Subsets of CD34$^+$ Cells and Rapid Hematopoietic Recovery After Peripheral-Blood Stem-Cell Transplantation

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Purpose: **To study whether there is a relationship between transplanted cell dose and rate of hematopoietic recovery after peripheral-blood stem-cell (PBSC) transplantation, and to obtain an indication whether specific subsets of CD34$^+$ cell populations contribute to rapid recovery of neutrophils or platelets.**

Patients and Methods: **Based on data from 59 patients, we calculated for each day after PBSC transplantation the dose of CD34$^+$ cells that resulted in rapid recovery of either neutrophils or platelets in the majority (> 70%) of patients. Using dual-color flow cytometry, subsets of peripheral-blood CD34$^+$ cells were quantified and the numbers of CD34$^+$ cells belonging to each of the reinfused subsets correlated with hematopoietic recovery following high-dose chemotherapy.**

Results: **The calculated threshold values with a high probability of engraftment showed a steep dose-effect relationship between CD34$^+$ cell dose and time to recovery of both neutrophils or platelets. Predominantly CD34$^+$ cells with the phenotype of myeloid precursors were mobilized. A minority of CD34$^+$ cells expressed the erythroid and megakaryocytic lineage-associated antigens and a low but distinct population of CD34$^+$ cells expressed antigens associated with multipotent stem cells. Analysis showed that the number of CD34$^+$CD33$^-$ cells ($r = -.74$, $P < .05$), as well as the number of CD34$^+$CD41$^+$ cells ($r = -.81$, $P < .005$), correlated significantly better with time to neutrophil and platelet recovery, respectively, than with the total number of CD34$^+$ cells ($r = -.55$ and $r = -.56$, respectively).**

Conclusion: **The numbers of CD34$^+$CD33$^-$ cells and CD34$^+$CD41$^+$ cells may help to predict short-term repopulation capacity of PBSCs, especially when relatively low numbers of CD34$^+$ cells per kilogram are reinfused.**

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AS COMPARED with bone marrow (BM) transplantation, reinfusion of mobilized peripheral-blood stem cells (PBSCs) accelerates hematopoietic recovery after bone marrow aplasia induced by high-dose chemotherapy.[1-3] By reconstitution of hematopoietic stem cells, multipotent progenitor cells and progenitor cells committed to the myeloid, lymphoid, erythroid, and megakaryocytic lineages have been found to reside within the CD34$^+$ cell population.[4-8] Further characterization of the CD34$^+$ cell populations by flow cytometry has identified early multipotent stem cells by the expression of MDR-1,[9] c-*kit*[10] and CD45RO[11] and by the lack of expression of CD38[12], human leukocyte antigen (HLA)-DR,[13,14] CD33,[15,16] and CD13.[17,18] Committed progenitor cells were shown to express antigens associated with myeloid lineage (CD33 and CD13),[16,17,19] erythroid lineage (CD71, transferrin receptor),[20,21] lymphoid lineage (CD7 and CD19),[22,23] or megakaryocytic lineage (CD41 and CD61).[24,25]

A major clinical issue is to define the minimum number of cells necessary for rapid and sustained reconstitution of hematopoiesis after high-dose chemotherapy. Numbers of nucleated cells, as well as granulocyte-macrophage colony-forming units (CFU-GM), have been used as indicators for the reconstitutive capacity of the PBSCs.[26-28] A better indication for the reconstitutive capacity of the graft is the measurement of CD34$^+$ cells.[14,29] This parameter correlates well with the peak levels of circulating CFU-GM mobilized by chemotherapy and hematopoietic growth factors.[14] Although the CD34$^+$ population is necessary for engraftment, the role of the various CD34$^+$ subsets in hematopoietic recovery is not well defined. The phenotypic characteristics of PBSCs and the relative proportions of specific subsets of CD34$^+$ cells may provide an explanation for the rapid engraftment observed with mobilized PBSCs. However, it cannot be excluded that the hematopoietic recovery is only due to the larger number of cells that are reinfused with PBSC transplantation in comparison with autologous BM transplantation.

The purpose of this study was to obtain a marker for a more accurate assessment of the reconstitutive capacity of the PBSC transplant. Therefore, we applied dual-color flow cytometry to analyze peripheral-blood–mobilized progenitor cells with a panel of 12 monoclonal antibodies

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(MoAbs) specific for the different cell lineages. Subsets of peripheral-blood CD34+ cells were quantified and the numbers of CD34+ cells belonging to each of the reinfused subsets were correlated with hematopoietic recovery after high-dose chemotherapy.

PATIENTS AND METHODS

MoAbs

In this study, the following antibodies were used: immunoglobulin G1 (IgG1) and IgG2a isotype control antibodies were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, the Netherlands). Phycoerythrin (PE)-labeled rat MoAb against the (kappa) light chain of mouse Ig and fluorescein isothiocyante (FITC)-labeled HPCA-2 (CD34) and Leu-17 (CD38) were purchased from Becton Dickinson (San Jose, CA). My9 (CD33) was purchased from Coulter (Hialeah, FL). MoAb against c-*kit* was purchased from Immunotech SA (Marseille, France). MoAbs against HLA-DR (CR3/43) and CD71 (OKT-9) were purchased from Dakopatts (Glostrup, Denmark). MoAb MRK-16 (CD117, MDR-1) was a generous gift of Dr P. Sonneveld (University of Rotterdam, Rotterdam, the Netherlands). MoAbs against CD7 (3A1), CD13 (Q20), CD19 (11G1), CD41 (CLB-thromb/7), CD45RO (UCHL-1), CD61 (CLB-thromb/1), and CD66 (B13.9) are all MoAbs produced in our laboratory (CLB) and clustered in the International Workshop on Leukocyte Differentiation Antigens.[30]

Patient Characteristics

The group of 59 patients studied (median age, 36 years; range, 18 to 57) included 28 patients with breast cancer, 15 with germ cell cancer, seven with Hodgkin's disease, five with non-Hodgkin's lymphoma, one with nasopharyngeal carcinoma, one with medulloblastoma, one with rhabdomyosarcoma, and one with neuroblastoma. The patients with breast cancer[31] were in their first chemotherapy-induced (near) complete remission, while the remaining patients were in second partial or complete remission. The patients with germ cell cancer underwent a tandem transplantation procedure with a 5-week interval. All recovery data presented here relate to the first transplantation procedure. All patients had a World Health Organization (WHO) performance status of 0 or 1, adequate renal and hepatic functions (creatinine clearance $\geq$ 50 mL/min, bilirubin level $\leq$ 25 μmol/L) and normal BM functions (WBC count $\geq$ 3.5 $\times$ 10^9/L, platelet count $\geq$ 100 $\times$ 10^9/L.

