Mobilization of Peripheral Blood Progenitor Cells by Sequential Administration of Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor Following Polychemotherapy With Etoposide, Ifosfamide, and Cisplatin

By Wolfram Brugger, Klaus Bross, Jürgen Frisch, Peter Dern, Boris Weber, Roland Mertelsmann, and Lothar Kanz

We report on the requirements that have to be met to combine a standard-dose chemotherapy regimen with broad antitumor activity with the mobilization of peripheral blood hematopoietic progenitor cells. Thirty-two cancer patients were given a 1-day course of chemotherapy consisting of etoposide (VP16), ifosfamide, and cisplatin (VIP; n = 46 cycles), followed by the combined sequential administration of recombinant human interleukin-3 (rhIL-3) and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF). Control patients received GM-CSF alone or were treated without cytokines. Maximum numbers of peripheral blood progenitor cells (PBPC) were recruited on day 13 to 17 after chemotherapy, with a median of 418 CD34+ cells/μL blood (range, 106 to 1,841) in IL-3/rhGM-CSF–treated patients, 426 CD34+/pL (range, 191 to 1,380) in GM-CSF–treated patients, and 46 CD34+/μL (range, 15 to 148) in patients treated without cytokines. In parallel, there was an increase in myeloid (10,490 colony-forming unit-granulocyte-macrophage [CFU-GM]/mL blood; range, 1,000 to 23,400), as well as erythroid (10,680 burst-forming unit-erythroid [BFU-E]/mL blood; range, 3,870 to 24,300) and multipotent (840 CFU-granulocyte, erythocyte, monocyte, megakaryocyte [CFU-GEMM]/mL blood; range, 160 to 2,070) progenitor cells in IL-3 plus GM-CSF–treated patients. In GM-CSF–treated patients, significantly less precursor cells of all lineages were mobilized, particularly multipotential progenitors (400 CFU-GEMM/mL blood; range, 200 to 2,150). Only small numbers of CD34+ cells and clonogenic progenitor cells could be recruited in intensively pretreated patients. Our data document that after standard-dose chemotherapy-induced bone marrow hypoplasia, IL-3 plus GM-CSF can be used to recruit PBPC, which might shorten the hematopoietic recovery after high-dose chemotherapy in chemosensitive lymphomas or solid tumors.

HIGH-DOSE CHEMOTHERAPY is potentially curative in some chemosensitive tumors,1,4 and a relationship between dose intensity of cytotoxic drugs and tumor response has been defined for several malignancies.5 Dose intensity should be adequate to overcome a threshold dose that produces response, and the average relative dose intensity received is probably a major factor determining outcome of chemotherapy.5 However, dose escalation is mostly limited by myelosuppression, as well as nonhematological organ toxicity. The risk of neutropenic infection and of bleeding complications requires expensive supportive care during this period. Therefore, hematopoietic growth factor treatment was studied by several investigators in conjunction with dose-intensive chemotherapy.2-13 However, hematopoietic growth factors are less effective in patients with reduced bone marrow reserve, either induced by preceding radiotherapy/chemotherapy or by the high-dose chemotherapy regimen itself.14 The more intensive the regimen, the higher the probability of destroying most of the committed progenitor cells. One approach to overcome these problems might be the combined application of hematopoietic growth factors and autologous peripheral blood committed precursor cells.

Several methods for mobilization of peripheral blood progenitor cells (PBPC) into circulation have been described. Chemotherapy-induced mobilization occurs during the recovery phase after bone marrow hypoplasia, particularly after cyclophosphamide treatment.15,17 Hematopoietic growth factors (eg, granulocyte colony-stimulating factor [G-CSF] or granulocyte-macrophage CSF [GM-CSF]) also expand the pool of circulating progenitors16,21; this effect is increased by combining chemotherapy with growth factors19,22 and is potentiated when GM-CSF is administered following high-dose cyclophosphamide.7,20

Here we report on our approach to study the requirements that have to be met to combine an effective cancer chemotherapy with mobilization of PBPC. The concept is to first treat patients with a standard-dose chemotherapy regimen using three non-cross-resistant cytotoxic drugs (etoposide [VP16], ifosfamide, and cisplatin = VIP), and at the same time to recruit PBPC. We describe the detailed characterization of mobilization requirements, as well as the phenotypic and functional analysis of progenitor cells induced under different conditions.

