High-dose therapy with autologous marrow or peripheral blood stem cell (PBSC) rescue has been extensively applied in the treatment of multiple myeloma (MM) patients during the past 10 years resulting in improved event-free and overall survival when compared with standard chemotherapy. However, relapses are common and cure is unlikely in the majority of patients. Because both bone marrow and PBSCs are contaminated with myeloma cells it is conceivable that relapse after autotransplantation originates at least in part from autografted tumor cells. In this study, mobilized PBSCs were examined for the presence of myeloma cells based on immunophenotyping and sensitive polymerase chain reaction (PCR)-based techniques. In addition, CD34+ Lin- Thy+ stem cells were purified from mobilized PBSC harvests of 10 MM patients by sequentially using counterflow elutriation centrifugation, treatment with phenytoin, and flow sorting, using 5-parameter gating (propidium iodide, forward scatter, side scatter, CD34+ v Lin- and CD34- v Thy+). Virtually all mobilized unsorted PBSC preparations contained myeloma cells in sufficient quantities (range, <0.1% to >10%) potentially causing a disease relapse. Stem cell purification led to an overall enrichment by about 50-fold in all 10 patients: ~90% of the final cell population expressed CD34+ Lin- Thy+ with no evidence of myeloma cell contamination based on flow cytometric analysis of CD38+MM cells (<0.1%). Quantitative PCR amplification of patient-specific complementarity determining region III (CDRIII) DNA sequences showed depletion of clonal B cells by 2.7 to 7.3 logs, with the highest log reduction noted in the samples initially containing the most tumor cells. Our results show that purification of CD34+ Lin- Thy+ cells depletes myeloma cells to undetectable levels from up to 10% present in unsorted PBSCs, thus offering a tool to investigate whether MM relapse after autotransplantation can be reduced markedly.

**A**utoologous transplantation is now frequently used in the treatment of patients with multiple myeloma (MM). A major advance in autologous transplantation was the observation that peripheral blood stem cells (PBSCs), mobilized with robust doses of high-dose cyclophosphamide and/or hematopoietic growth factor, markedly shortened the duration of bone marrow (BM) aplasia after myeloablative treatment. Since 1990, transplant protocols with PBSC support have been used for MM patients at our institution and elsewhere. The results proved encouraging, with a 40% to 50% complete remission (CR) rate in newly diagnosed MM and a 10% to 20% CR rate in refractory disease. A French prospective randomized study, including 200 newly diagnosed MM patients, showed a significantly better CR rate and event-free and overall survival with autotransplants when compared with standard chemotherapy. However, relapse remains a problem especially in high-risk patients.

Although the morphologically recognizable MM cells are mainly restricted to the BM, clonal tumor cells have been detected in the peripheral blood based on DNA aneuploidy, light-chain restriction, Ig gene rearrangement and polymerase chain reaction (PCR) for the hypervariable region of the heavy chain. Immunochemistry of these cells showed weak expression of CD19 and CD20, moderate positivity of CD10, PCA-1 and CD56, and strong reactivity with antibodies to CD45RO and CD38. A proportion of the circulating MM cells expresses the MDR gene. Therefore, PBSC infusions may contribute to relapse after transplantation. Supporting evidence for the role of the autograft as a potential source of relapse comes from reports in lymphoma, neuroblastoma, and breast cancer showing that the rate of relapse correlates with the presence of occult tumor cells in the marrow or in the peripheral blood. In these studies, gene marking of the autologous marrow cells, sensitive immunocytochemical or PCR-based methods, were applied.

Contaminating tumor cells can be depleted by purging the malignant cells, or by positive selection of hematopoietic stem cells. Human hematopoietic stem cells express the CD34 surface antigen and are lineage negative (Lin-). The more primitive hematopoietic stem cells in normal peripheral blood and BM are CD34+ Lin- Thy-. These cells are capable of multilineage differentiation with extensive self-renewal capacity.

