

# Engraftment, clinical, and molecular follow-up of patients with multiple myeloma who were reinfused with highly purified CD34+ cells to support single or tandem high-dose chemotherapy

Roberto M. Lemoli, Giovanni Martinelli, Elena Zamagni, Maria Rosa Motta, Simonetta Rizzi, Carolina Terragna, Roberto Rondelli, Sonia Ronconi, Antonio Curti, Francesca Bonifazi, Sante Tura, and Michele Cavo

**Eighty-two patients with advanced multiple myeloma (MM) were enrolled in 2 sequential clinical studies of 1 or 2 courses of myeloablative therapy with stem cell support. Conditioning regimens consisted of high-dose melphalan (MEL) with or without total body irradiation (TX1 = 35) and MEL as the first preparative regimen, followed within 6 months by busulfan and melphalan (TX2 = 47). On the basis of adequate stem cell harvest, 31 patients (TX1 = 13; TX2 = 18) were transplanted with highly purified CD34+ cells. Positively selected stem cells did not adversely affect hematopoietic recon-**

**stitution compared with unmanipulated peripheral blood stem cell. Overall, the complete remission (CR) rate of evaluative patients was 13.8% and 41% for single and double autotransplant, respectively ( $P = .04$ ). Moreover, 3 patients undergoing TX2 achieved molecular remission and 2 remain PCR-negative after 36 and 24 months from autograft. The median event-free survival (EFS) durations for TX1 and TX2 were 17 and 35 months, respectively ( $P = .03$ ). Actuarial 3-year overall survival for patients treated with 1 or 2 transplants are 76% and 92%, respectively ( $P = NS$ ). On multivariate analysis,**

**superior EFS was associated with low  $\beta 2$  microglobulin ( $\beta 2$ -M) level at diagnosis and TX2, whereas overall survival was correlated with  $\beta 2$ -M. Positive selection of CD34+ cells did not influence the achievement of clinical or molecular CR, as well as remission duration or survival of MM patients. Thus, whereas multiple cycles of high-dose therapy may be beneficial for patients with myeloma, the clinical impact of tumor cell purging remains highly questionable. (Blood. 2000;95:2234-2239)**

© 2000 by The American Society of Hematology

## Introduction

High-dose therapy followed by autologous stem cell transplantation has been shown to improve complete remission (CR) rate, event-free survival (EFS), and overall survival (OS) in patients with multiple myeloma (MM) when compared with conventional chemotherapy in a randomized study.<sup>1</sup> However, the lack of plateau in the survival curves of MM patients with long-term follow-up indicates that it may be unlikely to cure the disease by autologous stem cell transplantation. In this regard, the reinfusion of myeloma cells contaminating autologous grafts may contribute to disease relapse as demonstrated in other hematologic malignancies and solid tumors.<sup>2,3</sup> The issue of myeloma cell contamination in the leukapheresis products collected after mobilization protocols has recently been addressed.<sup>4-7</sup> These studies have consistently shown the presence of  $10^4$  to  $10^9$  tumor cells per autograft by immunophenotyping and molecular analysis of patient specific Ig heavy chain (IgH) complementary determining region III (CDR III).<sup>5,7</sup> Moreover, an inverse correlation between plasma cell concentration in peripheral blood stem cell (PBSC) collections and disease-free survival has recently been demonstrated with more than  $2 \times 10^5$  tumor cells per liter predicting for early relapse.<sup>8</sup>

Positive selection of hematopoietic CD34+ progenitor cells has been shown as a feasible approach for removing 2.5 to 4.5 log of myeloma cells from PBSC collections with a limited loss of normal

stem cells.<sup>5,7</sup> Recently, several phase II studies and 1 phase III randomized trial have shown that transplantation of positively selected CD34+ cells results in rapid and stable recovery of hematopoiesis, with no difference with respect to patients receiving unmanipulated PBSC.<sup>5,7,9</sup>

However, the clinical role of purging to prevent disease relapse or progression remains to be determined at this stage. Whereas, indirect evidence of the therapeutic benefit of purging has been accumulating over the years for different malignancies,<sup>2,3,10,11</sup> conclusive data are not currently available for MM. In fact, it may well be that residual chemotherapy-resistant cells are responsible for the clinical outcome and circulating myeloma cells may simply reflect overall tumor burden.

In this study, we report the engraftment, clinical, and molecular follow-up of 82 patients with advanced stage MM submitted to single (= 35) or double (= 47) transplantation of autologous stem cells. Among these 2 sequential cohorts of patients, we could separately analyze individuals who, on the basis of adequate stem cell mobilization, received 1 (= 13) or 2 (= 18) reinfusions of highly enriched CD34+ cells.

The results presented here suggest that reinfusion of positively selected CD34+ cells provides effective hematopoietic support for 1 or 2 subsequent courses of high-dose therapy. However, removal

From the Institute of Hematology and Medical Oncology "L. & A. Seragnoli" and Department of Pediatrics, University of Bologna, Bologna, Italy.

Submitted July 29, 1999; accepted December 1, 1999.

Supported by the Italian Association for the Research Against Cancer (AIRC), Milan, Italy; MURST; Project 60% and 40%; and University of Bologna (Funds for selected research topics).

**Reprints:** Roberto M. Lemoli, Institute of Hematology and Medical Oncology

"Seragnoli," Via Massarenti, 9-40100 Bologna, Italy; e-mail: rmlmoli@med.unibo.it

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2000 by The American Society of Hematology

of tumor cells does not seem to improve the serologic or molecular CR rate, EFS, and overall survival of MM patients.