MATERIALS AND METHODS

Patient selection and treatment regimen. Patient eligibility included small-cell lung cancer and advanced malignancies of different origin for which no standard treatment was available. The protocol was approved by the local ethics committee. Patients could not have received chemotherapy or radiotherapy within 4 weeks preceding protocol therapy, and should have a life expectancy of at least 4 months. Patients with central nervous system metastases and/or bone marrow involvement were excluded. After written informed consent, a total of 32 patients with 46 chemotherapy cycles were treated with a 1-day course of VP16 (500 mg/m²), ifosfamide (4,000 mg/m²) and cisplatin (50 mg/m²). Fifteen patients were treated with cyclophosphamide instead of ifosfamide, at a dose of 1,600 mg/m². Sufficient hydration with normal saline was performed to maintain a urine flow of 4 to 5 L/d. Mesna...
uroprotection was performed for 48 hours according to standard protocols. Antiemetic therapy consisted of ondansetron and dexamethasone.

Subcutaneous cytokine treatment was started 24 hours after the end of VIP-chemotherapy, either with recombinant human GM-CSF (rhGM-CSF; Behringwerke, Marburg, Germany), days 1 to 15, or with the combined sequential administration of recombinant human interleukin-3 (rhIL-3; yeast, Behringwerke), days 1 to 5 and rhGM-CSF, days 6 to 15, at a dose of 250 µg/m² each. Control patients received polychemotherapy without cytokines.

Patients were catagorized according to their pretreatment status into three different categories, one group of patients without prior treatment (n = 12), one group of patients with mild-moderate pretreatment (<6 chemotherapy cycles) (n = 11), and one group with intensive pretreatment (>6 chemotherapy cycles and/or radiotherapy to >20% of bone marrow) (n = 9), respectively.

Preparation of peripheral blood or bone marrow mononuclear cells for in vitro analysis. Heparinized blood samples were obtained daily, and bone marrow aspirates were obtained before and at day 15 following chemotheraphy. Mononuclear cells (MNC) were isolated by density gradient centrifugation over Ficoll/Hypaque (1.077 g/mL; Pharmacia, Freiburg, Germany), washed two times in phosphate-buffered saline (PBS; Biochrom, Berlin, Germany), and adjusted to 3 x 10⁶/mL in supplemented Iscove’s modified Dulbecco’s medium (IMDM) with 5 x 10⁻⁵ mol/L mercaptoethanol (GIBCO, Karlsruhe, Germany).

Culture assay for hematopoietic progenitor cells. Peripheral blood or bone marrow MNC were grown as previously described.²⁶,²⁷ MNC (1 x 10⁵) were immobilized in methylcellulose (0.9%) and cultured in supplemented IMDM with 30% fetal calf serum (FCS; Paesel, Frankfurt, Germany). Cultures were stimulated with 100 U/mL rhGM-CSF and 100 U/mL rhIL-3 (both provided by Behringwerke). Recombinant human erythropoietin (1 U/mL; Connaught, Willowdale, Ontario, Canada) was added to cultures at day 3 of incubation. Total volume per dish was adjusted to 1 mL. Cells were cultured in humidified atmosphere and 5% CO₂. The colonies were scored with an inverted microscope 12 to 14 days after initiation of culture. The count of circulating progenitor cells per microliter blood was determined by multiplying their frequency in culture with the absolute MNC count in the same sample of peripheral blood.

