dickinson [BD], Mountain View, CA). The CD38<sup>-</sup>CD45<sup>+</sup> cells were sorted using a FACStar Plus cell sorter (BD) as described previously. DNA extraction, generation of the CDRIII primers, and radiolabeled ASO-PCR reaction were performed as previously described. Dilution curves of myeloma cell DNA between 10% and 0.001% myeloma cell DNA were generated for each patient by diluting myeloma cell DNA into normal peripheral blood lymphocytes DNA, as previously described. Autoradiograms were scanned densitometrically and quantitation of myeloma cells in

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PBSC collections was performed by log linear regression as previously described.21–24

Quantitation of CD34+ Lin Thy' stem cells. Stem cells were detected by staining samples from apheresed blood (106 cells) for CD34 using anti CD34-PE (HPCA-2; Becton and for Thy (CD,90) using biotin-labeled GM 201 (Sysmexix, Palo Alto, CA), followed by RED 670-streptavidin (Systemix). Lin' cells were stained with FITC-conjugated antibodies to CD14, CD15, CD2, CD16, CD19, and antiglycoporphrin (all from BD). ISotypic controls corresponding to the antibody isotypes and the proper fluorochrome were used to determine the background staining. IgG1-biotin and avidin-RED 670 were used as a control for the Thy antibody. Propidium iodide was added to all tubes to exclude dead cells. Tubes were run on a FACScan flow cytometer (BD). Six parameters (FS, SS, PI, FITC, PE, and Red 670) were examined in listmode using the ListView software (Phoenix Flow System, San Diego, CA). The cells were sequentially gated from viable nucleated cells to the lymphoblastoid region and then to each of the two color combinations, ie. CD34 versus Lin and CD34 versus Thy. Each fluorochrome was color gated. Fifty thousand cells were analyzed.

Staining for myeloma cells. Staining of myeloma cells was performed on cytospin (50,000 cells from apheresis samples) after ethanol fixation (30 minutes at 4°C). Slides were stained for both 

Staining for immature myeloma cells and determination of plasma cell labeling index (PCLI). Several methods for determination of PCLI were tested using different anti BrdU antibodies and different cell denaturation protocols as described previously.22–24 The best results were obtained using the procedure described by Lokhorst et al.22 Briefly, samples from apheresed blood (106 cells) were allowed to incorporate bromodeoxyuridine (10 μmol/L BrdU for 1 hour at 37°C). Subsequently, aliquots of 50,000 cells were cytospun, fixed with ethanol, air-dried, and treated with 2N HCl (30 minutes at room temperature) to denature DNA. After neutralization in phosphate-buffered saline (PBS), slides were stained for κ and Λ light chains as described above and for BrdU with anti-BrdU antibodies (mouse monoclonal; Dako), followed by antiamouse-FITC (Jackson Immunoreagents, West Grove, PA). Double-stained cells were counted as cycling plasma cells. Cycling plasma cells were identified by positive staining for the relevant light chain and by their lymphoid/plasmacytoid morphology. Positive staining for the irrelevant light chain was always less than 10% of the relevant light chain. At least 500 cells were scored.

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Immature plasma cells were detected by cytoplasmic staining for κ/Λ light chain (rhodamine-conjugated antibodies), as described above, and for CD19 using FITC-anti-CD19 antibody (mouse, monoclonal; BD). Most CD19 cells were small lymphocytes, with predominant membrane staining for CD19. In 2 patients, CD19 cells had the appearance of plasmacytoid cells with weak staining for cytoplasmic CD19 antigen and positive staining for the relevant light chain. Positive staining of CD19+ cells with the irrelevant light chain was always less than 10% of the relevant light chain. At least 500 plasma cells were scored. Mouse FITC-IgG1 was used as isotypic control for background staining for the CD19 antibody.

