Plastic-Adherent Progenitor Cells in Mobilized Peripheral Blood Progenitor Cell Collections


The use of peripheral blood progenitor cells (PBPC) to reconstitute hematopoiesis after high-dose chemoradiotherapy is now commonplace in the treatment of malignancies. Attempts to characterize these cells have concentrated primarily on their phenotype and their content of clonogenic colony-forming cells (CFC). We have used a plastic-adherent cell assay to evaluate the quantity and quality of more primitive cells in addition to the conventional measurements of CFC and CD34-positive cells. The leukapheresis products from 20 patients mobilized using cyclophosphamide (Cy) and granulocyte colony-stimulating factor (G-CSF) were examined for progenitor cell content. The mean number of mononuclear cells (MNC), colony-forming units-granulocyte/macrophage (CFU-GM), and CD34-positive cells from two leukaphereses per patient were 7.3 x 10^6/kg, 47.3 x 10^3/kg, and 10.5 x 10^4/kg, respectively. The mean number of PΔ progenitors was 9.3 x 10^4/kg. Limiting dilution analyses showed the frequency of PΔ progenitors in PBPC to be between 1 and 5.3 per 10^6 MNC and that each PΔ progenitor has the proliferative capability to generate an overall mean of 4.5 CFU-GM. Of the 20 patients, 16 underwent autografting with PBPC alone. Fifteen patients engrafted neutrophils and platelets within 16 days. One patient had delayed engraftment associated with inadequate engraftment. Statistical analysis showed a strong correlation between numbers of CFU-GM and CD34 positivity. The numbers of plastic-adherent PΔ progenitor cells did not correlate with CFU-GM or CD34-positive cells. We conclude that the plastic-adherent PΔ progenitor cell assay is capable of measuring primitive hematopoietic cells and that it may be useful for the investigation of primitive progenitors in PBPC harvests.

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PERIPHERAL BLOOD progenitor cells (PBPC) mobilized by chemotherapy, with or without hematopoietic growth factors (HGFs), are being used increasingly to reconstitute hematopoiesis after high-dose chemoradiotherapy for a variety of hematologic and other malignancies. It has been known for some time that primitive progenitor cells circulate in low numbers in steady-state peripheral blood, and their numbers increase dramatically after various treatments. In mice, the numbers of repopulating stem cells have been shown to increase during the recovery phase after chemotherapy, and after the administration of an HGF such as granulocyte colony-stimulating factor (G-CSF). In humans, G-CSF and granulocyte-macrophage (GM)-CSF are the most widely used cytokines in mobilization protocols. The major advantage of PBPC transplantation over conventional autologous bone marrow transplantation (ABMT) is the more rapid recovery of peripheral blood neutrophil and platelet counts, thereby shortening the period of panmyeloablation that is responsible for the life-threatening infections and hemorrhage associated with transplantation. A reduction in the requirement for antibiotics and blood products and a shorter hospital stay can result in an overall cost saving as compared with ABMT, although the financial benefits remain somewhat controversial.

It is believed that the rapid recovery of peripheral blood cell counts could be due to the reinfusion of a large spectrum of differentiating progenitors in the leukapheresis product. However, little is known of the characteristics and kinetics of the cells with long-term repopulating ability that are harvested using this procedure. After cyclophosphamide treatment, with or without GM-CSF, the numbers of circulating CD34-positive cells and colony-forming cells (CFC) decrease to undetectable levels, and their reappearance together with the appearance of long-term culture-initiating cells (LTC-IC) parallels white blood cell (WBC) recovery. We have examined cells collected by apheresis after mobilization with cyclophosphamide and G-CSF to assess their progenitor content using a recently devised assay for plastic-adherent (PΔ) progenitor cells, in addition to the traditional colony-forming unit-granulocyte/macrophage (CFU-GM) assay and quantitation of CD34-positive cells.