All patients gave informed consent, and the separate protocols were approved by the Ethical and Scientific Review Committees of the Netherlands Cancer Institute and the Free University Hospital (Amsterdam, the Netherlands).

Mobilization Procedure, PBSC Harvest, and Reinfusion

Hematopoietic progenitor cells were mobilized by chemotherapeutic treatment followed by daily 5 μg/kg/d subcutaneous granulocyte colony-stimulating factor (G-CSF) (Filgrastim; Amgen Inc, Thousand Oaks, CA) until completion of the leukocytapheresis. In patients with breast cancer, the mobilizing regimen consisted of fluorouracil (500 mg/m^2), epirubicin (120 mg/m^2), and cyclophosphamide (500 mg/m^2) on day 1 with G-CSF started on day 2.[31] In the remaining patients, PBSCs were mobilized by ifosfamide (4 g/m^2 on day 1) and etoposide (100 mg/m^2 on days 1 to 3), followed by G-CSF onward from day 4.

From the seventh day of G-CSF administration, the percentage of CD34+ cells in the peripheral blood was determined daily. As soon as the WBC count was greater than 3.0 $\times$ 10^9/L and a clear increase in the CD34+ cell percentage was observed, leukocytapheresis procedures were started. At the start of each leukocytapheresis procedure, the number of platelets had to be $\geq$ 50 $\times$ 10^9/L. The leukocytapheresis was performed as an outpatient procedure with a continuous-flow blood-cell separator (Fenwal CS3000; Baxter Deutschland GmbH, Unterschleissheim, Germany). One leukocytapheresis procedure per day was performed. After each leukocytapheresis, the number of CD34+ cells was measured. Depending on the yield of CD34+ cells, further leukocytaphereses were planned. In a median of two (range, one to 10) leukocytapheresis procedures per patient, a median of 9.5 $\times$ 10^6 CD34+ cells/kg (range, 1.3 to 50.6 $\times$ 10^6/kg) were procured. Following leukocytapheresis, the cells were cryopreserved in saline, which contained 0.1% glucose, 0.38% trisodium citrate, 10% human serum albumin, and 10% dimethylsulfoxide at a cell concentration of approximately 50 $\times$ 10^6 mononuclear cells/mL. The cell suspensions were frozen at a controlled rate in a Kryo10 (Cryotech, Schagen, the Netherlands) and were subsequently stored in the vapor phase of liquid nitrogen until reinfusion.

Patients with nonhematologic malignancies received high-dose chemotherapy that consisted of 1,600 mg/m^2 carboplatin, 480 mg/m^2 thiotepa, and 6,000 mg/m^2 cyclophosphamide intravenously, divided over 4 days (CTC).[32] Patients with malignant lymphoma received the regimen of 300 mg/m^2 carmustine, 800 mg/m^2 etoposide, 800 mg/m^2 cytarabine, and 140 mg/m^2 melphalan (BEAM).[33] Forty-eight hours after the last dose of chemotherapy in the CTC regimen or 24 hours after the last dose of chemotherapy in the BEAM regimen, the cryopreserved apheresis products were thawed rapidly at the bedside and were reinfused via an indwelling subclavian catheter. Following transplantation, all patients received G-CSF 5 μg/kg/d, which was started on the day of PBSC transplantation and continued until the WBC count in the peripheral blood was greater than 5 $\times$ 10^9/L. No significant differences in the rate of neutrophil or platelet recovery were found with either high-dose chemotherapy regimens (CTC or BEAM) or in patients with different diagnoses (data not shown).

Transfusions of irradiated leukocyte-free RBCs were administered when the hemoglobin level decreased to less than 5.5 mmol/L. In case a low platelet number induced hemorrhagic diathesis or when platelets were $\leq$ 10 $\times$ 10^9/L, transfusion of 5 to 6 donor units of irradiated platelets was performed.

Hematopoietic Recovery

Primary graft failure was defined as continued requirement of platelet and/or RBC transfusion beyond 3 months after PBSC transplantation, or continued WBC counts less than 3.0 $\times$ 10^9/L at that time. Secondary graft failure was defined as a decrease in blood counts to subnormal levels of at least one lineage for at least 1 month, occurring at any time after initial full engraftment (hemoglobin level $\geq$ 7.0 mmol/L, WBC count $\geq$ 4.0 $\times$ 10^9/L, and platelet count $\geq$ 100 $\times$ 10^9/L).

Immunophenotyping

The percentage of cells that expressed the CD34 antigen was determined in a sample of the leukocytapheresis product by direct immunofluorescence just before cryopreservation. After lysis of the erythrocytes with isomolar amonium chloride buffer for 10 minutes, 1 $\times$ 10^6 cells were incubated with MoAb CD34-FITC. All incubations were performed at 4°C, and after each incubation, the cells

were washed with phosphate-buffered saline (PBS) that contained 0.2% (wt/vol) bovine serum albumin (BSA).

To facilitate further analysis of differentiation antigens on peripheral-blood $CD34^+$ cells, we enriched for immature progenitors by one or two rounds of immunomagnetic depletion of T cells and monocytes. Mononuclear cells (1×10^7/mL) were incubated for 40 minutes at 4°C with a mixture of CD2 and CD14 in the presence of DNAse (20 U/L) and 10 mmol/L magnesium chloride. Cells were washed twice and incubated with immunomagnetic beads coated with goat-antimouse Ig (ratio of beads to cells, 3:1; Dynal, Hamburg, Germany). This procedure did not affect the expression of differentiation antigens (MDR-1, c-*kit*, CD45RO, CD38, HLA-DR, CD7, CD19, CD33, CD13, CD71, CD41, and CD61) on $CD34^+$ cells. For example, the mean percentage ± SD of CD41 and CD33 on $CD34^+$ cells and mean fluorescence intensity (MFI) ± SD of specified antigens before and after immunomagnetic depletion of $CD2^+$ and $CD14^+$ cells (n = 3) were as follows: before depletion—CD41, 4.8% ± 1.5%; MFI, 10.3 ± 1.1; CD33, 41.9% ± 14.9%; MFI, 24.1 ± 3.5; after depletion—CD41, 4.5% ± 0.8%; MFI, 10.0 ± 0.2; CD33, 40.2% ± 14.3%; MFI, 24.4 ± 2.8.