RESULTS

The time course of stem cell mobilization for 4 representative patients is depicted in Fig 1. Maximum proportions of CD34+ and CD34+Lin Thy’ were observed on days 1 to 3, ranging from 1.8% to 22.2% and 0.48% to 8.2%, respectively. Figure 2 portrays the cumulative amount of CD34+ stem cells in the 12 MM patients studied. Sufficient quantities of CD34+ cells per kilogram for two autotransplants (>4 × 106) were obtained after two collections in 9 of the 12 patients. Eight patients mobilized greater than 10 × 106 kg of CD34+ cells within the first 3 days of collection. Three patients did not mobilize well.

The proportions of myeloma cells in PBSC harvests of 4 patients, including the percentages of light chain restricted B cells (PLC), their labeling index (PCLI), and the proportion of immature myeloma cells (CD19+) are depicted in Fig 3. In all 4 patients, mobilization of myeloma cells peaked on days 5 and 6, with PCLI values ranging between 3.1% and 7.5% on these days. Maximum concentrations of light chain-restricted CD19+ cells (presumed to represent plasmacytoid myeloma cells) usually coincided with peak values of PCLI. The cumulative amount of myeloma cells in the 12 myeloma patients is depicted in Fig 4. Ten patients mobilized greater than 5 × 107/kg and 6 greater than 1 × 107/kg myeloma cells during the 6 days of apheresis. One patient did not mobilize significant amounts of myeloma cells. In the other 11 patients, greater than 75% of myeloma cells were collected during the last 2 days of apheresis.

Figure 5 summarizes the proportions of CD34+, CD34+Lin Thy’, and myeloma cells during the consecutive days of PBSC apheresis. Median percentages of maximum CD34+ and CD34+Lin Thy’ cells were 5.5% (range, 0.2% to 22.2%) and 2.7% (range, 0.1% to 8.2%), respectively. Both cell populations were largest on days 1 to 3, with a subsequent significant decline reaching a minimum by day 6 (P < .001). In contrast, only 2 of 12 patients had ≥2.5% myeloma cells by immunochemistry in their PBSC collections on days 1 and 2, compared with 11 of 12 patients by day 6. A median of 4.4% myeloma cells (range, 1.6% to 10.9%) were cycling, most frequently on days 4 to 6 of PBSC collection. Similarly, CD19+ light chain-restricted cells peaked on day 6, with a median of 5.1% (range, 0% to 16%; results not shown). The least overlap between hematopoietic progenitor and myeloma cell mobilization was observed on days 1 and 2.

PCR-based quantitation of minimal residual disease is more sensitive (2 to 3 logs) than immunofluorescence-based quantitation of tumor cells. Therefore, quantitation of myeloma cells in PBSC collections was also performed by PCR amplification of the clone-specific CDR III sequence. Representative results from 6 patients (B.D., H.R., B.L., L.J., M.G., and D.P.) are shown in Fig 6. The dilution curves (left panels) indicate a sensitivity of detection of 1 myeloma cell in 10,000 to 100,000 nonmyeloma cells. A daily increase in the number of myeloma cells in PBSC collections (right panels) was seen. Densitometric scanning of the autoradiograms and quantitation by log-linear regression in 3 patients (E.D., H.R., and B.L.) showed a low content of myeloma
cells during the first days of apheresis (<0.01%, 1.2%, and 0.1%, respectively), with subsequent daily increases of up to one log, reaching 6.5%, 16.9%, and 22%, respectively, on day 6. Two patients (L.J. and D.P.) had already high levels of myeloma cells on day 1 (4.4% and 9.6%, respectively), with no obvious change during subsequent days of apheresis. One further patient (M.G.) had a low baseline level of myeloma cells (0.15%), with no further increase during subsequent days of apheresis. A significant correlation was observed between the levels of myeloma cell measured by the PCR method and by immunofluorescence with R values of 0.93 to 0.97.

**DISCUSSION**

Mobilization of PBSCs with high-dose chemotherapy alone or in combination with hematopoietic growth factors results in a 10- to 100-fold increase in the number of circulating hematopoietic progenitor cells and effects prompt, complete and durable engraftment after myeloablative treatment. Probably not unexpectedly, this approach also leads to mobilization of tumor cells, including myeloma cells.

