Peripheral blood progenitor cells (PBPCs) are increasingly used for hematopoietic support after high-dose chemotherapy and/or radiotherapy. PBPCs can be mobilized by chemotherapy, cytokines, or a combination of both. Multipotential and lineage-committed hematopoietic progenitor cells express the CD34 antigen. After filgrastim (R-metHuG-CSF, Amgen, Thousand Oaks, CA)-supported chemotherapy, CD34+ progenitor cell harvests differ individually by more than 100-fold. Factors adversely affecting the yield of the hematopoietic progenitor cells are the amount of previous cytotoxic chemotherapy and irradiation. However, a statistical analysis of clinical characteristics does not allow an individual prediction for a given patient.

We were interested in whether the analysis of hematopoietic progenitor cells before administration of cytotoxic therapy would allow us to establish a relationship to the individual mobilization efficiency.

During adult life, the bone marrow (BM) is the major hematopoietic organ in humans. On the other hand, primitive hematopoietic cells are also circulating in the PB. In mice it could be shown that cyclophosphamide followed by granulocyte colony-stimulating factor (G-CSF) leads to an increase of progenitor cells in PB, whereas their content in the BM decreased. Therefore, we looked whether during steady-state hematopoiesis the number of CD34+ cells or colony-forming cells in either of these compartments would be predictive for the number of circulating hematopoietic progenitor cells after filgrastim-supported chemotherapy.

In steady-state BM and in LP samples, the CD34+ cells were also assessed for coexpression of the myeloid-associated antigens CD33, CD45-RA, and the B-lymphoid antigen CD19. Phenotypically primitive CD34+ hematopoietic progenitor cells could be evaluated because they lack HLA-DR coexpression. The study showed that steady-state PBPC counts allowed to establish a relationship to the individual mobilization efficiency.

MATERIALS AND METHODS

Patients. Fifteen patients (2 men/13 women) were enrolled into this study. Their median age was 34 years (range, 22 to 58 years). Six patients had non-Hodgkin’s lymphoma (NHL). According to the working formulation, there were four high-grade NHL and two low-/intermediate-grade NHL. Two patients had Hodgkin’s disease and two multiple myeloma. There were three patients with breast cancer, one with ovarian tumor and one with a germ cell tumor. In all patients, the objective was to collect PBPC after chemotherapy plus granulocyte colony-stimulating factor mobilization. Before the mobilization regimen, multiparameter flow-cytometry analysis and clonogenic assays were used to examine the hematopoietic progenitor cells in bone marrow (BM) and peripheral blood (PB) before filgrastim (R-metHuG-CSF, Amgen, Thousand Oaks, CA)-supported chemotherapy and in PB and leukapheresis products (LPs) in the recovery phase. Fifteen patients (four with high-grade non-Hodgkin’s lymphoma [NHL], two with low-grade NHL, two with Hodgkin’s disease, two with multiple myeloma, three with breast cancer, one with ovarian cancer, and one with germ cell tumor) were included in this study. The comparison of immunofluorescence plots showed a homogenous population of strongly CD34+ cells in steady-state and mobilized PB whereas in steady-state BM, the CD34+ cells ranged from strongly positive with continuous transition to the CD34- population. Consistent with the similarity in CD34 antigen expression, a correlation analysis showed steady-state PB CD34+ cells (r = .81, P < .001) and colony-forming cells (CFCs; r = .69, P < .01) to be a measure of a patient’s mobilizable CD34+ cell pool. Individual estimates of progenitor cell yields could be calculated. With a probability of 95%, eg, 0.4 steady-state PB CD34+ cells × 10^6/L allowed to collect in six LPs 2.5 × 10^6 CD34+ cells/kg, the reported threshold-dose of progenitor cells required for rapid and sustained engraftment after high-dose therapy. For the total steady-state BM CD34+ cell population, a weak correlation (r = .57, P < .05) with the mobilized CD34+ cells only became apparent when an outlier was removed from the analysis. Neither the CD34+ immunologic subgroups defined by the coexpression of the myeloid lineage-associated antigens CD33 or CD45-RA or the phenotypically primitive CD34+/HLA-DR- subset nor the BM CFC count had a predictive value for the mobilization outcome. This may be caused by the additional presence of maturing progenitor cells in BM, which express lower levels of the CD34 antigen and do not circulate. Our results permit us to recognize patients who are at risk to collect low numbers of progenitor cells and those who are likely to achieve sufficient or high progenitor cell yields even before mobilization chemotherapy is administered.
bilation therapy, BM aspirates from the posterior iliac crest and PB samples were obtained. The interval from the last chemotherapy before the mobilization therapy was at least 34 days.