To detect minimal contamination with myeloma cells, a PCR assay was developed based on the patient-specific DNA sequence corresponding to the CDRIII region of the rearranged Ig heavy chain, thus providing a marker for detection of clonal B cells. Consensus oligonucleotide primers can be used to PCR-amplify the CDRIII region of the rearranged heavy chain and to detect the presence of small amounts of clonal B cells (1 clonal B-cell in a background of 10^6 normal cells).

Herein, we describe the purification of CD34+ Lin- Thy+ stem cells from PBSC harvests of 10 MM patients, after treatment with high-dose cyclophosphamide and granulocyte-macrophage colony-stimulating factor (GM-CSF). Although present in large quantities in virtually all unsorted PBSC, using flow cytometry and quantitative PCR, myeloma cells were undetectable in purified CD34+ Lin- Thy+ stem cells.

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MATERIALS AND METHODS

**Mobilization and procurement of peripheral blood stem cells.** Patients with newly diagnosed or refractory myeloma received 6 g/m^2^ cyclophosphamide, divided in doses of 1.2 g/m^2^ at 3-hour intervals. GM-CSF was initiated at a dose of 250 µg/m^2^ subcutaneously within 24 hours and continued daily until the completion of the PBSC collection. A Quinlon double-lumen catheter was inserted in the subclavian vein for stem cell apheresis. PBSCs were collected once the leukocytes exceeded 0.5 x 10^9/L, and platelets 50 x 10^9/L (untransfused) and collections were continued daily until a minimum of 6 x 10^7/kg mononuclear cells was obtained.

**Flow sorting of plasma cells.** Ficoll-Hypaque-separated BM mononuclear cells were stained for the CD38 and CD45 antigens (CD45-fluorescence isothiocyanate (FITC); CD38-phycerythrin (PE), Becton Dickinson [BD], Mountain View, CA). The CD38^+^CD45^-^ CD45^+^ CD56^+^ CD56^-^ and CD45^-^ mononuclear cells were stained for the CD38 and CD45 antigens (CD38-phycoerythrin (PE); CD38-fluorescence isothiocyanate (FITC); CD38-phycoerythrin). The sorted cells were then used for DNA extraction to generate the CDRIII probe.

**Depletion of Lin^-^ cells from PBSC.** PBSC harvests were shipped overnight to SyStemix (Palo Alto, CA) for further processing, consisting of counterflow elutriation centrifugation, followed by treatment with L-phenylalanine methylster (PME; GMP grade, Terumo, Elkton, MD). Red blood cells were lysed with isotonic ammonium chloride and the remaining cells stained for Lin^-^ cells with FITC-labeled CD14 (monocytes) and CD15 (granulocytes and myeloid cells). Anti-CD34 antibody (Tuk 3) was obtained from Dr A. Ziegler (University of Berlin, Berlin, Germany) and anti-Thy antibody (GM 201) was obtained from Dr W. Rettig (Ludwig Cancer Research Institute, New York, NY). Stem cells were detected by staining for CD34-sulfurhodamine (SR; SyStemix) and for Thy (CD,90) (followed by Thy-biotin-streptavidin and finally by biotin-PE, SyStemix). Isotypic controls were used to determine the background staining of red versus green fluorescence (CD34^-^Thy^-^ stem cells versus Lin^-^ cells). Propidium iodide (PI) was not used for stem cell sorting. Cell sorting was performed by using a fluorescence-activated cell sorter (FACS; model Vantage, BD).