## Patients and methods

### Patients

Eighty-two advanced stage MM patients aged between 18 and 65 years underwent autologous stem cell transplantation at the Institute "Seragnoli" from January 1994 to December 1998 and their clinical characteristics are shown in Table 1. Data were analyzed as of May 30, 1999. Up to December 1995, 31 individuals were submitted to a single autograft (see below) and were reinfused with either unmanipulated PBSC (= 18) or positively selected CD34+ cells (= 13). From January 1996, 47 patients were administered 2 subsequent lines of high-dose chemotherapy ("tandem" or "double" transplantation) (see below) supported by untreated PBSC (= 29) or enriched CD34+ cells (= 18). Because of the lack of adequate stem cell mobilization, in the same period, 4 additional patients were submitted to a single course of myeloablative chemotherapy with PBSC support. The diagnosis of MM was made using standard criteria<sup>12</sup> and the patients were staged according to Durie and Salmon classification.<sup>13</sup> Eligibility criteria included: Karnofsky status greater than 70%, creatinine clearance greater than 60 mL/min, cardiac ejection fraction and pulmonary function tests greater than 50% of predicted values; bilirubin and transaminases less than 2 times the normal upper limit; white blood cell and platelet counts greater than  $3 \times 10^9/L$  and  $100 \times 10^9/L$ , respectively; negative serology for human immunodeficiency virus (HIV) and hepatitis B surface antigen and no evidence of active infection. The protocol was approved by the University Hospital ethical committee and each patient gave written informed consent.

### Peripheral blood stem cell mobilization and collection

As previously reported,<sup>5</sup> patients were treated with cyclophosphamide (Cy; 7 g/m<sup>2</sup>), followed by the subcutaneous administration of 5 µg/kg of granulocyte colony-stimulating factor (G-CSF) starting on day +2 from chemotherapy and continuing until the completion of PBSC collection. Once the CD34+ cell count was greater than 20 000/mL of peripheral blood (PB), patients underwent leukaphereses using a Cobe Spectra (Cobe, Lakewood, CO). On the basis of our previous experience,<sup>5</sup> to obtain a target cell dose of at least  $2 \times 10^6$  CD34+ for each reinfusion, we established 4 or  $8 \times 10^6/kg$  CD34+ cells for single or tandem autograft, respectively, as the minimum number of hematopoietic cells, to be collected over 2 days, for proceeding to ex vivo manipulation. The patients (= 46) who failed to mobilize an adequate number of CD34+ cells to start the selection process were reinfused with unmanipulated PBSC. Five additional patients did not have their CD34+ stem cells enriched because of the loss of availability of the clinical grade device used in this study (Ceprate SC Concentrator, see below). During the study period, 12 additional patients (11%) mobilized less than  $2 \times 10^6$  CD34+ cells per kilogram and were not submitted to stem cell transplantation, whereas 10 patients were enrolled in a pilot trial on selection and transplantation of CD34+ B-lineage negative cells.<sup>14</sup>

### Stem cell enrichment and cryopreservation

One or 2 leukapheresis products were processed by the Ceprate SC Concentrator (CellPro, Bothell, WA) to positively select CD34+ cells as previously described,<sup>5</sup> while an additional apheresis was stored as an unmanipulated back-up. The CD34+ cell fraction was recovered and resuspended in phosphate-buffered saline (PBS) containing 7.5% dimethyl sulfoxide (DMSO) and 4% human serum albumine (HSA) to a final volume of 4.5 mL. Unmanipulated PBSC collections were stored in 10% DMSO. The cells were then cryopreserved using a controlled-rate freezing method and kept at -196°C. At the time of reinfusion, CD34+ cells were rapidly thawed in a water bath at 37°C and diluted by slowly adding 4.5 mL of heparinized PBS. The cell suspension was further diluted with PBS to 30 mL of final volume for each vial and reinfused via a central line.

### Cell phenotype analysis and colony assay

Flow cytometric analysis was carried out by direct immunofluorescence on blood cells from the apheresis products before and after CD34+ cell separation. The following human MoAb was used: anti-CD34 (HPCA-2) phycoerythrin (PE) (Becton Dickinson, Palo Alto, CA). Isotype control IgG1-PE was also purchased from Becton Dickinson. Data acquisition and analysis were assessed on a FACScalibur instrument by CellQuest software (Becton Dickinson) as earlier reported.<sup>5</sup>

Samples of PB cells were evaluated in tissue culture assay to determine myeloid progenitor cell growth as previously described.<sup>5</sup> Briefly,  $50 \times 10^4$  unseparated cells or 1000 to 5000 purified hematopoietic progenitors were plated in duplicate in culture medium consisting of 1 mL of Iscove's modified Dulbecco's medium (IMDM), supplemented with 24% fetal calf serum (FCS; Sera Lab, Crawley Down, Sussex, UK), 0.8% BSA (Sigma),  $10^{-4}$  mol/L 2-mercaptoethanol (Sigma). To measure the optimum clonogenic efficiency, 10% (vol/vol) of a selected lot of phytohemagglutinin-lymphocyte-conditioned medium (PHA-LCM) was added. Methylcellulose final concentration was 1.1%. Granulocyte-macrophage colony-forming unit (CFU-GM) was scored after 14 days of incubation at 37°C in a fully humidified 5% CO<sub>2</sub> atmosphere.