The patients with hematologic malignancies received cytotoxic chemotherapy such as HAM (high-dose cytosine arabinoside, 2 g/m² every 12 hours on days 1 and 2, and mitoxantrone, 10 mg/m²/d on days 2 and 3; six patients), dexaBEAM (dexamethasone 24 mg/m² every 12 hours on days 1, 2, and 3; 2, and mitoxantrone, 10 mg/m²/d on days 4 to 7; carmustine, 75 mg/m² on day 1; etoposide, 500 mg/m² on day 2; and cisplatin, 50 mg/m² on day 2 (PEI-2). The regimen for the patient with germ cell tumor consisted of cisplatin, 50 mg/m² on day 2; and melphalan, 30 mg/m² on day 3; two patients) or cyclophosphamide (4 g/m², one patient; 7 g/m², one patient). Patients with breast cancer were treated with ifosfamide, 1.2 g/m²/d, and ifosfamide, 1.2 g/m²/d, all on days 1 to 7; carbustine, 60 mg/m² on day 3; etoposide, 75 mg/m² on day 4 to 7; cytosine arabinoside, 200 mg/m² on days 4 to 7; and melphalan, 30 mg/m² on day 3; two patients) or cyclophosphamide (4 g/m², one patient; 7 g/m², one patient). Patients with breast cancer were treated with ifosfamide, 5 g/m²/d on day 1, and epirubicin, 100 mg/m² on day 2 (IE; three patients), whereas the patient with ovarian tumor received ifosfamide, 5 g/m²/d on day 1; etoposide, 500 mg/m² on day 2; and cisplatin, 50 mg/m² on day 2 (PEI-2). The regimen for the patient with germ cell tumor consisted of cisplatin, 20 mg/m²/d; etoposide, 75 mg/m²/d; and ifosfamide, 1.2 g/m²/d, all on days 1 to 5 (PEI-5). Subcutaneous administration of filgrastim was started 24 hours after chemotherapy at a dose of 300 μg/d. In two patients, the filgrastim-dose was increased to 1,200 μg/d when during the recovery phase, at a white blood cell (WBC) count of more than 5,000/μl, the percentage of CD34+ cells became detectable.

We used a forward scatter versus CD45 fluorescence dot plot to discriminate between the smallest lymphohematopoietic cell population and erythrocytes or debris. The percentage of CD34+ cells relative to the percentage of CD45+ cells and the absolute number of CD34+ cells were calculated as previously described.1

In BM, not more than 0.5% and in PB not more than 0.05% nonspecific positive events were allowed in control samples. The percentage of unspecific events was subtracted from positive events in stained samples. Examples of steady-state BM, PB, and mobilized PB of one patient are given in Fig 1.

To increase the sensitivity of progenitor cell detection in steady-state PB, a gated acquisition on CD34+ cells was performed. First, 10,000 cells were acquired ungated. Using the Lysis-II software (Becton Dickinson) a gate was set that included the CD34+ cells. The same sample was run again and 76 ± 10 CD34+ cells (mean ± SEM; values corrected for unspecific events) were acquired into this gate. The total number of cells in which the CD34+ cells were detected was 1,500 X 106/μl.

Table 1. Patient Characteristics I. Premobilization Features

<table>
<thead>
<tr>
<th>Patient</th>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Relapses</th>
<th>Cycles</th>
<th>Regimens</th>
<th>Irradiation</th>
<th>Mobilization</th>
<th>Status at</th>
<th>Bone Marrow Involvement</th>
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<td>Relapse</td>
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Abbreviations: NHL, non-Hodgkin's lymphoma; CR, complete remission; PR, partial remission; MIF, mediastinal involved field irradiation; MF, mantle field irradiation; IV, inverted Y field irradiation; S, splenic irradiation.