Dual-color flow-cytometric analysis was performed on 158 leukocytapheresis samples from 59 patients. Progenitor cells from 92 leukocytapheresis samples of 27 patients were studied for the expression of 12 antigens. With a limited panel of four MoAbs, antigen expression was studied on progenitor cells from an additional leukocytapheresis samples of 32 patients. For dual-color immunofluorescence analysis, the cells were incubated for 30 minutes with the primary MoAb, followed by incubation with PE-labeled rat-antimouse Ig. An isotype-matched mouse Ig served as control. Residual binding sites of rat-antimouse Ig were blocked with a mixture of irrelevant murine MoAbs of IgG1 and IgG2a subclasses. Subsequently, the cells were incubated with MoAb CD34-FITC. After each incubation, the cells were washed with PBS/BSA. The expression of the platelet antigens CD41 and CD61 was measured after removal of platelets by density centrifugation ($200 \times g$ for 20 minutes) and washing in PBS/BSA that contained 5 mmol/L edathamil (EDTA) to prevent platelet adhesion to $CD34^+$ cells (Dercksen MW, Weimar IS, Richel DJ, et al, submitted). Flow-cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). A minimum of 20,000 cells was acquired in list mode. Analysis of the five-dimensional data was performed with Consort 30 software (Becton Dickinson, San Jose, CA). The percentage of $CD34^+$ cells present in the leukocytapheresis product was assessed after correction for the percentage of cells reactive with an isotype control. For the determination of the phenotype of $CD34^+$ cells, a minimum of 2,500 $CD34^+$ cells was analyzed. A marker was set at the first log decade and the percentage of $CD34^+$ cells that coexpressed a specific antigen was assessed after correction for the percentage of cells reactive with an isotype control. Absolute numbers of $CD34^+$ progenitor cells were calculated by multiplication of the total number of nucleated blood cells in the leukocytapheresis product with the percentage of $CD34^+$ cells in the total leukocytapheresis product. Absolute numbers of a subset of $CD34^+$ cells were determined by multiplication of the total number of $CD34^+$ cells in the leukocytapheresis product with the percentage of $CD34^+$ cells that expressed a specific antigen.

Statistical Analysis

For nonnormal distributed values, data were summarized as medians and ranges; otherwise, the arithmetic mean ± SD was used. Differences were calculated by means of the Mann-Whitney *U* test. The correlations are Spearman-rank correlations. A *P* value less than $\alpha = .05$ was considered significant.

For the assessment whether a certain cell dose predicts for rapid or slow recovery threshold values were calculated for each day after PBSC transplantation. The following four subpopulations can be distinguished: quadrant A, patients who received relatively low cell numbers with a relatively slow recovery; quadrant B, patients who received relatively high cell numbers with a relatively slow recovery; quadrant C, patients who received relatively low cell numbers with a relatively rapid recovery; and quadrant D, patients who received relatively high cell numbers with a relatively rapid recovery. Threshold values for rapid hematopoietic recovery were defined by the optimal sensitivity (the number of patients in quadrant A divided by the sum of the number of patients in quadrant A plus B) and specificity (the number of patients in quadrant C divided by the sum of the number of patients in quadrant C plus D) of a tested parameter (Fig 1A), as determined in the receiver operating characteristic curve.

The Wilcoxon (Gehan) test was used to study the differences between the subpopulation of patients who received relatively high or low numbers of cells. To determine simultaneously the relative influence of parameters (multivariate analysis), a Cox's proportional hazards model was used. The models were built in a stepwise procedure.

RESULTS

Hematopoietic Recovery After PBSC Transplantation

Following high-dose chemotherapy, a median number of 6.4×10^6 $CD34^+$ cells/kg (range, 1.3 to 39.4×10^6/kg) was reinfused in 59 patients. Thirty-four of 59 patients had recovered to neutrophil counts of at least 0.5×10^9/L at day 10 after reinfusion (n = 59; median, 10 days; range, 8 to 28). Platelet transfusion independence (defined as platelet count remaining $\geq 20 \times 10^9$/L without platelet transfusions) was achieved within 37 days after PBSC transplantation in all patients. Thirty of 59 patients had recovered within 14 days (n = 59; median, 14 days; range, 7 to 37). A median number of four platelet transfusions (range, one to 14) and six RBC transfusions (range, two to 12) were administered.

Threshold Values for Rapid Neutrophil Recovery

For an assessment whether a certain cell dose of $CD34^+$ cells per kilogram predicts for rapid or slow recovery, we calculated threshold values of $CD34^+$ cells per kilogram. These calculations were based on the day that neutrophils reached counts greater than 0.5×10^9/L. As the calculated threshold value is dependent on the definition of rapid recovery, threshold values for days 9 to 14 after PBSC transplantation were calculated (Table 1). For days 9 to 11, the relationship between these threshold values of $CD34^+$ cells per kilogram with a high probability of engraftment and days of neutrophil recovery is represented by a steep curve, whereas for days 12 to 14, the curve reaches a horizontal plateau (Fig 2). However, this part

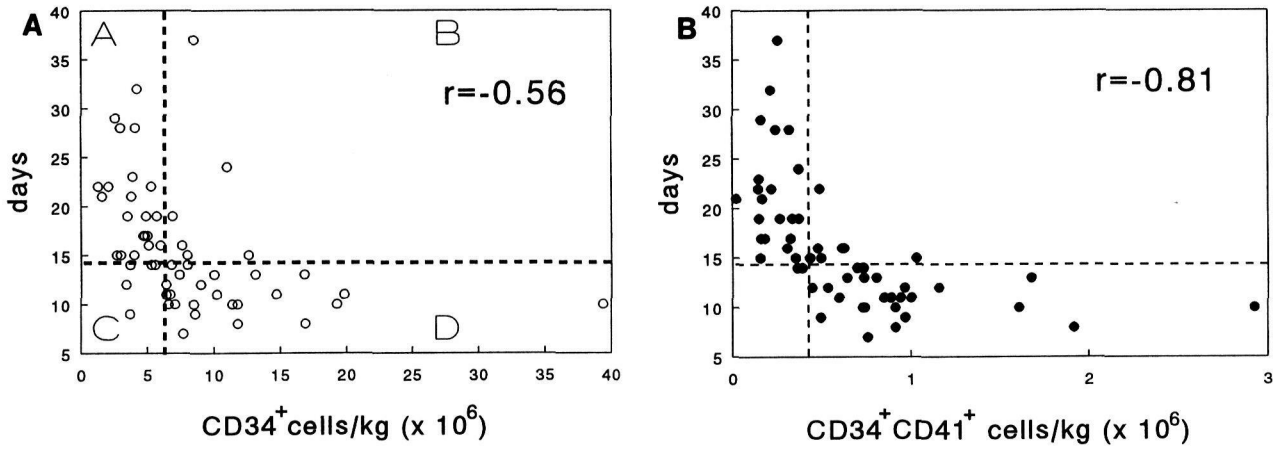

Fig 1. Correlation between number of reinfused (A) CD34+ cells/kg or (B) CD34+CD41+ cells and time to platelet transfusion independence. (---) Calculated threshold values of reinfused CD34+ cells or CD34+CD41+ cells for rapid platelet recovery (6.0 × 10⁶ CD34+ cells/kg and 0.57 × 10⁶ CD34+CD41+ cells/kg) and time for rapid platelet recovery.