### Stem cell transplantation

From January 1994 to December 1995, MM patients were enrolled in a clinical trial of positive selection of CD34+ cells to support a single course of high-dose chemotherapy (TX1). Untreated or CD34+-selected cells were reinfused on day 0 after a preparative regimen consisting of high-dose melphalan (MEL; 200 mg/m<sup>2</sup>; = 29) administered intravenously at day -1 or melphalan (140 mg/m<sup>2</sup>) at day -3 and total body irradiation (10 Gy; = 6) at day -1. G-CSF was administered at 5 µg/kg/d subcutaneously from day +6 until absolute neutrophil count (ANC) reached more than  $0.5 \times 10^9/L$  for 3 consecutive days. From January 1996, we started a pilot trial of "tandem" transplantation of purified CD34+ cells for advanced stage MM patients (TX2). The first conditioning regimen was MEL 200 mg/m<sup>2</sup> as reported previously. The second course of myeloablative therapy was scheduled within 6 months from MEL and consisted of busulfan (12 mg/kg total dose) given orally 4 times daily from day -4 to day -2 and melphalan 120 mg/m<sup>2</sup> on day -1 (Bu/Me). Stem cell reinfusion and G-CSF treatment were the same as above.

**Table 1. Study population**

Characteristics	No. of patients	
	TX1	TX2
Median age in years (range)	51 (29-64)	52 (32-60)
Sex (M/F)	20/15	30/17
Tumor stage		
I	7	10
II	4	6
III	24	31
Disease status		
Responsive	12	19
Refractory	8	17
Progressive	15	11
Median β-2 microglobulin (mg/L) (range)	2.1 (0.3-8)	1.9 (1.1-5.9)
Median C-reactive protein (mg/dL) (range)	0.5 (0.1-3.4)	0.9 (0.1-3.1)
Previous therapy		
Alkylating agents	20	26
VAD + alkylating	9	13
VAD or VAD-like therapy	15	20
α-IFN + other	3	3
Median time from diagnosis to transplant in months (range)	15 (6-78)	13 (4-58)

TX, treatment; VAD, vincristine, doxorubicin, and dexamethasone; IFN, interferon. Statistical analysis did not show any difference between the 2 series of patients. Thirty-one patients (TX1 = 13; TX2 = 18) were reinfused with highly purified CD34+ cells. When analyzed separately, we did not find any difference with patients receiving unmanipulated PBSC as for the major prognostic factors listed in the Table. Patients were staged according to Durie and Salmon classification.

Patients were nursed in single or double rooms in reverse isolation and received antimicrobial prophylaxis consisting of oral nystatin and ciprofloxacin. Packed red blood cells (RBCs) and single-donor platelet transfusions were administered to maintain a hemoglobin level more than 8 g/dL and a platelet count greater than  $10 \times 10^9/L$ . Patients were treated with broad spectrum antibiotics when fever developed and ANC was less than  $0.5 \times 10^9/L$ . Amphotericin B was added if the patients had persistent fever after 4 to 7 days of intravenous antimicrobial therapy. No prophylactic antipneumocystis and antiviral therapy was administered. The severity of side effects that occurred in the peritransplant period was assessed according to the World Health Organization (WHO) scoring system. Documented infection was defined in febrile patients as the occurrence of a single blood culture that was positive for any microorganism. Patients with invasive infection required histologic documentation or culture.

Patients who achieved CR or partial remission (PR) (see below) after completion of the transplantation program received  $\alpha$ -interferon ( $\alpha$ -IFN) subcutaneously ( $3 \times 10^6$  IU/m<sup>2</sup> 3 times a week) beginning at the time of full hematologic recovery and continued until evidence of disease progression.

The primary end point of the clinical study was time to hematopoietic reconstitution, which was defined as the number of days taken to achieve an ANC greater than  $0.5 \times 10^9/L$  (first of 3 consecutive days) and an unsupported platelet count greater than 20 and  $50 \times 10^9/L$ . Secondary end points were the evaluation of tumor cell purging, incidence of serologic and molecular CR (see below), rate of EFS, and OS.

### Response criteria

Assessment of tumor response to transplantation was generally performed 30 days after reinfusion of autologous stem cells and was planned every 3 months thereafter. MM patients were considered responsive to treatment if they showed at least 50% reduction in tumor mass. PR was defined as a tumor mass reduction of at least 75%. CR required the absence of monoclonal gammopathy in serum and urine by immunofixation (IF) analysis, and a normal BM aspirate and biopsy. In all cases, these findings had to be present on at least 2 occasions 3 months apart. New lytic lesions and/or any increase in plasma cell infiltration in BM and monoclonal gammopathy were considered as disease progression or relapse. Patients with at least 2 negative IF analysis were monitored for the presence of residual disease at the molecular level.

### Molecular analysis of minimal residual disease (MRD)

BM and PB specimens were prepared for DNA and RNA analysis as described.<sup>14,15</sup> The molecular study was performed at diagnosis on BM samples, on unmanipulated apheresis products, after CD34+ cell selection, and during clinical follow-up every 3 months for the first 6 months, twice a year for the first 2 years, and once a year thereafter. Molecular CR was defined as PCR-negative results in at least 2 subsequent evaluations.

VDJ gene rearrangement amplification was performed with a panel of VH family-specific primers,<sup>4</sup> together with a JH consensus primer.<sup>14,15</sup> To determine the VH segment used in VDJ gene rearrangement, 7 amplifications were performed for each patient. The reaction mixture (50  $\mu$ L) contained 200  $\mu$ mol/L of dNTPs, 1  $\times$  PCR buffer (10 mmol/L BME, 6.7  $\mu$ mol/L EDTA pH 8, 67  $\mu$ mol/L Tris pH 8.8, 170 mg/mL BSA), 7.7 mmol/L MgCl<sub>2</sub>, 50 pmol/L of each primer, 2% DMSO and 0.3 U Taq DNA polymerase (Boehringer Mannheim, Monza, Italy). Thirty cycles of amplification were performed. Denaturation at 95°C for 30 seconds, annealing at 61°C for 40 seconds and extension at 72°C for 50 seconds were then followed by a 7-minute final extension at 72°C.