Table 2. Patient Characteristics II. Mobilization Features

<table>
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<tr>
<th>Patient</th>
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<th>Maximum Filgrastim-Dose (μg/d)</th>
<th>CD34+ Cells/μl Harvested (total)</th>
<th>No. of LPs</th>
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</table>

Abbreviation: CY, cyclophosphamide (4 g/m² or 7 g/m²).
Bone Marrow

Day 0

Peripheral Blood

Day 0

Mobilized

CD34

Side-Scatter Characteristics

Fig 1. Representative immunofluorescence analysis of CD34 antigen expression in steady-state (day 0) BM, PB, and mobilized PB after filgrastim-supported chemotherapy. For assessment of circulating CD34⁺ cells in steady-state hematopoiesis, 123 CD34⁺ cells were acquired in a life gate from a total population of 171,000 mononuclear cells stained with the HPCA2-PE antibody. From the premobilization, BM and mobilized PB 10,000 events stained with the HPCA2-FITC antibody were acquired ungated. As opposed to BM, PE contained a homogenous population of CD34⁺ cells. The threefold difference in CD34 fluorescence intensity between the PE samples may be attributed to the different staining intensity of the PE-conjugated (steady-state PB) and the FITC-conjugated HPCA2-antibody (mobilized PB).

contained was recorded. From a sample stained with an isotype-specific control antibody the same total number of cells was acquired using the same gate. The gated CD34 stained and control samples where then displayed in a forward- versus side-scatter dot plot. A second gate was drawn to encompass the CD34⁺ cell population, which displayed a low to intermediate side scatter, thus falling into the blast/lymphocyte region as reported by others.¹⁵,¹⁶ Unspecific events of the control sample falling into this region were subtracted to obtain the corrected CD34⁺ cell number; the CD34⁺ cell frequency was calculated by dividing by the total number of acquired events. As above, the percentage of CD34⁺ cells relative to the percentage of CD45⁺ cells in the ungated sample was calculated. The resulting percentage of CD34⁺ cells was multiplied with the mononuclear cell concentration to obtain the steady-state PB CD34⁺ cell count per liter mononuclear cells. In the analysis presented here, 132,000 ± 18,653 events per gated steady-state PB CD34⁺ cell sample were measured. The expected coefficients of variation (CV) were 13% ± 1%. In an independent series of three steady-state PB samples stained for CD34⁺ cells, the measured CV of 13% ± 6% was matched by the expected CV of 12% ± 3%. This was considered sufficiently accurate for CD34⁺ cell determinations from steady-state PB.¹⁵

For dual immunofluorescence analysis, 1 × 10⁶ cells from BM or LP samples were incubated for 30 minutes at 4°C with the FITC-conjugated MoAb HPCA2 (CD34) and one of the following PE-conjugated MoAbs: My-9 (CD33), Leu-12 (CD19), or HLA-DR. PE-conjugated HPCA2 was used for double staining with CD45-RA. With the exception of CD33 (RD1, Coulter Clone, Hialeah, FL), all antibodies were obtained from Becton Dickinson. CD34 subset analysis was performed on cells acquired in a fluorescence versus side-scatter gate. From BM and LP samples, 234 ± 33 (mean ± SEM) and 274 ± 31 CD34⁺ cells were acquired, respectively. The proportion of false-positive events in the double-staining was 1.02% ± 0.16% of CD34⁺ cells in BM and 0.92% ± 0.24% of CD34⁺ cells in LP samples.

Clonogenic assays for hematopoietic progenitor cells. The concentration of hematopoietic progenitor cells in each BM sample, PB sample, and LP product was assessed using a semisolid clonogenic culture assay (Stem Cell Technologies Inc, Vancouver, Canada). The culture medium consisted of 30% fetal calf serum, 10% medium conditioned by phytohemagglutinin-stimulated leukocytes, 1 IU recombinant human erythropoietin, 5 × 10⁻⁵ M 2-mercaptoethanol, and 0.9% methylcellulose. BM cells (1.5 × 10⁶), premobilization PB mononuclear cells (5 × 10⁷), or 5 × 10⁴ LP cells were plated in duplicate and incubated at 37°C and 5% CO₂ in humidified atmosphere. After 14 days, colonies were scored using an inverted microscope.