![Fig 1. Phenotyping of plasma cells in the mobilized PBSC products. (A) patient with non-Hodgkin's lymphoma. (B) Myeloma patient.](image-url)
Fig 2. Analysis of myeloma (CD38<sup>neg</sup>CD56<sup>neg</sup>) and hematopoietic stem cell (CD34<sup>-</sup>Lin<sup>-</sup>Thy<sup>-</sup>) phenotype by multiparameter flow cytometry. (A) CD56PE (green) versus CD38-FITC (blue). (B) Thy APC (yellow) versus CD38-FITC. (C) CD34 SR (red) versus CD38-FITC. (D) Thy-APC versus CD56 PE. (E) CD34-SR versus CD66-PE. (F) CD34-SR versus Thy-APC. CD34<sup>-</sup> Lin<sup>-</sup>Thy<sup>-</sup> cells are represented by magenta. The sorted CD34<sup>-</sup> Lin<sup>-</sup>Thy<sup>-</sup> cells were restained with isotype controls (IgG1-FITC and IgM-FITC), or with CD38-FITC or CD56-FITC. (G) Lin<sup>-</sup>FITC versus CD34-SR dot plot before sorting. (H) Lin<sup>-</sup>FITC versus CD34-SR dot plot after sorting. (I) Thy-PE versus CD34-SR dot plot showing the reanalysis of sorted CD34<sup>-</sup> Lin<sup>-</sup>Thy<sup>-</sup> stem cells. (J) Sorted cells restained with FITC isotype controls. (K) Sorted cells restained with CD56-FITC. (L) Sorted cells restained with CD38-FITC. CD34<sup>-</sup> Lin<sup>-</sup>Thy<sup>-</sup> stem cells express CD38 weakly; the level of CD38<sup>neg</sup> cells (as detected in [C]) is < 0.1%.
Fig 3. Purification of CD34+ Lin- Thy+ cells by counterflow elutriation centrifugation, treatment with PME, and flow-sorting. (A through C) Apheresis cells. (D through F) After elutriation. (G through I) After PME-treatment. (J through L) Reanalysis after sorting for CD34+ Lin- Thy+ stem cells. Panels A, D, G, and J are light scatter dot plots in the various fractions. Panels B, E, H, and K are two-color immunofluorescence dot plots and show the progressive increase in CD34+ Lin- cells. Panels C, F, I, and L are also two-color dot plots and show the relative enrichment for CD34+ Lin- Thy+ stem cells during the purification process.
DNA (0.5 μg) from purified plasma cells and 16 pmoles of each primer (JH and Vg-FR3) in a cocktail containing 2.5 U of Taq polymerase, 200 μmole/L of each deoxynucleotide triphosphate (dNTP), 20 mmole/L TRIS buffer (pH 8.3), 50 mmole/L KCl and 1.2 mmole/L MgCl2. Two consecutive amplification protocols were used: 5 minute denaturation at 94°C followed by 5 cycles of 30 seconds at 94°C, 30 seconds at 45°C, and 1 minute at 74°C. This protocol was followed by 30 cycles of 30 seconds at 94°C, 1 minute at 53°C, and 2 minutes at 74°C. One fifth of the reaction mixture (10 μL) was run on 6% polyacrylamide gels (BioRad minigels; BioRad, Hercules, CA). Bands corresponding to the CDRIII fragments were identified by staining of gels with ethidium bromide and bands were eluted from the gel by the crush-and-soak method. Purified DNA fragments were used for sequencing.

DNA sequencing and synthesis of the allele-specific (ASO) primer. DNA sequencing was performed by the dideoxy termination method (Applied Biosystems, Foster City, CA), using cycle sequencing with fluorescently labeled JH primer and the allele-specific CDRIII fragment (Applied Biosystems labeling kit). After sequencing, the ASO primer was designed by deducing the CDRIII sequence using primer oligonucleotide (15 to 19 bp) was performed by automated sequencing system (model 373A; Applied Biosystems). The actual percentage of the product was done by scanning of developed x-ray films with the Perkin Elmer Cetus DNA-PCR kit. The reaction mixture containing 30 pmoles of the primer and 30 to 60 pmoles of the ASO primer was added to tubes containing the various genomic DNAs and a cocktail containing 200 μmole/L of each dTNP, 20 mmole/L TRIS buffer (pH 8.3), 50 mmole/L KCl, 1.2 mmole/L MgCl2, 2.5 U of Taq polymerase and 5 to 10 μCi of l3P-deoxycytidine triphosphate (3P-dCTP; NEN, Boston, MA). After a denaturation step (5 minutes at 95°C), amplification was performed by 30 cycles of 30 seconds; at 95°C, 30 seconds; at 56°C, 45 seconds; at 75°C, followed by 10 minutes; at 75°C, for extension of the PCR products. The labeled products were then separated on a polyacrylamide gel as before. Gels were dried and exposed for 1 to 3 hours to x-ray film (Fuji RX GCU film; Fuji Photo film, Tokyo, Japan) in a Fisher Biotech cassette with L plus intensifying screens (Fisher Biotech, Pittsburgh, PA). Quantitation was done by scanning of developed x-ray films using a computing densitometer model 300A equipped with image quantitation program (Molecular Dynamics, Sunnyvale, CA). The actual percentage of contaminating myeloma cells in patients samples was extrapolated from a dilution curve made separately for each patient. The correlation coefficients for the log/linear regression (linear density versus log dilution) for the 10 patients studied ranged between r = .87 and r = .99. Individual extrapolations were calculated between adjacent dilution points. The specificity of the ASO primers was confirmed by crossing primers of different patients. Amplification of the CDRIII band was observed only when a proper primer used for the corresponding patient. Furthermore, in three cases, the band amplified in the ASO-PCR reaction was sequenced and was found to contain identical sequence to the original CDRIII fragment.