An aliquot of 15  $\mu$ L was analyzed on ethidium bromide stained 3% agarose gel: a single, discrete band of approximately 300 base pairs (bp) was obtained at diagnosis. A 30  $\mu$ L aliquot of the amplification product corresponding to the VH family-specific gene rearrangement was loaded on a 1.25% low-melt preparative grade agarose gel (BioRad, Segrate, Italy). The 300-bp band was excised from the gel and purified with a Gel Nebulizer Micropure Separator (Amicon, Milan, Italy), according to the manufacturer's instructions. An aliquot of purified DNA was directly sequenced with the family-specific VH primer using the Thermo Sequenase

DNA cycle-sequencing kit (Amersham, Milan, Italy). Sequence analysis was performed using the PC-GENE software (IntelliGenetics). To generate patient-specific amplifications, patient-specific primers were designed on the CDRII and CDRIII region identified.

Clonally expanded B cells were detected by amplifying 1  $\mu$ g of DNA or 10  $\mu$ L of cDNA using the patient-specific primers directed to the CDRII and CDRIII regions. Fifty cycles of amplification were performed consisting of denaturation at 96°C for 30 seconds, annealing at the best tested temperature for 30 seconds, and extension at 72°C for 40 seconds, followed by a 7-minute final extension at 72°C. The reaction mixture (50  $\mu$ L) contained 200  $\mu$ mol/L dNTPs, 1  $\times$  PCR buffer, 500 mmol/L KCl, 100 mmol/L Tris pH 8.3, 2.5 mmol/L MgCl<sub>2</sub>, 50 or 100 pmol/L of patient-specific primers, and 1 U of AmpliTaq Gold (Parkin Elmer, Milan, Italy). An aliquot of 15  $\mu$ L was analyzed on agarose gel as reported above. A rearrangement band of approximately 150 bp was obtained from each patient. The sensitivity of each set of primers ( $1:10^5$ - $10^6$ ) was assessed on the DNA or RNA from the patient initial marrow specimen serially diluted in an appropriate amount of DNA or RNA from normal PB cells.

### Statistical analysis

The results are presented as median values and ranges where applicable. EFS and OS among categorical prognostic variables measured before start of therapy were compared using the log-rank test.<sup>16</sup> To avoid bias in favor of patients submitted to tandem transplant, landmark analysis was used to compare EFS and OS between the 2 groups.<sup>17</sup> The analysis was performed by determining the time (6 months) within which 90% of the patients received the second cycle of high-dose therapy. Only the individuals event-free and alive at that landmark were compared in the 2 studies. The probabilities of neutrophil and platelet recovery and achievement of CR and PR of the different series of patients were compared by means of the Kaplan and Meier method. Cox regression was used to examine continuous and categorical univariate and multivariate effects of prognostic features on EFS and OS.<sup>18</sup> Variables measured after start of therapy were incorporated in the Cox models as time-dependent covariates.

## Results

Between January 1994 and December 1998, 82 patients with advanced MM were considered eligible for 1 or 2 courses of high-dose chemotherapy supported by stem cell transplantation and their clinical characteristics are reported in Table 1. On the basis of adequate stem cell mobilization, 31 patients (37.8%) were submitted to positive selection and transplantation of CD34+ cells, whereas the remaining 51 individuals were reinfused with unselected PBSC. Overall, the median interval between diagnosis and transplantation was 14 months (range 4-78). All individuals had received 1 or more lines of treatment before transplant and none of them was in CR at time of study. The median duration of alkylating agents therapy was 9 months (0-18). Thirty-one patients (37.8%) showed responsive disease at the time of autotransplant (greater than 50% reduction of tumor mass), whereas 51 patients (62.1%) were enrolled in the trial with refractory or progressive disease. Although this was a prospective nonrandomized study, the 2 groups of patients (TX1 and TX2) were well balanced for the clinical parameters considered (Table 1).

### Positive selection of CD34+ cells, engraftment results, and toxicity

A median of 2 aphereses (range 1-2) were performed. Similar to our previous results,<sup>5</sup> stem cell processing resulted in the median recovery of 65% (range 10%-100%) of the initial content of CD34+ cells with a purity of 89% (range 48%-98%). Clonogenic assays demonstrated the recovery of 44.5% (range 6%-100%) of CFU-C. Overall, 31 patients were reinfused after MEL with a

median of  $4.6 \times 10^6$  purified CD34+ cells/kg (range 1.6-10.4) and  $12.1 \times 10^4$  CFU-C/kg (range 1-64) and showed a rapid and sustained reconstitution of hematopoiesis. None of the patients required reinfusion of unselected back-up cells. The median time to an ANC greater than  $0.5 \times 10^9/L$  and to 20 and  $50 \times 10^9$  platelet per liter was 11, 12, and 15 days, respectively. Transfusion requirement was minimal and the median time to hospital discharge after reinfusion was only 13 days (range 11-28). When analyzed separately, no difference was found between patients who received MEL as the only preparative regimen (= 13) and individuals for whom MEL represented the first of 2 sequential lines of myeloablative chemotherapy (= 18). Bu/Mel conditioning regimen was administered at a median time of 4 months (range 3-15) from MEL. The second course of high-dose chemotherapy (supported with a median of  $5.5 \times 10^6$  enriched CD34+ cells/kg; range 2.8-11.9) did not result in a delayed hematopoietic recovery, compared with TX1 (median time of 10.5 days to ANC greater than  $0.5 \times 10^9/L$ ; range 9-12, and median time of 11 days to  $20 \times 10^9$  platelet/L; range 10-100). Hospital discharge occurred at a median of 14 days (range 12-21) after transplant.