Collection of PBPC and cryopreservation. Harvesting was performed with a Fenwal CS 3000 (Baxter Deutschland GmbH, Munich, Germany) using a small volume collection chamber. PB CD34⁺ cell measurements were started after a WBC count greater than 1,000 × 10⁹/L was reached. The first leukapheresis was started when more
Individual estimations of progenitor cell harvests for a stated PB CD34+ cell count were calculated with the Statistical Analysis System software (SAS Institute Inc., Cary, NC).

RESULTS

CD34 antigen expression on steady-state BM and PB hematopoietic progenitor cells. BM aspirates of patients contained a median of 1.33% CD34+ cells with a range of 0.1% to 2.93%. This reflects a 17-fold inter-individual difference. The corresponding median concentration was 31.49 CD34+ cells × 10^6/L (0.72 to 169.44 × 10^6/L). In contrast, the median proportion of CD34+ cells in PB was approximately 100-fold smaller compared with BM (median, 0.06%; range, 0.03% to 0.12%). Accordingly, 0.46 CD34+ cells × 10^6/L (0.14 to 1.86 × 10^6/L) were measured. Neither proportions nor absolute numbers of BM and PB CD34+ cells correlated. When only patients without BM infiltration or only patients without prior irradiation were analyzed, this did not improve.

To further analyze the difference between BM and PB CD34+ cells, the level of CD34 expression was compared. CD34+ cells from BM showed a relatively wide range of antigen density, with gradual transition from bright to dim (Fig 1). In contrast, circulating CD34+ cells had a distinctly high expression of the CD34+ antigen, which distinguished them clearly from cells not expressing the antigen.

The immunologic data were complemented by the colony assay results. In BM, the cloning efficiency for CFU-GM was 568/10^6 (430/10^6 - 3,333/10^6) mononuclear cells (MNC) and for BFU-E 1887/10^6 cells (897/10^6 - 3,333/10^6). In PB 14 CFU-GM colonies/10^6 MNC (2/10^6 - 39/10^6) and 142 BFU-E/10^6 plated cells (63/10^6 - 525/10^6) were observed. Different from BM, a strong correlation of CD34+ cells and colonies was observed in steady-state PB (r = 0.90, P < .001). In comparison, a correlation of CD34+ cells and colonies was also found in leukapheresis products (r = .76, P < .05, n = 10). However, here colony data of five patients are not available, because the high plating efficiency did not allow to count or discriminate single colonies.

Correlation analysis of steady-state progenitor cell values and mobilization outcome. As parameter for the mobilization potential, we used the individual peak PB CD34+ cell counts out of a series of 76 measurements (median, 3 per patient; range, 1 to 14). The peak values of CD34+ cells in the 15 patients spread from 6.42 to 505.68 CD34+ cells × 10^6/L (median, 43.7 × 10^6/L). Mobilized PB contained a homogenous population of CD34+ cells that was similar to steady-state PB, but not steady-state BM, which showed a continuous transition from CD34+ bright to CD34+ dim cells (Fig 1). When all steady-state BM CD34+ cell values were plotted against the corresponding peak CD34+ cell counts, no statistically significant correlation was found (r = .25, P > .05, Fig 2A). When the outlier patient no. 8, who had a steady-state BM CD34+ cell count of 169.4 × 10^6/L and a peak CD34+ cell count of 6.42 × 10^6/L, was removed from the analysis a weak correlation (r = .57, P < .05) became apparent. If steady-state BM colony counts were used for the calculation instead of CD34+ cell counts, this was not seen (r = .24, P > .05). Consistent with the similarities in CD34

Fig 2. Premobilization BM CD34+ cell and colony counts did not have a predictive value for the peak CD34+ cell count in mobilized PB (A) (n.s., not significant). However, steady-state PB CD34+ cells (r = .81, P < .001) and colony-forming cells (r = .69, P < .01) showed a close relationship to the number of CD34+ progenitor cells mobilized (B).
antigen expression, the steady-state PB CD34+ cell count correlated strongly ($r = .81, P < .001$, Fig 2B) with the peak PB CD34+ cell value as did the steady-state PB colony count ($r = .69, P < .01$).