of the curve is based on few observations. The most informative part of the curve (days 9 to 11) indicates that the reinfusion of four times more CD34+ cells per kilogram will only result in 1 to 2 days earlier recovery of neutrophils. The steep end of the dose-effect curve suggests that it is hard to enhance neutrophil recovery by increasing cell dose.

The median time to neutrophil recovery in this group of patients was 10 days and the threshold value for this day was calculated to be 6.0 × 10⁶ CD34+ cells/kg. Patients who received more CD34+ cells than this threshold value reached neutrophil counts of 0.5 × 10⁹/L significantly faster than patients who received relatively low numbers of CD34+ cells ($P = .001$). However, the difference in median time to reach neutrophil recovery for these two groups was only 1 day and did not differ significantly (≥ 6.0 × 10⁶ CD34+ cells/kg, n = 31 [median, 10 days, range, 8 to 21], v < 6.0 × 10⁶ CD34+ cells/kg, n = 28 [median, 11 days, range, 9 to 28]).

Table 1. Threshold Values of the Number of CD34+ Cells per Kilogram for Rapid Neutrophil Recovery (> 0.5 × 10⁹/L)

Rapid Recovery (within × days)	Threshold Value (no. of CD34+ cells 10⁶/kg)	Patients With Rapid Recovery After Reinfusion of More CD34+ Cells/kg Than (or equal to) the Threshold Value		Patient With Rapid Recovery After Reinfusion of Fewer CD34+ Cells/kg Than the Threshold Value	
		No.	%	No.	%
9	11.7	8/10	80.0	5/49	10.2
10	6.0	24/31	77.4	10/28	35.7
11	2.9	46/54	85.2	0/5	0.0
12	1.7	51/57	89.5	0/2	0.0
13	1.7	52/57	91.2	1/2	50.0
14	1.7	53/57	93.0	1/2	50.0

Threshold Values for Rapid Platelet Recovery

The threshold values of the number of CD34+ cells per kilogram for rapid platelet transfusion independence are shown in Fig 2 and Table 2. These results demonstrate that by increasing the number of CD34+ cells from 2.5 × 10⁶ CD34+ cells/kg to 10.7 × 10⁶ CD34+ cells/kg (~ fourfold), the majority of patients had platelet recovery within 11 days instead of 17 days.

A threshold value of 6.1 × 10⁶ CD34+ cells/kg was calculated for platelet transfusion independence within 14 days, which is the median time to platelet transfusion independence of all 59 patients (Fig 1A). Reinfusion of

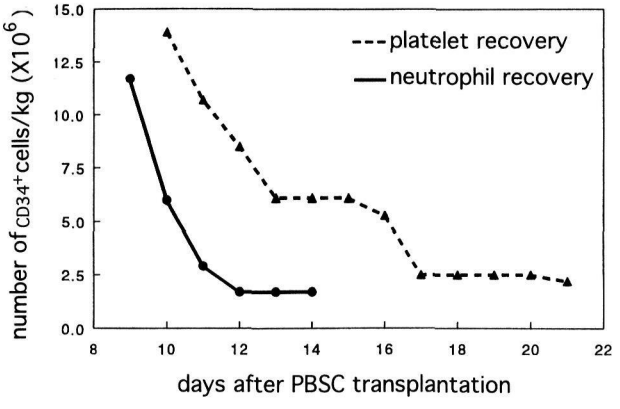

Fig 2. Threshold values for rapid neutrophil (——) and platelet recovery (----).

Table 2. Threshold Values of the Number of CD34$^+$ Cells per Kilogram for Rapid Platelet Transfusion Independence

Rapid Recovery (within × days)	Threshold Value (no. of CD34$^+$ cells × 10^6/kg)	Patients With Rapid Recovery After Reinfusion of More CD34$^+$ Cells/kg Than (or equal to) the Threshold Value		Patients With Rapid Recovery After Reinfusion of Fewer CD34$^+$ Cells/kg Than the Threshold Value	
		No.	%	No.	%
10	13.9	3/6	50.0	9/53	17.0
11	10.7	8/12	66.7	9/47	19.1
12	8.5	11/16	68.8	10/43	23.3
13	6.1	22/31	71.0	3/28	10.7
14	6.1	24/31	77.4	6/28	21.4
15	6.1	26/31	83.9	10/28	35.7
16	5.3	30/34	88.2	10/25	40.0
17	2.5	43/56	76.8	0/3	0.0
19	2.5	47/56	83.9	0/3	0.0
21	2.2	48/57	84.2	0/2	0.0

more than this threshold value was associated with a significantly shorter time to platelet transfusion independence ($P < .001$, n = 31; median, 11 days, range, 11 to 37) as compared with the group of patients who received relatively low numbers of CD34$^+$ cells (n = 28; median, 17 days; range, 9 to 32).

Subpopulations of CD34$^+$ Cells

Dual-color flow-cytometric analysis on leukocytapheresis samples of 59 patients was performed to determine the percentage antigen expression on CD34$^+$ cells present in the leukocytapheresis product and the absolute number of that subset. The expression of 12 antigens was measured on progenitor cells from 27 patients. These MoAbs define the markers that are linked to early multipotent stem cells (MDR-1, c-*kit*, CD45RO, CD38$^-$, HLA-DR$^-$, CD33$^-$, and CD13$^-$), as well as the differentiation of hematopoietic cells of different lineages, ie, lymphoid (CD7 and CD19), myeloid (CD33 and CD13), erythroid (CD71), and megakaryocytic (CD41 and CD61). With a selected panel of four informative MoAbs, antigen expression was studied on progenitor cells of an additional 32 patients.