We then compared the engraftment of MM patients reinfused with selected CD34+ cells with that of patients who received unmanipulated PBSC containing a median of 3.7 (range 1.3-9.7; TX1) and 6.4 (range 2.5-13.2; TX2)  $\times 10^6$  CD34+ cells/kg. As reported in Figure 1, the time to hematologic reconstitution was not statistically different in the 2 groups of patients, both after the first and the second autograft. Long-term complete hematopoietic reconstitution as defined as ANC and platelet count greater than 2.5 and  $100 \times 10^9/L$ , respectively, was documented in all patients.

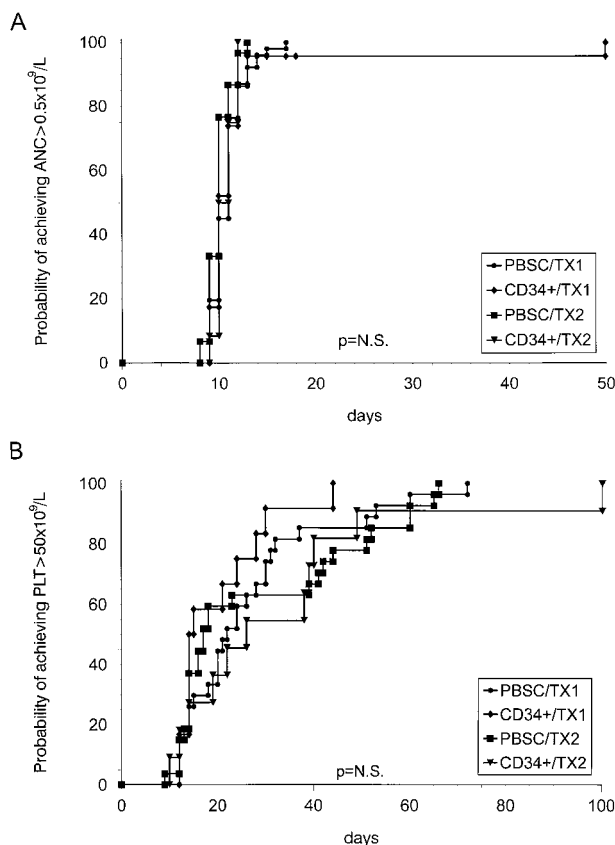


Figure 1. Kaplan-Meier plot of probability of recovery of neutrophils to  $0.5 \times 10^9/L$  (A) and recovery to an unsupported platelet count of  $50 \times 10^9/L$  (B) in patients undergoing 1 (TX1) or 2 (TX2) courses of high-dose chemotherapy with selected or unselected CD34+ cells.

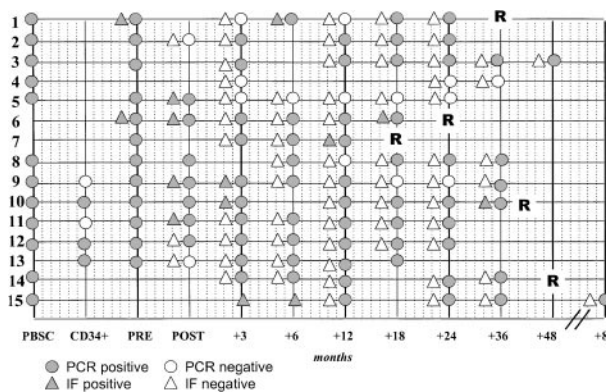


Figure 2. Molecular follow-up of myeloma patients who achieved serologic and clinical CR after TX1 or TX2. Nos. 1 to 8 and 9 to 13 represented double autotransplants supported with PBSC or CD34+ cells, respectively, whereas Nos. 14 and 15 were patients who were submitted to a single course of high-dose therapy with unmanipulated stem cells.

Tandem transplant procedure either supported by CD34+ cells or by unselected PBSC was well tolerated and severe extrahematologic toxicity (more than grade 2 according to WHO scoring system) was mainly limited to oral mucositis. There were 2 treatment-related deaths (4.2%) (veno-occlusive disease-VOD = 1 and interstitial pneumonia = 1) after Bu/Mel and reinfusion of PBSC. Other toxicities included hemorrhagic cystitis (4 patients), pulmonary toxicity requiring steroid therapy in 1 patient, and severe life-threatening VOD (data not shown). One patient who was reinfused with purified CD34+ cells after TBI-containing conditioning regimen (TX1) died in the peritransplant period because of interstitial pneumonia.

**Clinical outcome and molecular follow-up**

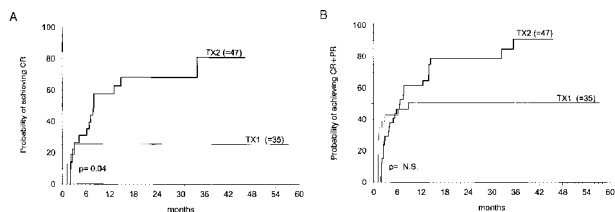
PBSC collections and CD34+ cell fractions were analyzed for the presence of myeloma cells by PCR reaction for CDRIII. Consistent with our previous results,<sup>5</sup> we found that all the leukaphereses and 22/31 of the selected cell products did contain residual clonal B cells (data not shown).

Eight of 13 patients who received 1 course of myeloablative therapy and CD34+ selected cells were responsive to transplantation (more than 50% reduction of tumor burden). However, none of them achieved clinical and serologic CR. Conversely, 5/18 (28%) patients with advanced disease who underwent double autotransplant achieved CR. Only 2/5 patients were reinfused with tumor-free autografts (Figure 2) and only 1 showed a transient molecular remission. To date, 4 individuals are still in clinical and serologic CR, despite detection of molecular MRD.