RESULTS

Plasma cells (CD38high/CD56low) in PBSC harvests of MM patients do not overlap with hematopoietic stem cells (CD34+ Lin-Thy1). Figure IA shows the relative paucity of CD38high/CD56low (0.9% of the lymphoblastoid gated region) and CD38high/CD56low (0.5%) in the apheresis sample from a patient with non-Hodgkin’s lymphoma, whereas in a patient with MM high levels of both CD38high/CD56low (5.8%) and CD38high/CD56low (10.1%) cells were present. Mobilized PBSC of 5 additional MM patients showed percentages of CD38high/CD56low cells ranging from 1.0 to 6.3% (average, 4.8%) and of CD38high/CD56low cells from 0.7% to 10.1% (average, 3.6%). Calculated for the total number of cells (3 x 10^6 to 9 x 10^6), 2 x 10^5 to 8 x 10^5 CD38high cells were present in a single PBSC collection. At least 45% of these CD38high cells expressed the same heavy and light chain as the BM plasma cells.

The phenotypic relationship between CD34+ Lin-Thy1 stem cells and CD38high/CD56low myeloma cells was examined by 7-parameter flow cytometric analysis (Fig 2, A through F). Color gating was used to distinguish each cell population in the multiple analyses. Thy1 and CD34+ cells express the CD38 antigen only weakly (Fig 2, B and C) and are negative for the CD56 antigen (Fig 2, D and E). CD34+
Lin− Thy+ cells can be completely separated from CD38bright CD56− cells (Fig 2F).

Flow-sorted CD34+ Lin− Thy+ cells were restained with CD38 FITC or CD56 FITC (Fig 2, G through L). The percentages of CD38bright CD56− or CD38bright CD56− cells calculated from the original PBSC harvest were (0.1%), similar to the background level of staining obtained with isotypic controls (<0.1%).

**Purification of CD34+ Lin− Thy+ stem cells by flow sorting**. Samples from each purification step were analyzed for the presence of CD34+ Lin− Thy+ cells to evaluate the relative purity of the stem cell preparations. A representative analysis of one patient is shown in Fig 3. After apheresis, elutriation, PME treatment and flow sorting, the total cell numbers were 2.2 × 10^6, 6.8 × 10^6, 4.2 × 10^6, and 1.1 × 10^6, respectively; the percentages of CD34+ Lin− cells were 3.4, 8.2, 20, and 94; and the percentages of CD34+ Lin− Thy+ cells were 0.9, 2.7, 9.3, and 88, respectively. Cell viability was checked after each of the above outlined purification steps and was greater than 95%.

The average recovery of stem cells from all of the enrichment steps up to the flow-sort was 65%. The average recovery from the flow-sort was 60%, hence the average overall recovery was about 40%. This overall recovery is comparable with the recovery obtained by other methods such as the immunomagnetic beads and the avidin columns. Factors that did affect the recovery of stem cells were the degree of contamination with red blood cells and extent of prior therapy (results not shown). However, no special effort was done in these studies to improve the yield of stem cells. Careful selection of patients and apheresis collections should result in an improved recovery of stem cells for clinical trials. However, it is important to note that the shipment of the PBSC harvests per se, did not result in loss of stem cells, and only modest decrease in granulocytes was observed (10% to 15%, results not shown). From the flow analysis, it is clear that the CD34+ Lin− Thy+ stem cell population was free of myeloma cells to the sensitivity of 0.1% (the background staining of the isotypic controls). To test for lower levels of contamination, we used the PCR-based amplification of the CDRIII region. This method results in a sensitivity of 0.001% myeloma cells (see below).