ImmunocytoLOGY of surface antigens on peripheral blood mononuclear cells. Peripheral blood MNC were attached to Alcan blue-coated slides and prefixed in 0.05% glutaraldehyde at room temperature for 5 minutes. For the evaluation and characterization of CD34⁺ MNC, the following mouse monoclonal antibodies (mAb) were used: anti-CD34 (clone My10, HPCA-1, IgG1 from Dianova, Hamburg, Germany), anti-CD33 (My9, Coulter, IgG1, Krefeld, Germany), anti-HLA-DR (IgG2a) and anti-CD38 (Dianova, Hamburg, Germany), anti-CD33 (My9, Coulter, IgG1, Krefeld, Germany), anti-HLA-DR (IgG2a) and anti-CD38 (Leu-17, IgG1; all from Becton Dickinson, Rödermark, Germany). An irrelevant, isotype-matched (IgG1) anti-rota virus mAb (Dianova) was used as a control antibody for nonspecific binding of anti-CD34 mAb. A peroxidase-antiperoxidase (PAP) technique was applied followed by postfixation with OsO₄ as described previously.²⁹ The percentage of CD34⁺ MNC was determined and multiplied by the absolute MNC count in the same blood sample to determine the absolute CD34⁺ count per microliter peripheral blood.

Dual-color flow cytometry analysis. Peripheral blood MNC were incubated with anti-CD34, fluorescenic isothiocyanate (FITC)- conjugated mAb (Dianova) and/or anti-CD33, phycoerythrin (PE)- conjugated mAb, anti-CD38, PE-conjugated mAb, or anti-HLA-DR, PE-conjugated mAb (all from Becton Dickinson) for 30 minutes at 4°C. Cells were analyzed by flow cytometry using a FACScan analyzer (Becton Dickinson), equipped with a filter set for FITC-PE dual-color fluorescence. Data acquisition was performed with FACScan Lysis II research software. Forward light-scattering, orthogonal light-scattering, and dual-fluorescence signals were determined for each cell and stored in list mode data files. Each measurement contained 20,000 to 40,000 cells; the frequency of cells expressing CD34 and/or CD33, CD38, or HLA-DR antigens was calculated as percentage of all cells analyzed.

Statistics. The statistical significance of the data obtained was analyzed by the Wilcoxon rank sum test and by the Student’s t test using the CSS computer software program (Statsoft, Tulsa, OK). A P value less than .05 was considered significant. Linear regression analyses and correlation analyses were performed using LOTUS software program.

RESULTS

Mobilization of PBPC was studied in 32 cancer patients with a total of 46 cycles following polychemotherapy with or without administration of either GM-CSF or IL-3 plus GM-CSF. Patient characteristics and responses to study treatment are summarized in Table 1 and hematological recovery data after VIP-chemotherapy are shown in Fig 1.

Hematopoietic progenitor cell numbers were evaluated by analysis of CD34 expression on peripheral blood MNC and by simultaneously studying the colony-forming capacity of these cells (in vitro culture assays for colony-forming unit-granulocyte, macrophage [CFU-GM], burst-forming unit-erythroid [BFU-E], and CFU-granulocyte, erythocyte, monocyte, megakaryocyte [GEMM]). CD34 expression was assessed by the PAP slide technique with a sensitivity of 0.01%.²⁸ Flow cytometry (sensitivity of ~1%) was used for dual-color analysis.

Under steady-state hematopoiesis (baseline), we were able to detect minimal amounts of circulating CD34⁺ cells (median, 0.3/µL blood; range, 0 to 10/µL; n = 20) and a

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<th>Table 1. Patient Characteristics and Outcome</th>
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<td>Total no. of patients</td>
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<td>Total no. of cycles</td>
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<td>Median age (range)</td>
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<td>Treatment (no. of cycles)</td>
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<td>+ rhGM-CSF</td>
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<td>+ rhIL-3 + rhGM-CSF</td>
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<td>Pretreatment (no. of cycles)</td>
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<td>Without pretreatment</td>
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<td>Mild to moderate</td>
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<td>Diagnosis (no. of patients)</td>
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<td>SCLC</td>
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<td>NSCLC</td>
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<td>Tumor response</td>
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<td>CR + PR</td>
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Abbreviations: SCLC, small-cell lung cancer; NSCLC, non–small-cell lung cancer; CR, complete response; PR, partial response.
number of days

![Graph showing hematopoietic recovery after 46 cycles of VIP combination chemotherapy with or without growth factors. Data are presented as median days of less than 500 neutrophils (ANC)/µL and less than 100,000 platelets/µL blood, respectively. Neutrophil recovery was significantly shortened by GM-CSF and by IL-3 plus GM-CSF (P < .01); platelet recovery was not significantly different in these three groups.](image)

Following VIP-chemotherapy with or without administration of hematopoietic growth factors, there was an increase in CD34+ cells and clonogenic progenitor cells. The kinetics of PBPC mobilization of three representative patients treated with or without growth factors are exemplified in Fig 2 by the number of circulating CD34+ cells and circulating progenitor cells. The highest numbers of precursor cells were recruited by the sequential administration of IL-3 plus GM-CSF.