Coexpression of multipotent stem-cell–associated antigens. The multipotent stem-cell–associated antigens MDR-1 and c-*kit* were expressed on CD34$^+$ cells in low frequencies (MDR-1 [n = 27]: median, 1.9% [range, 0.7% to 6.0%); c-*kit* [n = 27]: median, 2.2% (range, 0.2% to 9.0%]), whereas a considerable fraction of the reinfused CD34$^+$ cells expressed the CD45RO antigen (n = 59; median, 16.5%; range, 4.6% to 33.7%). The majority of CD34$^+$ cells strongly expressed the CD38 antigen (n = 59; median, 79.5%; range, 36.4% to 98.2%) and the HLA-DR antigen (n = 27; median, 90.8%; range, 28.2% to 95.6%) (Fig 3A). Small populations of CD34$^+$CD38$^-$ and CD34$^+$HLA-DR$^-$ cells were identified. The median total numbers of reinfused subsets of CD34$^+$ cells are listed in Table 3. The results show that a median of approximately 0.5 × 10^6 CD34$^+$ cells/kg that expressed the multipotent stem-cell–associated antigens MDR-1$^+$, c-*kit*$^+$, or HLA-DR$^-$ were reinfused, whereas about twice as many CD34$^+$CD45RO$^+$ cells per kilogram and CD34$^+$CD38$^-$ cells per kilogram were detected in the PBSC transplant. These data indicate that in every PBSC transplant, CD34$^+$ cells were present with the phenotype of multipotent stem cells.

Coexpression of myeloid lineage-associated antigens. The majority of reinfused peripheral-blood CD34$^+$ cells strongly expressed the pan-myeloid antigen CD13 (n = 27; median, 87.0%; range, 48.3% to 98.0%), whereas a considerable proportion of CD34$^+$ cells expressed low levels of the CD33 antigen (n = 59; median, 25.7%; range, 0.8% to 85.8%) (Fig 3B). As a result, high numbers of CD34$^+$CD13$^+$ cells per kilogram were reinfused in most patients, whereas a broad range of CD34$^+$CD33$^+$ cells per kilogram was found in the PBSC transplant.

Coexpression of erythroid and lymphoid lineage-associated antigens. The fraction of CD34$^+$ cells present in a transplant that expressed the erythroid lineage-associated antigen CD71 varied considerably, ranging from 3.1% to 41.2% (n = 27; median, 15.2%). Lymphoid-associated antigens were detected on few CD34$^+$ cells. In fact, in most of the transplants, less than 2% of the CD34$^+$ cells expressed the T-cell lineage-associated antigen CD7 (n = 27; median, 1.8%; range, 0.3% to 9.7%) or the B-cell lineage-associated antigen CD19 (n = 27; median, 0.8%, range, 0% to 6.7%) (Fig 3B).

Coexpression of megakaryocytic lineage-associated antigens. The expression of megakaryocytic lineage-associated antigens, including CD41 and CD61, was measured in the presence of EDTA to prevent platelet adhesion to CD34$^+$ cells, which may hamper the accurate detection of endogenously expressed platelet glycoproteins (Dercksen MW, Weimar IS, Richel DJ, et al, submitted). Under these conditions, a small but distinct population of CD34$^+$ cells that coexpressed the platelet antigens CD41 and CD61 was observed in the PBSC transplant. A median of 8.1% of the CD34$^+$ cells expressed the CD41 antigen (n = 59; range, 1.4% to 17.0%), and CD61 was detected on a median of 2.6% of CD34$^+$ cells (n = 27; range, 0.3% to 8.2%) (Fig 3B). The results show that in most of the patients less than 1.0 × 10^6 CD34$^+$CD41$^+$ cells/kg and in all patients fewer than 1.0 × 10^6 CD34$^+$CD61$^+$ cells/kg were reinfused (Table 3).

In summary, a small but distinct subpopulation of CD34$^+$ cells present in the leukocytapheresis product expressed antigens associated with multipotent stem cells.

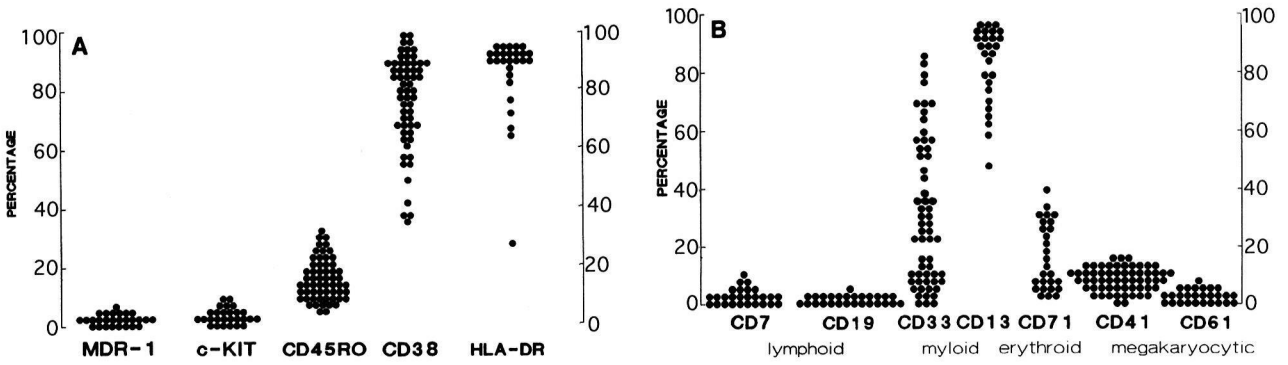

Fig 3. Antigen expression on CD34+ cells of 27 or 59 patients. Dots represent (A) percentage of CD34+ cells present in leukocytapheresis product expressing MDR-1, c-*kit*, HLA-DR (n = 27) or CD45RO, and CD38 (n = 59), or (B) percentage of CD34+ cells expressing CD7, CD19, CD13, CD71, CD61 (n = 27) or CD33, and CD41 (n = 59).

A predominant expression of myeloid lineage-associated antigens on mobilized CD34+ cells was found, while a minority of CD34+ cells expressed the erythroid and megakaryocytic lineage-associated antigens. In contrast, low or undetectable expression of lymphoid lineage-associated antigens was detected on mobilized CD34+ cells.