It is worth noting that the strong correlation was observed in a heterogeneous group of patients receiving different cytotoxic mobilization regimens. A statistically significant relation between prior cytotoxic therapy or BM involvement and the degree of progenitor cell mobilization was not found here. However, an inverse correlation between prior cytotoxic therapy and the mobilization yield could be observed if a larger, more homogenous patient group was analyzed.

Steady-state PB CD34+ cell counts allow us to estimate the progenitor cell yield. For individual estimations, a specific steady-state PB CD34+ cell count needs to ensure with 95% probability a certain minimum progenitor cell yield (Fig 3). This takes into account the scatter around the regression line, ie, the average yield. To estimate these mobilization yields, calculations were based on the average CD34+ cell contents per leukapheresis product that were calculated from the number of leukaphereses and the total CD34+ cells harvested (Table 2). As expected, a strong correlation ($r = .77, P = .001$) was found between the steady-state PB CD34+ cell counts and the corresponding average CD34+ cell contents per leukapheresis product. The lower 95% confidence limit for the individual yield can in turn be used to estimate the number of leukaphereses required for a certain target CD34+ cell harvest. As an example, in Table 3 a target quantity of $2.5 \times 10^6$ CD34+ cells/kg body weight, the reported "threshold dose" for rapid and sustained engraftment after myeloablative therapy, was chosen. For example, at $0.4 \times 10^6$ steady-state PB CD34+ cells/L, six leukaphereses will be necessary to collect a sufficient harvest. With this estimation, we would have been able to reassure four patients (steady-state PB CD34+ cell counts: patient no. 1, $0.69 \times 10^6$/L; no. 2, $0.46 \times 10^6$/L; no. 6, $1.86 \times 10^6$/L; no. 9, $0.67 \times 10^6$/L) in whom we expected low progenitor cell harvests because of a clinical history of four or more cycles of prior chemotherapy (Table 1) to have a 95% chance of collecting sufficient progenitor cells in a clinically acceptable number of six leukapheresis products before the start of mobilization therapy.

CD34+ cell subsets do not improve the predictive value of steady-state BM. As shown for the whole BM CD34+ cell population before, neither of the absolute numbers of myeloid-associated CD34+/CD33+, CD34+/CD45-RA+, early B-lymphoid CD34+/CD19+ nor the phenotypically primitive CD34+/HLA-DR- cells had a predictive value for the peak CD34+ cell count or the PBPC harvest. This did not change when the outlier patient no. 8 was removed from the analysis. A comparison of the proportional distribution of the different CD34+ subgroups between steady-state BM and LP products (Fig 4) showed a significantly smaller proportion of CD34+/CD33+ cells in steady-state BM than in LP ($P < .05$). For CD34+/CD19+ B-lymphoid progenitors the reverse was seen ($P < .05$). The number of CD34+/CD45-RA- cells that represent committed myeloid progenitor cells and B-lymphoid progenitors tended to be higher in BM than in LP. The proportion of phenotypically immature CD34+/HLA-DR- cells within the total CD34+ cell population was not different between BM and LP.

**DISCUSSION**

This study was performed to look for predictive parameters of hematopoietic progenitor cell mobilization into the PB. Progenitor cells can be assessed immunologically by staining for the CD34 antigen or functionally using colony- assays. CD34+ cells comprise hematopoietic progenitor cells at various developmental stages with different capacity of self-renewal and differentiation that can be assessed in BM and PB in steady-state hematopoiesis. The immunologic assessment of CD34+ cells was introduced by Siena et al to monitor progenitor cell harvesting. The relevance of CD34+ cells for PB progenitor cell transplantation has re-
The mobilization of PB CD34+ cells was only found when an outlier progenitors abrogated mobilization of multipotent and lineage-committed hematopoietic cells to peripheral sites. The predictive quality of premobilization PB CD34+ cell counts has also been noted when after mobilization chemotherapy granulocyte-macrophage CSF was given instead of G-CSF.

Where do mobilized CD34+ cells originate? Investigations in mice using chemotherapy plus G-CSF for mobilization showed an increase of multipotent and lineage-committed hematopoietic progenitor cells in the PB and spleen that was accompanied by a marked loss of these cells in the BM. In another murine study using interleukin-7 that showed a similar effect on progenitor cell mobilization as reported with G-CSF, the depletion of BM by administration of Sr, which led to a greater than 99% reduction in BM myeloid progenitors abrogated mobilization of multipotent and lineage-committed hematopoietic cells to peripheral sites.