As shown in Fig 3, there was a significant difference in the time to reach peak values of PBPC between patients treated without cytokines and patients treated with GM-CSF or IL-3 plus GM-CSF. In patients treated without cytokines, peak levels of CD34+ cells, as well as myeloid and erythroid progenitor cells, were reached simultaneously at a median of 18 days after chemotherapy, whereas in patients treated with GM-CSF or IL-3/GM-CSF, peak levels occurred 4 days earlier (median, day 14 [range, 11 to 20] in the GM-CSF group and day 14.5 [range, 10 to 20] in the IL-3/GM-CSF group). Multilineage progenitors appeared 1 day later at maximal levels (median, day 15; range, 11 to 20). The differences in the kinetics between GM-CSF- and IL-3/GM-CSF–treated patients were not significant.

Median values of peak levels of CD34+ cells and circulating clonogenic progenitor cells are summarized in Table 2 and Fig 4. There was a significant difference in the recruitment of CD34+ cells between patients treated without cytokines (median, 46 CD34+/µL; range, 15 to 148) and patients treated with GM-CSF (median, 426 CD34+/µL; range, 191 to 1,380; P < .01) or IL-3 plus GM-CSF (median, 418 CD34+/µL; range, 106 to 1,841; P < .01). The difference in the numbers of CD34+ cells was not significant between GM-CSF– and IL-3/GM-CSF–treated patients.

The numbers of circulating clonogenic cells were significantly lower in patients treated without cytokines when compared with patients treated with GM-CSF or IL-3 plus GM-CSF. As compared with GM-CSF alone, more clonogenic progenitor cells of different lineages could be mobilized without cytokines.
presented as median days (27 patients).

Data are presented as median days (27 patients).

In comparison to baseline values, sequential IL-3 plus GM-CSF
resulted in a 400-fold increase in circulating CFU-GEMM.

GM-CSF treatment induced an 80- to 120-fold increase in
multipotential progenitor cells. In patients treated with GM-CSF alone, there was a
400-fold increase in circulating multipotent progenitor cells. In previously untreated patients, the highest number of
CD34+ cells was mobilized with both GM-CSF and IL-3
plus GM-CSF; no significant difference between GM-CSF
alone and the combination of IL-3 plus GM-CSF could be
found. In mildly to moderately pretreated patients, significantly less CD34+ cells could be recruited (P < .02). In
these patients, the combined IL-3 plus GM-CSF treatment
was more effective than GM-CSF alone (P < .05).

In intensively pretreated patients, only a median of less than
20 CD34+ cells/μL could be induced. Only in three of nine
intensively pretreated patients considerable numbers of
PBPC could be recruited (up to 171 CD34+/μL) by the
combined IL-3 and GM-CSF treatment (not shown in detail).

Myeloid and erythroid progenitor cells in the bone
marrow of the patients studied remained unchanged before
and after IL-3 plus GM-CSF treatment, whereas multipotent
progenitor cells (CFU-GEMM) slightly increased
(Table 4). However, GM-CSF treatment reduced all types
of hematopoietic progenitor cells to approximately 50% of
pretreatment values, probably due to a predominance of
mature myeloid cells in these samples. Chemotherapy
alone had marginal effects on bone marrow progenitor
cells.

**DISCUSSION**

PBPC have been used with increasing frequency as an
alternative to autologous bone marrow, and more recently
in addition to marrow grafts for shortening the period of
severe pancytopenia following myeloablative therapy. Moreover, these cells might be used in conjunction with
hematopoietic growth factors to support hematopoietic
recovery after high-dose chemotherapy. The advantages of
this modality include the possibility of autografting when
bone marrow is infiltrated with malignant cells or damaged
by prior radiotherapy/chemotherapy, the reduced possibility
tumor cell contamination in disseminated cancer, and
a more rapid recovery of neutrophils and platelets, probably due to the high number of committed progenitor
cells infused.