Correlation Between Subsets of Reinfused CD34+ Cells and Neutrophil Recovery After PBSC Transplantation

Spearman-rank correlation assays were applied to assess the relationship between the total number of CD34+ cells or the number of CD34+ cells belonging to each of the

Table 3. Correlation of Subsets of CD34+ Cells With Time to Neutrophil and Platelet Recovery

Subset of CD34+ Cells	n	No. of Reinfused Cells ($\times 10^6$/kg) Median	Range	Time to Neutrophil Recovery (> 0.5 × 10^9/L) Correlation Coefficient	95% CI	Time to Platelet Transfusion Independence Correlation Coefficient	95% CI
All cells	27	6.0	1.6-39.4	−.51	−.75-−.16	−.55	−.77-−.21
	59	6.4	1.3-39.4	−.55	−.71-−.34	−.56	−.71-−.35
Subset of cells							
Multipotent							
CD34+MDR-1+	27	0.12	0.01-0.72	−.32	−.62-.07	−.39	−.67-−.01
CD34+c-*kit*+	27	0.12	0.01-0.72	−.36	−.65-.02	−.58	−.45-.13
CD34+CD45RO+	59	0.94	0.14-8.79	−.48	−.66-−.26	−.42	−.61-−.18
CD34+CD38−	59	1.12	0.13-7.91	−.24*	−.47-−.02	−.30*	−.52-−.05
CD34+HLA-DR−	27	0.56	0.16–4.62	−.34	−.64-.05	−.41	−.68-−.04
CD34+CD33−	59	4.14	0.37-30.64	−.74*	−.84-−.60	−.70	−.81-−.54
CD34+CD13−	27	0.88	0.07-8.66	−.28	−.60-.11	−.42	−.69-−.05
Differentiated							
CD34+CD38+	59	4.61	1.09-35.58	−.53	−.69-.32	−.54	−.70-−.33
CD34+HLA-DR+	27	5.45	1.35-37.11	−.56	−78-−.23	−.50	−.74-−.15
Myeloid							
CD34+CD33+	59	1.49	0.03-9.90	−.05†	−.30-.21	.06‡	−.20-.31
CD34+CD13+	27	5.17	1.35-30.74	−.60	−.80-−.28	−.58	−.79-−.26
Erythroid							
CD34+CD71+	27	2.32	0.16-9.87	−.31	−.62-.08	−.42	−.69-−.05
Megakaryocytic							
CD34+CD41+	59	0.50	0.02-2.92	−.61	−.75-−.42	−.81†	−.89-−.71
CD34+CD61+	27	0.19	0.01-0.79	−.32	−.62-.07	−.78	−.89-−.57

Abbreviation: 95% CI, 95% confidence interval.

*Significantly different from the correlation between the total number of CD34+ cells and time to neutrophil recovery or time to platelet transfusion independence at $P < .05$.

†$P < .005$.

‡$P < .0005$.

reinfused subsets and the time to neutrophil recovery (neutrophil count $> 0.5 \times 10^9$/L) after PBSC transplantation. In a group of 27 patients, the correlation for the total number of $CD34^+$ cells with time to neutrophil recovery was −0.51 (Table 3). Although the correlation coefficient was improved for several subsets of $CD34^+$ cells (CD45RO, CD38, and CD33), in this small group of patients none of these subsets correlated significantly better with time to neutrophil recovery than the total number of $CD34^+$ cells.

Based on the results in the group of 27 patients, the study was extended, and on $CD34^+$ cells from an additional 32 patients, the expression of a limited number of markers (CD45RO, CD38, and CD33) was measured. In this population of 59 patients, the correlation between the numbers of $CD34^+$ cells and time to neutrophil recovery ($r = -.55$) was significantly improved when the numbers of $CD34^+$ cells that lacked expression of the CD33 antigen were correlated with time to neutrophil recovery ($r = -.74$, $P = .04$) (Table 3).

Fifty percent of patients reached a neuthophil count greater than 0.5×10^9/L at day 10. For a better understanding of the clinical implications of the calculated threshold values, we used these 50% points at day 10 to illustrate the clinical significance of reinfusion of more or fewer cells than the calculated threshold values. The threshold value of the number of $CD34^+CD33^-$ cells per kilogram for neutrophil recovery within 10 days was calculated at 2.79×10^6 $CD34^+CD33^-$ cells/kg. Reinfusion of more than this threshold value was associated with a significant shorter time to neutrophil recovery ($P = .001$) as compared with the group of patients who received relatively low numbers of $CD34^+CD33^-$ cells ($\geq 2.79 \times 10^6$ $CD34^+CD33^-$ cells/kg: n = 40 [median, 10 days; range, 8 to 17], $v < 2.79 \times 10^6$ $CD34^+CD33^-$ cells/kg: n = 19 [median, 12 days; range, 10 to 28]). Thirty-two of 40 patients (80%) who received more than the threshold value had recovered within 10 days, whereas only two of 19 (10.5%) who received fewer $CD34^+CD33^-$ cells had recovered in this time. In addition, the difference in median time to reach neutrophil recovery within 10 days for these two groups ($<$ or $\geq 2.7 \times 10^6$ $CD34^+CD33^-$ cells/kg) was 2 days and was highly significant ($P < .001$) (Fig 4A), whereas no statistically significant difference in median time to reach neutrophil recovery was found between the patients who received more or fewer than 6.0×10^6 $CD34^+$ cells/kg.

Correlation Between Subsets of Reinfused $CD34^+$ Cells and Platelet Recovery After PBSC Transplantation

When subsets of $CD34^+$ cells were correlated with the time to platelet transfusion independence (n = 27), the $CD34^+CD33^-$ cells and $CD34^+$ cells that expressed the megakaryocytic lineage-associated antigen CD41 correlated significantly better ($r = -.82$, $P < .05$ and $r = -.83$, $P < .05$, respectively) than did the total number of $CD34^+$ cells ($r = -.55$) (Table 3). The expression of the CD33 and CD41 antigens was measured on progenitor cells from an additional 32 patients. In the population of 59 patients, a significantly better correlation with time to platelet transfusion independence in comparison with the total number of $CD34^+$ cells ($r = -.56$) was found for $CD34^+CD41^+$ cells ($r = -.81$, $P = .004$). Although the number of $CD34^+$ cells that lacked expression of the CD33 antigen also correlated better with time to platelet transfusion independence ($r = -.70$) than did the total number of $CD34^+$ cells, at this time this difference failed to reach statistical significance ($P = .11$).