The fraction of bone marrow mononuclear cells that adheres to plastic contains progenitors capable of generating substantial numbers of CFU-GM in a 1-week-long delta assay system. The plastic-adherent progenitors have, therefore, been called PΔ progenitors. Their early position in the hematopoietic hierarchy has been confirmed by their ability to generate long-term in vitro hematopoiesis, burst-forming units-erythroid (BFU-E), and cobblestone area-forming cells (CAFC) in addition to CFU-GM; their resistance to 5-fluorouracil; their coexpression of the early phenotypic antigens CD34 and Thy-1; and the absence of expression of HLA-DR, CD33, and CD38. Removal of adherent cells from murine marrow compromises its ability to reconstitute irradiated recipients, suggesting that adherence depletes repopulating cells. This study has assessed the frequency and proliferative potential of PΔ progenitors in mobilized PBPC collections and compared them with CFU-GM and CD34-positive cells as markers of progenitor cell content and engraftment kinetics.

MATERIALS AND METHODS

Patients and PBPC collection. Twenty consecutive patients underwent PBPC collection and subsequent autografting in Cambridge, UK, between April and August 1994. All gave informed consent. Diagnoses included non-Hodgkin’s lymphoma (NHL; 11 patients),
PLASTIC-ADHERENT CELLS IN PBPC COLLECTIONS

### Table 1. Patient Details and Progenitor Cell Yields

<table>
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<tr>
<th>UPN</th>
<th>Disease</th>
<th>No. of Cells/Progenitors Harvested</th>
<th>Engraftment</th>
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<td>MNC x 10⁶/kg</td>
<td>CFU-GM x 10⁶/kg</td>
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<td>MM</td>
<td>6.3</td>
<td>48.0</td>
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<td>225</td>
<td>MM</td>
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<tr>
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<td>HD</td>
<td>6.5</td>
<td>16.2</td>
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Reinfusion doses given as per kilogram body weight. Engraftment data were excluded from patients 213 and 246 who received autologous bone marrow in addition to PBPC. Patient 235 who received CD34-purified cells, and patient AB who did not undergo autografting and was not assigned a UPN. There were no statistical differences between the numbers of progenitor cells collected from the patients with myeloma and the patients with lymphoma (CFU-GM, \( P = .49 \); CD34, \( P = .49 \); PA, \( P = .47 \)).

Abbreviations: MM, multiple myeloma; NA, results not available.

Hodgkin's disease (HD; one patient), and multiple myeloma (eight patients). Patient characteristics are listed in Table 1. The patients with lymphoma were a heavily pretreated cohort, while the myeloma group had received induction chemotherapy with vincristine, Adriamycin, and dexamethasone (VAD) but no other chemotherapy. All patients were primed with cyclophosphamide (Cy; Farmitalia Carlo Erba Ltd, St Albans, UK) 3 g/m² and G-CSF (Amgen Ltd, Cambridge, UK) 5 μg/kg/d to mobilize their PBPC. Cells were harvested on 2 consecutive days when the recovering WBC count surpassed 2%. The procedure took between 3 and 4 hours and used three times the patient's estimated blood volume was processed via an anticoagulant-to-blood ratio of 1:14. Flow rates varied between 60 and 80 mL/min, and a separation factor of 900 was used. Throughout the collection, the hematocrit of the harvested cells was maintained between 2% and 4%, and the resultant mononuclear cell volume ranged from 140 to 180 mL. Red cell contamination was less than 5% in all cases. The cells were frozen in a rate-controlled freezer and stored in the vapor phase of liquid nitrogen. Cryopreserved-thawed cells were reinfused after conditioning therapy using the BEM regimen.29 Briefly, this consists of BCNU 300 or 450 mg/m² (according to lung function tests), etoposide 2 g/m², and melphalan 140 mg/m². No hematopoietic growth factors were administered after the autograft. Venous blood was taken from 10 laboratory volunteers as controls. CFU-GM assay. Mononuclear cells (MNC) from each leukapheresis were isolated by density-gradient separation over Histopaque-1.077 (Sigma Diagnostics, Poole, UK). The cells from the interface were harvested and washed twice in Hank's buffered salt solution (HBSS; GIBCO, Paisley, UK). They were reuspended in 1 mL of HBSS and counted. Fresh viable MNC were plated in duplicate in 35-mm Petri dishes (Nunc, Life Technologies, Ltd, Paisley, UK) in semisolid medium containing 1.0% methylcellulose (Fluka, Buchs, Switzerland) and Iscove's modified Dulbecco's medium (GIBCO) 30% fetal calf serum (FCS; GIBCO), 1% bovine serum albumin (Sigma Diagnostics), β-mercaptoethanol (5 x 10⁻⁴ mol/L; Sigma Diagnostics), and recombinant GM-CSF (50 IU/mL; Schering-Plough, Mildenhall, UK) as a source of hematopoietic growth factor. The MNC were incubated at 37°C in humidified 5% CO₂ in air for 14 days. Colonies of more than 50 cells were scored on day 14. Results were expressed as the number of CFU-GM per 10⁶ MNC plated and converted to a reinfusion dose per kilogram of body weight.