Here we describe our approach for the mobilization of
PBPC. The rationale is to combine a standard-dose chemo-
therapy regimen (VIP-regimen) with broad antitumor activity with the simultaneous recruitment of circulating progenitor cells that can be collected and retransfused to the patients after they have received high-dose chemotherapy.

Thirty-two patients—with a total of 46 cycles—were given a 1-day course of chemotherapy, followed by the sequential administration of IL-3 plus GM-CSF. The rationale to use IL-3 plus GM-CSF is based on the finding that these cytokines act synergistically to mobilize progenitors. Moreover, the sequential administration of IL-3 and GM-CSF resulted in a synergistically enhanced stimulation of hematopoiesis in primate models. In addition, patients given chemotherapy might profit clinically from these growth factors, since IL-3 was shown to induce thrombocytosis and GM-CSF stimulates rapid onset and high levels of neutrophilia.

Our data document that VIP-chemotherapy and IL-3 plus GM-CSF administration allows the recruitment of a median of 418 CD34+ cells/μL blood (range, 106 to 1,841), and in parallel, induces high numbers not only of myeloid (median, 10,490 CFU-GM/μL blood), but also erythroid (median, 10,660 BFU-E/μL blood) and multipotential (median, 840 CFU-GEMM/μL) progenitor cells.

Hematopoietic growth factors have been shown by several other groups to be able to mobilize myeloid progenitor cells. Socinski et al19 and Gianni et al24 were the first to introduce growth factors following chemotherapy for the recruitment of circulating progenitors. Socinski et al used a polychemotherapy regimen including doxorubicin, ifosfamide, and dacarbazine and GM-CSF, but only low numbers of PBPC, as analyzed by CFU-GM colonies, could be induced (median, 2,250 CFU-GM/μL blood). Gianni et al were able to mobilize circulating CFU-GM effectively by treating patients with high-dose cyclophosphamide (7 g/m²) and GM-CSF (median, 14,000 CFU-GM/μL blood).

We have used a standard-dose chemotherapy regimen (VIP) for the recruitment of PBPC. Etoposide, ifosfamide, and cisplatin have been proven to be effective in many tumors. Moreover, with the use of the VIP-regimen in our study, there has been no severe thrombopenia, which would impede leukapheresis at the time of maximal levels of progenitor cells in the peripheral blood. Our first patients were treated with an equitoxic dose of cyclophosphamide instead of ifosfamide, but no striking differences in the release of PBPC were seen (data not shown). VIP chemotherapy was well tolerated with no major side effects observed, although neutropenia occurred in all patients for 3 to 6 days, depending on their prior treatment status. Patients were discharged from hospital 1 day after chemotherapy. The overall response rate following VIP-chemotherapy was 62%, although the patient population was heterogenous. The best results were obtained in small-cell lung cancer patients and lymphoma patients, and no responses were seen in refractory sarcoma patients.

VIF chemotherapy and subcutaneous IL-3 plus GM-CSF treatment allowed to recruit numbers of CFU-GM/μL blood comparable to data published by Gianni et al24 and Siena et al,25 although the overall amount of CD34+ cells is higher in our patients (median, 136 CD34+/μL blood [Gianni] vs 418 CD34+/μL blood [our results]). Being aware that different methods for quantitation of PBPC were applied, a comparison of CD34+ cells and CFU-GM shows that there is a ratio of 15:1 in Gianni's study, whereas in our study the ratio is 40:1 for CFU-GM and 20:1 for all clonogenic progenitors. CD34+ cells include progenitor cells of all lineages. Our observation that the increase in
circulating CD34+ cells on stimulation with cytokines is not accompanied by a parallel increase in clonogenic progenitor cells possibly indicates that part of the CD34+ cells recruited either are more mature precursors that are no longer clonogenic in vitro, or represent very immature precursors and/or progenitors of lymphoid lineages that cannot be cultured by the techniques used. Another explanation might be that CD34 antigen expression is induced on peripheral blood MNC on cytokine stimulation, and we cannot exclude that VIP-chemotherapy might have damaged part of the committed CD34+ progenitor cells. It is of interest that the number of CD34+ cells is not different between the GM-CSF– and IL-3/GM-CSF–treated groups of patients, in contrast to the number of clonogenic progenitors, which are significantly increased in the IL-3 plus GM-CSF group. This finding indicates that IL-3 plus GM-CSF induces more clonogenic progenitors that are not reflected in the number of CD34+ cells.