**Amplification of the CDRIII region by PCR with consensus primers**. Amplification by PCR of the CDRIII region of the Ig heavy chain was achieved using JH and FR3 consensus primers that resulted in DNA fragments of 70 to 90 bp. Representative examples of the CDRIII bands derived from genomic DNA of sorted plasma cells from seven patients are depicted in Fig 4. DNA from one patient (lane 1; R.J.) was extracted from a nonsorted population of plasma cells (<10%) and two bands were amplified. In this case, the lower band gave the proper sequence for the ASO primer. Lanes 2 through 7 depict the CDRIII bands obtained from patients H.A., T.D., H.R., H.O., H.D., and L.T., respectively, with each yielding a single amplified CDRIII band. DNA from one patient (Y.M.) did not show any CDRIII band using the above primer system, but the CDRIII band could be amplified when the seven VH family consensus primers were used resulting in a band of 289 bp (results not shown). Two patients (F.D., M.R.) had no detectable plasma cells in their marrow aspirates before treatment with cyclophosphamide and, therefore, plasma cells were sorted from the PBSC collection (CD38bright CD56− cells). Amplification of genomic DNA from these patients resulted in a single CDRIII band (results not shown).

**Quantitation of residual myeloma cells in flow sorted CD34+ Lin− Thy+ stem cells with ASO-PCR**. Figure 5 depicts composite photographs of four autoradiograms of ASO-
PCR products obtained from genomic DNAs of unsorted (Fig 5A) and flow-sorted CD34+ Lin− Thy+ PBSC (Fig 5B). The dilution curve for each patient is displayed. The level of contamination in all purified stem cell samples was below 1 clonal B cell in 10^5 sorted cells. Log linear regression was performed on the values obtained from the four last dilutions of each patient’s DNA sample (1% to 0.001% plasma cells). The calculated log depletion of clonal B cells varied from more than 2.7 to greater than 6.5 (Table 1).

In 6 patients, the presence of residual clonal B cells was determined in both CD34+ Lin− Thy− and CD34+ Lin− Thy+ cells. As shown in Fig 6, the level of contamination was less than 1 clonal B cell in both 10^5 CD34+ Lin− Thy+ (B) and CD34+ Lin− Thy− cells (C).

The calculated number of residual clonal B cells in the CD34+ Lin− Thy+ cells of the 10 patients analyzed was between less than 1 and less than 312 cells in a PBSC collection of 6 × 10^6 to 9 × 10^6 total cells (Table 1). Recoveries of both CD34+ Lin− Thy+ and CD34+ Lin− Thy− cells were between 3.5 × 10^5 and 3.1 × 10^6 cells, with an input between 8.4 × 10^5 and 89.7 × 10^5 apheresed PBSC.

**DISCUSSION**

This study shows the feasibility of purification of an early hematopoietic progenitor cell population (CD34+ Lin− Thy−) from mobilized PBSC harvests of MM patients as a means to markedly deplete clonal B cells.

Mobilized PBSC of MM patients contained between 0.1% to 10% myeloma cells expressing CD38nght CD56−, previously shown to be present on circulating MM cells. No overlap was observed on flow cytometry between MM cells (CD38nght CD56−) and stem cells (CD34+ Lin− Thy−). In contrast with MM cells, flow-sorted CD34+ Lin− Thy+ cells stain only weakly for the CD38 antigen and are negative for CD56.