Pooling together PBSC and CD34+ cells-supported transplants, CR and PR + CR rates for evaluative patients who were administered 1 or 2 lines of high-dose chemotherapy, respectively, are 13.8%, 41% ( $P = .04$ ) and 44%, 71.8%. Figure 3 shows a Kaplan-Meier type plot for the probability of achieving CR (A) and CR + PR (B) for TX1 and TX2. Moreover, molecular CR was only observed in 3 MM patients submitted to tandem autotransplantation (CD34+ = 1; unmanipulated PBSC = 2) and 2 of these individuals (= PBSC) remain PCR-negative after 24 and 36 months from completion of the program (Figure 2).

**Prognostic factors**

On univariate analysis of 10 pretreatment variables (Table 1), 2 ( $\beta$ -2 microglobulin level at diagnosis  $\leq 4$  mg/L and responsive disease at time of transplant) had significant ( $P < .05$ ) association



**Figure 3. Kaplan-Meier plot of probability of achieving CR (A) and CR + PR (B) for TX1 and TX2.** The time, in months, is calculated from the date of the (first) transplant. CD 34+-selected transplants were pooled together with unmanipulated autografts. Disease complete response is significantly correlated with TX2 (see text). One single patient (TX2) reached CR after 32 months from transplant after  $\alpha$  IFN treatment.

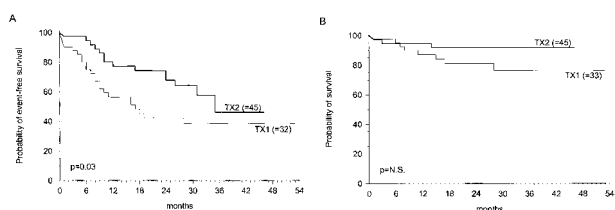
with EFS and OS, whereas age, sex, disease stage, M component isotype, C-reactive protein level, BM plasmacytosis, time from diagnosis to transplant, and reinfusion of selected CD34+ cells did not have any correlation. Only  $\beta$ -2 microglobulin level maintained independent significance on multivariate analysis for both EFS (relative risk = 0.5,  $P$  = .005) and OS (relative risk = 0.6,  $P$  = .02). Moreover, to assess the potential impact of response and treatment (ie, TX1 and TX2) on EFS and OS, a time-dependent covariate analysis was conducted accounting for a second cycle of high-dose therapy, time to high-dose therapy, CR, time to CR, CR + PR, and time to CR + PR. A statistically significant association was only found between EFS and any second transplant (relative risk = 0.3,  $P$  = .02).

Figure 4 compares the probability of EFS (A) and OS (B) for MM patients submitted to single or tandem autograft according to landmark analysis. The median follow-up from transplant is 34 (range 5-84) and 28 (range 2-53) months for patients receiving TX1 or TX2, respectively, and their median EFS durations were 17 and 35 months ( $P$  = .03). Actuarial 3-year OS for patients treated with 1 or 2 transplants are 76% and 92%, respectively ( $P$  = NS).

## Discussion

Positive selection of CD34+ cells is widely applied to remove CD34-cancer cells from autologous grafts. This is based on early results<sup>5,7,9,19-22</sup> showing the ability of selected cells to restore autologous hematopoiesis after myeloablative therapy as well as untreated PBSC. However, whether purified stem cell can be used to support multiple lines of high-dose therapy and whether extensive purging of myeloma cells would represent a significant improvement in the patient outcome has yet to be demonstrated. In this regard, Vescio et al<sup>9</sup> showed the same probability of progression-free survival at 1 year of MM patients reinfused with selected and unselected stem cells in a randomized study mainly focused on engraftment capacity of enriched CD34+ cells.

In this paper, we report a single Center experience of 82 patients



**Figure 4. Probability of EFS (A) and OS (B) for patients undergoing 1 or 2 courses of myeloablative therapy assessed according to landmark analysis (see "Patients and methods").** Also in this case, selected and unselected transplants were pooled together. Superior EFS is significantly associated with TX2.

with intermediate to advanced-stage MM who were enrolled from January 1994 to December 1998 in 2 sequential phase II trials to determine (1) the feasibility of positive selection of CD34+ cells in pretreated myeloma patients; (2) the indirect purging of tumor cells; and (3) the ability of positively selected CD34+ cells to support the hematopoietic reconstitution after 1 or 2 courses of myeloablative chemotherapy. Of note, the choice of a more aggressive therapy ("tandem transplant") from 1996 was mainly based on the promising results reported by the University of Arkansas group<sup>23</sup> and the lack of plateau in the survival curve of MM patients submitted to a single course of high-dose therapy. Thus, we reasoned that by coupling effective ex vivo purging with the delivery of 2 lines of myeloablative chemotherapy we could benefit myeloma patients with otherwise poor prognosis. We set 4 or  $8 \times 10^6$  cells/kg (single or double autotransplant, respectively) as the minimum number of CD34+ elements to be collected from PB to proceed to stem cell enrichment. This was based on our and others previous experience<sup>5,7,9,24</sup> showing delayed platelet engraftment after the reinfusion of less than  $2 \times 10^6$  CD34+ cells/kg and prediction of approximately 50% loss of progenitor cells during processing. Notably, only 40% of heavily pretreated myeloma patients met the requirements for stem cell selection. Thus, autologous transplantation and stem cell selection should be planned shortly after diagnosis to avoid long-term exposure to alkylating agents that prevent optimal mobilization of CD34+ cells.