Delta assay of plastic-adherent (PA) progenitor cells. MNC from the PBPC collections were isolated by density-gradient separation. Ten million MNC were suspended in 10 mL α-medium (GIBCO), supplemented with 15% fetal calf serum and incubated at 37°C in humidified 5% CO₂ in air for 2 hours in 25-cm plastic tissue culture flasks (Nunc). Preliminary experiments showed that increasing the incubation period to 18 hours did not increase the number of progenitor cells. The MNC were then diluted in α-medium supplemented with 10% FCS, 10% 5637 conditioned medium, and 2 x 10⁻⁴ mol/L methylprednisolone (Upjohn, Crawley, UK) to a final concentration of 10⁶ MNC/mL. The cultures were centrifuged at 37°C in humidified 5% CO₂ in air for 7 days. The supernatant containing the residual adherent cells. The flasks were then washed at 37°C in humidified 5% CO₂ in air for 1 hour. The nonadherent cells harvested and assayed for CFU-GM. Colonies of more than 50 cells were scored on day 7; of more than 200 cells, on day 14; and of more than 500 cells, on day 21.13 Colonies were expressed as the number of CFU-GM per flask per 10⁴ cells plated and back-calculated to give the number of PA progenitor cells present per milliliter of blood; and hence, the numbers reinfused.

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**RESULTS**

**PBPC harvesting.** The median time taken for the recovering WBC count to reach \(4 \times 10^9/L\) was 10 days (range, 9 to 13 days). The mean number of MNC collected from two leukaphereses was \(7.9 \times 10^9/kg\), of which \(10.5 \times 10^9/kg\) were CD34-positive. Mean numbers of day-14 CFU-GM and PA progenitors were \(47.3 \times 10^9/kg\) and \(9.3 \times 10^9/kg\), respectively. The numbers of MNC and progenitors harvested are listed in Table 1. There was a significant correlation (Fig 1) between the numbers of CFU-GM and CD34-positive cells \((r = .86; P = .001)\), but the numbers of plastic-adherent PA progenitor cells did not correlate with CFU-GM or CD34-positive cells.