Conventional flow-sorting is a slow and labor-intensive procedure. Therefore, it was essential to remove accessory cells to reduce sorting time and increase the purity of the final preparations. Debunking was accomplished by counterflow elutriation centrifugation and treatment with PME, resulting in a depletion greater than 99% of Lin+ cells within 3 hours. CD34+ Lin− Thy− cells were enriched from 0.9% in the initial PBSC collection to 88% post flow-sorting. These cells appeared fully viable and gave rise to all hematopoietic lineages in various in vivo and in vitro stem cell assays.

PCR amplification of the CDRIII sequence has been applied to quantitate minimal residual disease in various B-cell malignancies, including MM. We used this technique to quantitate residual clonal B cells in the final stem cell preparation. The consensus (JH, FR3) primer amplification reaction, performed with DNA from highly purified (>90%) flow-sorted plasma cells (CD38nght CD45), was successful in over 80% of cases attempted. Flow-sorted CD38nght CD56− myeloma cells from PBSC collections containing greater than 45% light-chain restricted B cells, were also successfully used to amplify the CDRIII band.

Residual clonal B cells in the final stem cell preparations were quantitated using flow cytometry and ASO-PCR. The level of detection of minimal residual disease with flow cytometry is limited (0.1%). By flow cytometry, all PBSC collections were negative for myeloma cells; however, 6 of 10 collections contained significant amount of myeloma cells by the ASO-PCR technique. In all 10 cases, a log depletion of clonal B cells by greater than 2.7 was obtained with less than 1 clonal B cell detectable in 10^5 stem cells (CD34+ Lin− Thy−). However, it is important to note that not all the dilution curves were linear for all 5 logs of dilution; in some cases, linearity was limited to the last 3 logs of dilution. Therefore, the limit of sensitivity is about 1 clonal B cell in 10^5 cells and the accuracy of our measurements may be off by one-half log. Based on this technique, unsorted mobilized PBSC harvests contained variable numbers of clonal B cells (0.001% to 10%). In the purified fractions a greater depletion of clonal cells (up to 7 logs) was obtained in patients with a higher initial load of tumor cells (0.1% to 10%).

The procedure described herein results in highly purified preparations of CD34+ Lin− Thy− stem cells (88%). Such a degree of purity could not be achieved with other available CD34 selection methods (40% to 80%), using avidin biotin immunoadsorbent columns, magnetic microspheres, or monoclonal antibodies covalently immobilized on polystyrene surfaces. Furthermore, these CD34+ cells exhibit a lineage commitment. CD19 coexpression has been reported on 40% to 60% of CD34+ cells in the peripheral blood of MM patients. More importantly, the majority of these CD34+ CD19+ cells belongs to the malignant clone, based on DNA aneuploidy and CDR III PCR, and show the same N-ras mutation as the malignant plasma cells in the BM. Furthermore, diploid myeloma cells expressing the CD19 antigen were described. Hence, selection only for CD34+ expression is unlikely to provide tumor-free grafts.

Our study has not addressed the important practical aspects of collecting, in a timely manner, adequate numbers of CD34+ Lin− Thy+ stem cells to ensure engraftment. The availability of a high-speed fluorescence-activated cell sorter, processing up to 20,000 cells/second, makes it feasible to complete the clinical sorting step within 2 to 3 hours, with an overall processing time of 7 to 8 hours. Engraftment has been reported with as few as 0.27 to 0.6 × 10^6 cells/kg CD34+ cells with an average purity of 42% and 62%, respectively. Approximately 25% of CD34+ cells are CD34+ Lin− Thy+. Therefore, 2 × 10^6/kg of CD34+ Lin− Thy+ cells should be sufficient to ensure complete engraftment after myeloablative therapy. Hence, the total number of CD34+ Lin− Thy+ stem cells necessary for one transplant will vary between 1 × 10^7 and 2 × 10^7 cells (assuming body weight of 50 to 100 kg) with a maximum number of MM cells infused of less than 200.

The presence of between 1% and 15% CD34+ Thy− Lin− cells in mobilized PBSC suggests that enough stem cells can be collected for two autotransplants in patients with limited prior exposure to stem cell--damaging alkylating agents.

Finally, the stem cell purification technique outlined in this paper should also provide tumor-free grafts in low-grade B-cell lymphoma and B-cell chronic lymphocytic leukemia.

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