In this paper, we confirmed that reinfusion of positively selected CD34+ cells does not adversely affect the ability of hematopoietic stem cells of restoring BM function after 1 course of myeloablative therapy. Moreover, we demonstrated that purified CD34+ cells could be used to support multiple cycles of high-dose chemotherapy ("tandem" transplantation) as well as unmanipulated PBSC. Despite maintenance treatment with  $\alpha$ -IFN, we did not observe any late engraftment failure or delayed infections in these patients. The absence of late viral or fungal infections is important as extensive removal of T and B cells from autografts may increase the risk of long-lasting immunodeficiency in MM patients undergoing 2 or more courses of high-dose chemotherapy. In this regard, the randomized phase III trial<sup>9</sup> did not show any difference between patients reinfused with selected CD34+ cells or unselected PBSC as for the number and the type of infections within the first 3 months from transplantation. Furthermore, TBI- or cyclophosphamide-containing preparative regimens<sup>25</sup> may be more immunosuppressive than MEL and Bu/Mel used in our study.

As previously reported,<sup>5</sup> PCR-based monitoring of MRD allowed the detection of myeloma cells in the majority of CD34+ cell products. This finding may be due to either CD34- tumor cells contaminating the CD34+ cell population or to CD34+CD19+ myeloma elements.<sup>26</sup> Thus, strategies to achieve tumor-free autografts have recently been developed by purification of CD34+Thy1+Lin- cells<sup>27</sup> or by positive/negative selection of CD34+ B-lineage negative progenitors.<sup>14</sup> However, when CD34-selected autotransplants were analyzed as part of a multivariate analysis for EFS and OS, we did not find any clinical advantage in favor of tumor cell purging. This was a prospective nonrandomized study and may not be sufficiently powered to assess small but still clinically significant differences. However, despite the consistent involvement of PBSC collections from large numbers of malignant cells in myeloma patients with advanced disease, the high cost of stem cell selection, the relatively low percentage of patients who could be submitted to this procedure, and the potential of delayed platelet engraftment in case of reinfusion of less than  $2 \times 10^6$

CD34+ cells/kg suggest that positive selection of CD34+ cells should not be routinely performed until an update of the phase III trial, comparing selected versus unselected cells, will conclusively assess the role of tumor cell purging in MM.

Noteworthy, tandem autotransplant (regardless of whether enriched stem cell were reinfused) resulted in a CR rate of 41% which was significantly better than that of a single course of myeloablative therapy. Moreover, molecular follow-up demonstrated that 3 patients achieved at some point PCR negativity and 2 of them remain in molecular and clinical CR after 2 and 3 years from transplantation. These results extend early observations from Corradini et al<sup>28</sup> and Bjorkstrand et al<sup>29</sup> who reported few cases of molecular CR in patients undergoing double but not single autotransplant.<sup>28</sup> Although a longer follow-up and a larger number of patients are needed to determine the role of molecular evaluation in predicting relapse in myeloma, in other B-cell malignancies, PCR negativity has been correlated with a better clinical outcome.<sup>30</sup>

The impact of the second transplant and other prognostic features on EFS and OS of patients with intermediate and advanced stage MM was also evaluated. On multivariate analysis, TX2 and  $\beta 2$  microglobulin level at diagnosis were associated with superior EFS. Again, this was not a randomized study and the results should be taken with caution. However, these data may confirm previous results indicating the clinical benefit of multiple cycles of high-dose therapy with stem cell support for pretreated MM patients.<sup>23</sup> In this respect, Palumbo et al<sup>31</sup> have recently demonstrated the superiority of 2 to 3 cycles of submyeloablative doses of melphalan in comparison with standard chemotherapy in elderly myeloma patients. The issue of 1 versus 2 autotransplants for MM patients at diagnosis is currently being addressed in 2 randomized studies.<sup>32,33</sup>

In conclusion, whereas the clinical impact of tumor cell removal from autografts remains highly questionable at this stage, PBSC-supported MEL-based tandem transplant may be an attractive alternative for myeloma patients with symptomatic disease.