Myeloma cells are present in virtually all mobilized PBSC harvests with a frequency of 0.01% to greater than 10%. Depletion of myeloma cells can be achieved by positive selection for CD34+ cells or, more completely, by selection for CD34+Thy-Lin- stem cells. This is the first detailed study assessing, simultaneously, the kinetics of mobilization of both hematopoietic progenitor cells and myeloma cells, including their proliferative capacity and the immature myeloma cell phenotype. The highest proportions of progenitor cells (CD34+ and CD34+Thy-Lin-) were observed during the first 2 days of apheresis, when the total white blood cell (WBC) count was still low (0.5 to 2 × 10^9/L). More than 4 × 10^6/kg CD34+ cells, sufficient for two autotransplants, were collected during the first 2 days in 9 of the 12 patients. Two of the three patients...
who did not mobilize adequate amounts of CD34+ cells had prolonged prior treatment with alkylating agents (>12 months), resulting in permanent stem cell damage, as reported before.7 The proportions of CD34+ cells in these 3 patients were comparable to those observed in peripheral blood of unmobilized MM patients (<1%).

In contrast to stem cells, the highest concentrations of myeloma cells (>75% of total tumor cells) were collected during days 5 and 6 of apheresis, when the percentage and absolute number of progenitor cells were already rapidly declining. Hence, an ideal window for PBSC collection with high progenitor cell content and minimal myeloma cell contamination exists during the first few days after WBC and platelet counts start to recover.

Moreover, during the last days of apheresis, a significant proportion of myeloma cells was cycling and exhibited a more immature phenotype (CD19+). Both immunocytochemical and PCR-based methodologies detected comparable amounts of myeloma cells in the daily collections (R values of 0.93 to 0.97). The more sensitive PCR-based assay showed a daily increase in concentration of myeloma cells by 1 log in several patients.

Whether mobilization of myeloma cells is the result of cyclophosphamide treatment or the administration of hematopoietic growth factor remains unknown. We are currently conducting a study comparing the yield of CD34+ cells and degree of myeloma cell contamination in MM patients mobilized with either G-CSF or G-CSF plus cyclophosphamide. A direct comparison between G-CSF and cyclophosphamide would have been preferable, but cyclophosphamide as a sole mobilizing agent results in lower quantities of CD34+ cells per kilogram, CD34+CD38- cells per kilogram, and CFU-GM per kilogram when compared with G-CSF alone.29 The fact that most of the multilineage hematopoietic growth factors are capable of mobilizing hematopoietic stem cells as well as tumor cells suggests a greater sensitivity of hematopoietic progenitor cells and myeloma cells to cytokines compared with myeloma cells.

The molecular basis of homing of hematopoietic progenitor cells and tumor cells to the BM and their release into the peripheral blood is not well understood.35 Sustained high...
levels of circulating growth factors may downregulate the expression of adhesion molecules such as VLA-4, ICAM-1, and ELAM-1 on the surface of stem cells, endothelial cells, and myeloma cells. In support of this hypothesis is the recent observation of high levels of circulating hematopoietic growth factors in umbilical cord blood\textsuperscript{38} and their effect on the expression of the adhesion molecules, ELAM-1 and ICAM-1.\textsuperscript{37} Umbilical cord blood induces rapid hematopoietic engraftment after myeloablative treatment. Interestingly, CD34\textsuperscript{+} cells express increased levels of L-selectin after PBSC mobilization strategies, which correlated with the speed of hematopoietic engraftment.\textsuperscript{38} While depletion methods for myeloma cells from PBSCs are currently being evaluated,\textsuperscript{13,29,39} we recommend that, in the absence of such methods, large volume exchanges early after leukocyte and platelet recovery are performed.\textsuperscript{40,41} Our conclusions are supported by the absence of breast cancer cells, as measured by sensitive immunocytochemistry and clonogenic assays, in single day large volume PBSC collections.\textsuperscript{42}

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Differential mobilization of myeloma cells and normal hematopoietic stem cells in multiple myeloma after treatment with cyclophosphamide and granulocyte-macrophage colony-stimulating factor

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