The threshold value of the number of $CD34^+CD41^+$ cells per kilogram for platelet transfusion independence within 14 days was calculated at 0.54×10^6 $CD34^+CD41^+$ cells per kilogram. In the 28 patients who received more than this threshold value, the time to platelet recovery was significantly faster (median, 11 days; range, 7 to 16) as compared with time to platelet recovery of the patients who received fewer $CD34^+CD41^+$ cells per kilogram (n = 31; median, 19 days, range, 9 to 37) ($P < .0001$; Figs 1B and 4B). Twenty-five of 28 patients (89.3%) who received more than the threshold had recovered within 14 days, whereas only five of 31 (16.1%) who received fewer $CD34^+CD41^+$ cells per kilogram were platelet transfusion–independent at that time.

Multivariate Analysis

When the subsets of $CD34^+$ cells that correlated better with neutrophil and platelet recovery ($CD45RO^+$, $CD33^-$, and $CD41^+$) as compared with the total number of $CD34^+$ cells were analyzed in a Cox's model together with the total $CD34^+$ cell population, the only parameter predictive for rapid neutrophil recovery was the number of $CD34^+$ that lacked expression of the CD33 antigen ($P < .0001$). The most predictive parameter for rapid platelet recovery was the number of $CD34^+$ cells that expressed CD41 ($P < .0001$).

Graft Failures

Primary graft failures (see Patients and Methods for definition) were not observed. However, in two patients, secondary graft failure occurred. In one patient, reinfusion of 3.9×10^6 $CD34^+$ cells/kg resulted in a neutrophil recovery to values greater than 0.5×10^9/L within 16 days and platelet transfusion independence within 23 days. After primary engraftment with platelet recovery

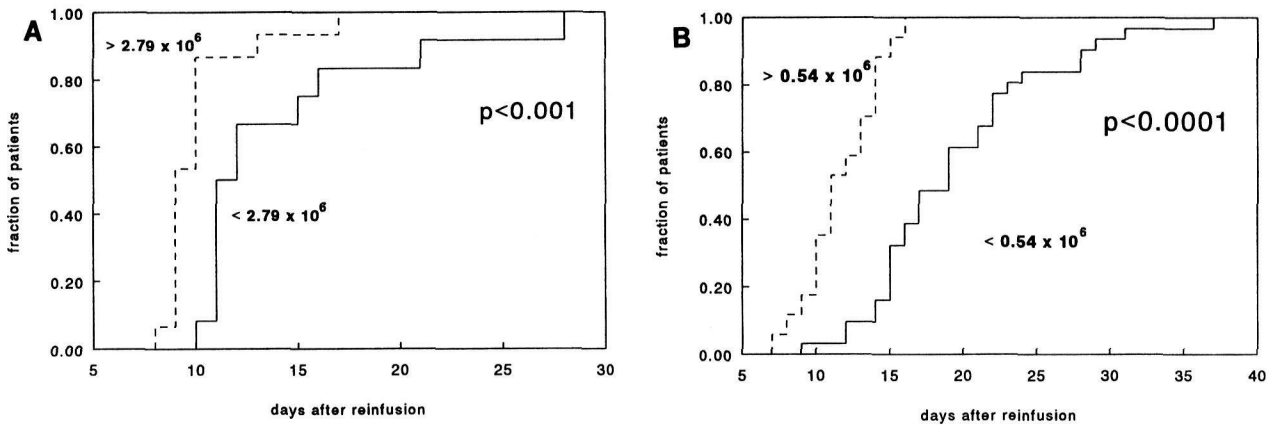

Fig 4. (A) Neutrophil count recovery to 0.5 × 10^9/L after PBSC transplantation; (----) ≥ 2.79 × 10^6 $CD34^+CD33^-$ cells/kg grafted (n = 40; median, 10 days; range, 8-17); (——) < 2.79 × 10^6 $CD34^+CD33^-$ cells/kg grafted (n = 19; median, 12 days; range, 10-28). (B) Platelet recovery to platelet transfusion independence after PBSC transplantation; (----) ≥ 0.54 × 10^6 $CD34^+CD41^+$ cells/kg grafted (n = 28; median, 11 days; range, 7-16); (——) < 0.54 × 10^6 $CD34^+CD41^+$ cells/kg grafted (n = 31; median, 19 days; range, 9 to 37).

up to 75 × 10^9/L and a maximum WBC count of 4.5 × 10^9/L, cytopenia reoccured. From day 57 after PBSC transplantation, the number of platelets decreased to levels of 20 × 10^9/L, while the WBC counts declined to 2.6 × 10^9/L. This secondary graft failure resolved 78 days later. The other patient was reinfused with 1.6 × 10^6 $CD34^+$ cells/kg, and neutrophil and platelet recovery were observed on days 15 and 21, respectively. After 3 months, the secondary graft failure occurred (platelet count, 15 × 10^9/L; WBC count, 2.0 × 10^9/L).[34] Reinfusion of autologous BM, which had been stored before high-dose chemotherapy, 197 days after transplantation resulted in a rapid and sustained hematologic reconstitution. Both patients had received low numbers of $CD34^+$ cells that expressed MDR-1 (0.10 × 10^6 and 0.01 × 10^6 $CD34^+$MDR-1^+ cells/kg, respectively), CD45RO (0.32 × 10^6 and 0.14 × 10^6 $CD34^+CD45RO^+$ cells, respectively), or CD41 (0.15 × 10^6 and 0.02 × 10^6 $CD34^+CD41^+$ cells, respectively), and had received the lowest number of $CD34^+$ cells that expressed c-*kit* antigen (0.03 × 10^6 and 0.01 × 10^6 $CD34^+$ c-kit^+ cells/kg, respectively) of all 59 patients. Both patients had received a moderate number of $CD34^+CD33^-$ cells per kilogram (0.66 × 10^6 and 1.19 × 10^6 $CD34^+CD33^-$ cells, respectively).

DISCUSSION

An important clinical issue is whether a relationship exists between cell dose and the rate of hematopoietic recovery after PBSC transplantation. Because a threshold value for rapid recovery is dependent on the definition of rapid recovery, we calculated a threshold value for the number of $CD34^+$ cells per kilogram with a high probability of engraftment for each day after PBSC transplantation. For days 9 to 11, a steep dose-effect relationship between $CD34^+$ cell dose and day of recovery was found for neutrophils. These data indicate that, even at high cell doses, a dose-response effect still exists. Also, because of the steep form of the curve, it will be hard to achieve a further reduction in neutrophil recovery below 9 days only by increasing the cell dose. For platelet recovery, the dose-effect relationship was less steep, and a fourfold increase in the number of $CD34^+$ cells, from 2.5 × 10^6 to 10.7 × 10^6 $CD34^+$ cells/kg, resulted in a 6-day earlier recovery of platelets. These data suggest that reinfusion of at least 10 × 10^6 $CD34^+$ cells/kg may result in fewer days of neutropenia and fewer days that require platelet transfusion as compared with 2.5 × 10^6 $CD34^+$ cells/kg.