## References

- Attal M, Harousseau JL, Stoppa AM, et al. A prospective randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. *N Eng J Med*. 1996;335:91.
- Brenner MK, Rill DR, Moen RC, et al. Gene-marking to trace the origin of relapse after autologous bone-marrow transplantation. *Lancet*. 1993;341:85.
- Deisseroth AB, Zu Z, Claxton D, et al. Genetic marking shows that Ph+ cells present in autologous transplants of chronic myelogenous leukemia (CML) contribute to relapse after autologous bone marrow in CML. *Blood*. 1994;83:3068.
- Corradini P, Voena C, Astolfi M, et al. High-dose sequential chemoradiotherapy in multiple myeloma: residual tumor cells are detectable in bone marrow and peripheral blood harvests and after autografting. *Blood*. 1995;85:1596.
- Lemoli RM, Fortuna A, Motta MR, et al. Concomitant mobilization of plasma cells and hematopoietic progenitors into peripheral blood of multiple myeloma patients: positive selection and transplantation of enriched CD34+ cells to remove circulating tumor cells. *Blood*. 1996;87:1625.
- Gazitt Y, Tian E, Barlogie B, et al. Differential mobilization of myeloma cells and normal hematopoietic stem cells in multiple myeloma following treatment with cyclophosphamide and GM-CSF. *Blood*. 1996;87:805.
- Schiller GJ, Veschio RA, Freytes C, et al. Transplantation of CD34+ peripheral blood progenitor cells after high-dose chemotherapy for patients with advanced multiple myeloma. *Blood*. 1995;86:390.
- Gertz MA, Witzig TE, Pineda AA, Greipp P, Kyle RA, Litzow MR. Monoclonal plasma cells in the blood stem cell harvest from patients with multiple myeloma are associated with shortened relapse-free survival after transplantation. *Bone Marrow Transplant*. 1997;337:19.
- Veschio R, Schiller G, Stewart AK, et al. Multicenter Phase III trial to evaluate CD34+ selected versus unselected autologous peripheral blood progenitor cell transplantation in multiple myeloma. *Blood*. 1999;93:1858.
- Gribben JG, Freedman AS, Neuberg D, et al. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. *N Eng J Med*. 1991;325:1525.
- Laporte JP, Douay L, Lopez M, et al. One hundred twenty-five adult patients with primary acute leukemia autografted with marrow purged by mafosfamide: a 10-year single institution experience. *Blood*. 1994;84:3810.
- Chronic Leukemia-Myeloma Task Force, National Cancer Institute. Proposed guidelines for protocol studies-II. Plasma cell myeloma. *Cancer Chemother Rep*. 1973;4(part 3):724.
- Durie BGM, Salmon SE. A clinical staging system for multiple myeloma: correlation of measured myeloma cell mass with presenting clinical features, response to treatment and survival. *Cancer*. 1975;36:842.
- Lemoli RM, Martinelli G, Olivieri A, et al. Selection and transplantation of autologous CD34+B-lineage negative cells in advance phase multiple myeloma patients: a pilot study. *Br J Haematol*. 1999;107:419-428.
- Martinelli G, Terragna C, Lemoli RM, et al. Clinical and molecular follow up by amplification of the CDR-III IgH region in multiple myeloma patients after autologous transplantation of hematopoietic CD34+ stem cells. *Haematologica*. 1999;84:397.
- Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep*. 1966;50:163.
- Anderson JR, Cain KC, Gebler RD. Analysis of survival by tumor response. *J Clin Oncol*. 1983;1:710.
- Cox DR. Regression models and life-tables (with discussion). *J Royal Stat Soc, Series B*. 1972;34:187.
- Shpall EJ, Jones RB, Bearman SI, et al. Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy: influence of CD34-positive peripheral blood progenitors and growth factors on engraftment. *J Clin Oncol*. 1994;12:28.
- Berenson RJ, Bensinger WI, Hill RS, et al. Engraftment after infusion of CD34+ marrow cells in patients with breast cancer and neuroblastoma. *Blood*. 1991;77:1717.
- Brugger W, Henschler R, Heimfeld S, Berenson RJ, Mertelsmann R, Kanz L. Positively selected autologous blood CD34+ cells and unseparated peripheral blood progenitor cells mediate identical hematopoietic engraftment after high-dose VP 16, ifosfamide, carboplatin and epirubicin. *Blood*. 1994;84:1421.
- Gorin NC, Lopez M, Laporte JP, et al. Preparation and successful engraftment of purified CD34+ bone marrow progenitor cells in patients with non-Hodgkin's lymphoma. *Blood*. 1995;85:1647.
- Vesole DH, Barlogie B, Jagannath S, et al. High-dose therapy for refractory multiple myeloma: improved prognosis with better supportive care and double transplants. *Blood*. 1994;84:950.
- Schiller G, Veschio R, Freytes C, et al. Autologous CD34-selected blood progenitor cell transplants for patients with advanced multiple myeloma. *Bone Marrow Transplant*. 1998;21:141.
- Rossi JF, Legouffe E, Fegoux N, et al. Autologous transplantation (AT) of CD34+ peripheral blood progenitor cells (PBPC) after double (D) high dose chemotherapy (HDC) in multiple myeloma (MM) is followed by severe immunodeficiency (ID) and high production of interleukin-6 (IL-6) related-C reactive protein (CRP) requiring additive immunotherapy [abstract]. *Blood*. 1996;88(suppl 1):142a.
- Szczepek AJ, Bergsagel PL, Axelsson L, Brown CB, Belch AR, Pilarski LM. CD34+ cells in the blood of patients with multiple myeloma express CD19 and IgH mRNA and have patient-specific IgH VDJ gene rearrangements. *Blood*. 1997;89:1824.
- Tricot G, Gazzit Y, Leemhuis T, et al. Collection, tumor contamination and engraftment kinetics of highly purified hematopoietic progenitor cells to support high-dose therapy in multiple myeloma. *Blood*. 1998;91:4489.
- Corradini P, Voena C, Tarella C, et al. Molecular and clinical remissions in multiple myeloma: role of autologous and allogeneic transplantation of hematopoietic cells. *J Clin Oncol*. 1999;17:208.
- Bjorkstrand B, Ljungman P, Bird JM, Samson D, Garthon G. Double high-dose chemotherapy with autologous stem cell transplantation can induce molecular remissions in multiple myeloma. *Bone Marrow Transplant*. 1995;15:367.
- Gribben JG, Neuberg D, Freedman AS, et al. Detection by polymerase chain reaction of residual cells with the bcl-2 translocation is associated with increase risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. *Blood*. 1993;81:3449.
- Palumbo A, Triolo S, Argentino C, et al. Dose-intensive melphalan with stem cell support (MEL 100) is superior to standard treatment in elderly myeloma patients. *Blood*. 1999;94:1248.
- Attal M, Payen C, Facon T, et al. Single versus double transplant in myeloma: a randomized trial of the "InterGroup Francais du Myeloma" (IFM) [abstract]. *Blood*. 1997;90:418a.
- Tosi P, Cavo M, Zamagni E, Ronconi S, Benni M, Tura S. A multicentric randomized clinical trial comparing single vs double autologous peripheral blood stem cell transplantation for patients with newly diagnosed multiple myeloma: results of an interim analysis. [abstract] *Blood*. 1999;94:715a.