During steady-state hematopoiesis, PB progenitor cells showed a strong correlation (r = .81, P < .001) to the mobilized CD34+ cells and allowed us to estimate the minimum CD34+ cell yield in a PBPC harvest, whereas for steady-state BM progenitor cells, a weak correlation to the mobilized PB CD34+ cells was only found when an outlier was removed. The predictive quality of premobilization PB progenitor cell counts has also been noted when after mobilization chemotherapy granulocyte-macrophage CSF was given instead of G-CSF.

The number of colony-forming cells in steady-state PB was reported to be approximately 0.23 x 10⁶/L, which is consistent with our data. A sensitive and reproducible method of CD34+ cell determination facilitates the rapid quantitation of this small cell population. The biologic relevance of the measured CD34+ cell proportions is validated by the strong correlation with cloning efficiencies also reported by others. Furthermore, the predictive value of CD34+ cell counts to the mobilized CD34+ cell numbers was higher than if the estimation was based on colony formation. On the other hand, as described previously, BM CD34+ cells during steady-state hematopoiesis did not show a relation to the colony formation in BM. This may partially be explained by the variable proportion of nonclonogenic CD34+/CD19+ B-lymphoid progenitors in BM (0.1% to 61% of CD34+ cells; Fig 4) that do not normally circulate.

Why does the steady-state PB progenitor cell count have a higher predictive value than BM? We assume that circulating progenitor cells may be a subgroup of progenitors from those found in the marrow. When progenitor cells differentiate, they gradually loose the CD34+ antigen, which may be associated with a loss of the ability to circulate in the PB. This hypothesis is supported by the finding that circulating steady-state and mobilized CD34+ cells have a high and distinct expression of the CD34 antigen, whereas BM CD34+ cells display a broad range of antigen density, ranging from strongly positive with continuous transition to CD34 negativity. This line is followed by a report of Delia et al on the reciprocal regulation of adhesion molecules and the CD34 antigen on vascular endothelial cells, suggesting that CD34 may have a negative modulating role on adhesion functions.

A predictive value for steady-state BM CD34+ cell subsets could not be shown. This was expected for the lineage-committed myelomonocytic CD34+/CD33+ and CD34+/CD45-RA subsets as well as the B-lymphoid CD34+/CD19+ precursor populations, which all may contain CD34-dim cells. The CD34+/HLA-DR subset was considered to be pheno-
typically primitive. However, recent reports showed CD34+/HLA-DR- cells to be heterogeneous in proliferative capacity, whereas only the progeny of CD34+/CD38- cells could give rise to each of the hematopoietic lineages. Therefore, the lacking correlation between BM CD34+/HLA-DR- cells and the number of mobilized PB CD34+ cells may not allow conclusions about the role of the most primitive BM hematopoietic cells in progenitor cell mobilization. Further antigens that need to be studied are CDw90 (Thy-1), which is present on CD34+ bright but not CD34+ dim cells, and the CD49d (very late antigen 4 [VLA-4])- subpopulation in BM as most circulating CD34+ cells are CD49d-.

The data presented show that the minimum progenitor cell yield can be reliably estimated before chemotherapy plus G-CSF is started. Patients being at risk of not achieving sufficient CD34+ cell numbers for a sufficient autotransplant may thus be recognized. It is also possible to detect patients who are likely to achieve high progenitor cell yields and who may thus be eligible for ex vivo techniques that go along with an expected loss of progenitor cells such as CD34 selection or purging.

ACKNOWLEDGMENT

We thank Professor D.W. van Bekkum (TNO-Institute, Rijswijk, The Netherlands) for critical reading of the manuscript and helpful suggestions. We thank Margit Pfoehler, Kirsten Flentje, Evi Holdermann, and Magdalena Volk for their excellent technical assistance; the expertise in their work and high reproducibility of assays were the basis for establishing the predictive values reported here.

REFERENCES


Peripheral blood progenitor cell (PBPC) counts during steady-state hematopoiesis allow to estimate the yield of mobilized PBPC after filgrastim (R-metHuG-CSF)-supported cytotoxic chemotherapy [see comments]

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