Transplantation of purified $CD34^+$ cells can restore hematopoiesis after high-dose chemotherapy.[4] To obtain an indication whether specific subpopulations of $CD34^+$ cells contribute to rapid recovery of platelets or neutrophils, the expression of a variety of cell-surface markers, including myeloid, erythroid, lymphoid, and megakaryocytic lineage-associated antigens, on $CD34^+$ cells was assessed, and the number of $CD34^+$ cells belonging to each of the reinfused subsets was quantified. The phenotypic pattern of antigen expression on the $CD34^+$ cells collected during the recovery from myelosuppression-inducing chemotherapeutic regimens followed by G-CSF is in agreement with reports by others,[18,35-38] as we demonstrated a dominant expression of myeloid antigens, distinct $CD34^+$ cell populations that expressed erythroid and megakaryocytic lineage-associated antigens, and a limited number of T- and B-lymphoid progenitor cells present in

the PBSC transplant. Only low percentages of mobilized $CD34^+$ cells expressed the multipotent stem-cell–associated antigens c-*kit,* MDR-1, and CD45RO. In addition, small populations of $CD34^+CD38^-$ and $CD34^+$HLA-DR^- were clearly identified. These observations suggest the presence of primitive progenitors in the PBSC transplant and are consistent with clinical reports that PBSCs are capable of providing long-term reconstitution.[39] In two patients, secondary graft failure developed after reinfusion of PBSCs. In one patient, the reinfusion of the relatively low number 1.6×10^6 $CD34^+$ cells/kg could have caused this secondary graft failure. The observation that both patients received low numbers of $CD34^+$ cells that expressed MDR-1 or CD45RO and the lowest number of $CD34^+$ cells that expressed c-*kit* of all 59 patients suggests that one or more of these subpopulations of $CD34^+$ cells with the phenotype of early progenitor cells could be involved in the durable engraftment after PBSC transplantation. Our finding that none of $CD34^+$c-kit^+, $CD34^+$MDR-1^+, $CD34^+CD38^-$, or $CD34^+$HLA-DR^- subpopulations correlated significantly better with neutrophil or platelet recovery does not exclude that these populations are critically important for engraftment, as it may be possible that the analysis techniques do not reliably measure these small numbers of cells and therefore these measurements lack statistical power.

According to one hypothesis, committed progenitor cells are responsible for short-term repopulating ability of the graft. Furthermore, the transplantation of primitive, early progenitor cells will result in delayed engraftment, because these cells have to undergo more cycles before becoming a mature granulocyte, platelet, or erythrocyte. The first part of the hypothesis is supported by several murine studies, which demonstrated that transplantation of isolated committed progenitor cells results in a transient engraftment, but not in sustained engraftment.[40] The second part of the hypothesis is under discussion. Jones et al[41,42] separated murine BM cells by flow-density centrifugation and identified a population that resulted in long-term engraftment, without short-term repopulating ability. However, other studies have demonstrated that few (10 to 100) very early purified stem cells can protect mice from death caused by lethal irradiation.[43-45] Moreover, Uchida et al[46,47] showed that in mice very early progenitor cells attribute to early reconstitution. In man, no data are available about the cells that contribute most to the rapid hematopoietic recovery after PBSC transplantation. Our finding that the number of $CD34^+CD33^-$ cells correlates significantly better with neutrophil recovery than the total number of $CD34^+$ cells suggests that, within this relative immature subpopulation, the cells reside that are important for rapid neutrophil recovery after PBSC transplantation. The pluripotent $CD34^+$ cells probably do not contribute to the rapid recovery, because the correlation between the $CD34^+$ cells that coexpress the multipotent stem-cell–associated antigens and time to neutrophil recovery was lost.

For platelet recovery, the number of $CD34^+CD41^+$ cells correlated significantly better with time to platelet transfusion independence after PBSC transplantation than did the total number of $CD34^+$ cells. When the number of $CD34^+CD41^+$ cells per kilogram was used as a threshold value, the difference in median time to platelet recovery between the groups of patients reinfused with either relative high or low numbers of $CD34^+CD41^+$ cells was increased by 2 days as compared with the difference in median time to platelet recovery when the number of $CD34^+$ cells per kilogram was used as a parameter. Therefore, our results indicate that the determination of CD41 on $CD34^+$ cells may help to predict short-term repopulation capacity of the PBSCs. These data suggest that the putative megakaryocytic progenitor cells are important in platelet recovery.

The practical benefit of additional phenotyping for neutrophil recovery seems limited, because the time to neutrophil recovery showed only little variation. Only five patients had not recovered their neutrophil counts within 14 days after PBSC transplantation. However, in contrast to neutrophil recovery, the time to platelet recovery was widely distributed, especially when relatively low numbers of $CD34^+$ cells per kilogram were reinfused. Therefore, in this group of patients, the determination of CD41 on $CD34^+$ cells may help to select patients who are likely to have slow recovery.

In addition to PBSC transplantation, phenotyping of $CD34^+$ cells can be used for other applications. It can be used to optimize stem-cell–mobilizing regimens or purging techniques. Furthermore, these data are important for ex vivo expansion of progenitor cells, because our data suggest that selective expansion of $CD34^+CD33^-$ cells and $CD34^+CD41^+$ cells could result in rapid neutrophil, as well as platelet, recovery after transplantation.

In summary, our data indicate that the numbers of reinfused $CD34^+CD33^-$ cells per kilogram and $CD34^+$ $CD41^+$ cells per kilogram result in a significantly improved correlation with neutrophil recovery and platelet recovery, respectively, as compared with the total number of $CD34^+$ cell per kilogram. Moreover, these subsets are important for rapid recovery after PBSC transplantation. Our results also suggest that the more rapid recovery after PBSC transplantation in comparison to BM transplantation is due to higher numbers of reinfused cells rather

than to overpresentation of more mature stem cells in the peripheral blood. Furthermore, additional phenotyping and quantification of subsets of $CD34^+$ cells may help to predict short-term repopulation capacity of the PBSCs, especially when relatively low numbers of $CD34^+$ cells are reinfused. This procedure can be useful in the establishment of a practical minimum of progenitor cells required for rapid engraftment.

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