Correlation analyses of circulating CD34+ cells with clonogenic progenitors have shown that there is no strong correlation in patients treated without cytokines or in patients treated with IL-3 plus GM-CSF. However, in GM-CSF–treated patients, CD34+ cells correlate well with myeloid (CFU-GM) and erythroid (BFU-E) progenitors. Interestingly, peripheral blood MNC correlated well with clonogenic CFU-GM (r = .82), independently of any cytokine administration. Therefore, for practical reasons concerning PBPC harvest and for estimation of harvested CFU-GM, this correlation might be useful.

Considering a threshold dose of circulating CFU-GM for successful engraftment between 2 to 3 × 10⁶ CFU-GM in a 70-kg human, it might be possible to restore hematopoiesis after a myeloablative chemotherapy with approximately 2,000 mL of peripheral blood collected at the time of peak levels of PBPC under the conditions described. If a recovery of 50% after leukapheresis and a freezing loss of 60% is calculated, only one cytapheresis with a total volume of approximately 5 L of blood processed would be sufficient for a successful engraftment. However, such calculations assume the functional identity of PBPC and bone marrow progenitors cells, an implication that has not yet been proven.

Based on dual-color flow cytometry in some of our patients, the vast majority of CD34+ cells simultaneously express CD33, CD38, and HLA-DR antigens, indicating that most of these cells are committed rather than very immature progenitor cells. However, lineage-negative CD34+ cells are thought to comprise pluripotent stem cells,

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<th>Table 3. Correlation Analysis Between CD34 Cells and Colony-Forming Cells of 145 Blood Samples</th>
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<td>Correlations between CD34+ cells/µL peripheral blood (independent variable; x) and CFU-GM/µL, BFU-E/µL and CFU-GEMM/µL, in all patients treated without or with SC application of rhGM-CSF or rhIL-3 + rhGM-CSF, respectively. Data are presented as the correlation coefficients, r.</td>
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GM-CSF–treated patients, CD34+ cells correlate well with myeloid (CFU-GM) and erythroid (BFU-E) progenitors.

Table 4. Bone Marrow Progenitor Cells Before and After VIP-Chemotherapy With or Without Cytokines in Initially Untreated Patients

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<th>Table 4. Bone Marrow Progenitor Cells Before and After VIP-Chemotherapy With or Without Cytokines in Initially Untreated Patients</th>
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<td>Postchemotherapy Without + GM-CSF + IL-3/GM-CSF</td>
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<td>CFU-GM</td>
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Data are given as median (range) of colonies per 1.5 × 10⁶ bone marrow MNC (n = 14).
which are necessary for long-term engraftment.\textsuperscript{30} Three-color flow cytometry combined with sorting and culturing of subsets of CD34\textsuperscript{+} cells is necessary to establish whether IL-3/GM-CSF–recruited PBPC meet criteria for “putative” stem cells.

In conclusion, we have shown that standard-dose chemotherapy with VIP, followed by IL-3 plus GM-CSF application, recruits large numbers of myeloid, erythroid, and multilineage progenitors into circulation. These cells are thought to be responsible for early recovery after bone marrow damage. Therefore, we started to use these cells to shorten hematopoietic recovery after high-dose chemotherapy consisting of a 3-day course of the VIP-regimen with a total cumulative dose of 1,500 mg/m\textsuperscript{2} etoposide, 12 g/m\textsuperscript{2} ifosfamide, and 150 mg/m\textsuperscript{2} cisplatin.

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and multipotent hematopoietic colony-forming cells and their
Mobilization of peripheral blood progenitor cells by sequential administration of interleukin-3 and granulocyte-macrophage colony-stimulating factor following polychemotherapy with etoposide, ifosfamide, and cisplatin [see comments]

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