**Quantitation of PA progenitors.** We first examined PA cultures from the peripheral blood of 10 normal volunteers. Four of the 10 volunteers produced less than one CFU-GM per flask per \(10^7\) mononuclear cells plated. The remainder produced between 10 and 50 CFU-GM. A single limiting dilution experiment on normal blood gave a frequency of \(1/10^3\) mononuclear cells. Four limiting dilution analyses of PBPC gave frequencies of PA progenitor cells of between \(1 \pm 0.3\) (mean \(\pm\) SE; 95% range, 0.3 to 1.6) and \(5.3 \pm 0.7\) \((3.8 \pm 0.6)\) per \(10^7\) MNC, which is similar to that of normal bone marrow \((2.5 \pm 10^7\) per \(10^7\) MNC). The fifth analysis gave a frequency of \(0.3 \pm 10^6\), which is similar to normal blood, and because only 3% of the wells were positive, this analysis was excluded from further calculations. The proliferative potential of individual PA progenitors was heterogeneous with respect to CFU-GM production within experiments, but the distributions for different samples were very similar \((P = .8, \text{Kruskal-Wallis Test; Fig 2})\). Consequently, it seemed justified to calculate the coefficient of PA progenitor cells present in the bulk cultures by dividing the total number of CFU-GM produced by the mean of the individual values \((2.4, 3.4, 5.1, \text{and } 7.1)\) for CFU-GM per PA progenitor cell \((ie, 4.5 \pm 1.8)\). The derived number of PA cells infused is listed in Table 1.

**Engraftment kinetics.** Of the 20 patients who underwent PBPC mobilization and collection, four were excluded from further evaluation. The cells from one patient (unique patient number [UPN] 235) were further manipulated to obtain purified CD34-positive cells, two patients (UPN 213 and 246) yielded low numbers of CFU-GM and subsequently received stored-up autologous bone marrow together with their PBPC, and one patient (AB) relapsed and did not undergo autografting.

All patients who received PBPC alone were given more than our threshold number of \(10 \times 10^6\) CFU-GM and more than \(2.7 \times 10^5\) PA cells per kilogram. Fifteen of 16 patients engrafted rapidly, with a median of 12 days to a neutrophil count of greater than \(0.5 \times 10^9/L\) and 12 days to a sustained platelet count of greater than \(50 \times 10^9/L\). The remaining patient (UPN 230) had delayed engraftment \((25 \text{ and } 45 \text{ days})\), which was associated with poor renal clearance of etoposide. Infusion of more than the threshold number of cells and consistent rapid engraftment precluded evaluation of an association between progenitor cell numbers and engraftment kinetics.

**DISCUSSION**

Small numbers of primitive hematopoietic progenitor cells are known to circulate in steady-state peripheral blood. In the recovery phase after chemotherapy or after administration of certain HGFs, numbers increase to levels that allow collection and subsequent autografting. For reasons that are not entirely clear, engraftment after PBPC transplantation is

![Fig 1. Correlation between the numbers of CFU-GM and CD34 positive cells in the leukapheresis harvests (n = 18).](image-url)
Fig 2. Cumulative distribution of CFU-GM numbers (days 7, 14, and 21 and total) produced by individual PΔ progenitors cultured at limiting dilution. The data plotted were taken from the cell concentration that corresponded to a probability of greater than .75 that the contents of the wells were monoclonal. The results are from three separate experiments. The y axis shows the percentage of positive wells yielding between 1 and n CFU-GM; results from the negative wells are not plotted. The individual curves represent results from 7 to 12 wells. The frequencies of negative wells were 0.63, 0.75, and 0.78; the probabilities of monoclonality were .80, .75, and .78. In one experiment (data not plotted) consisting of 96 replicate wells, 11.5% of the wells were positive (probability of monoclonality = .94) and produced a total of 1 to 11 CFU-GM.

More rapid than with bone marrow, presumably as a result of infusing a spectrum of differentiating cells. This procedure has radically altered the clinical approach to high-dose therapy and stem cell rescue. The kinetics and characteristics of PBPC are under intensive investigation and are as yet not fully understood. Much of this work has focused on CD34-positive cells and CFU-GM, but the long-term culture system, which requires bone marrow stroma as a feeder layer, has been used to study more primitive progenitor cells. A recently described assay takes advantage of the adherent nature of primitive hematopoietic cells in bone marrow. When cultured in a plastic tissue culture flask, the nonadherent component that contains CFU-GM is removed, leaving earlier progenitors adherent to the side of the flask. Subsequent CFU-GM production can, therefore, be attributed to the pre-CFU-GM that adhered to the flask. It is possible to measure CFU-GM after 7 days because preexisting CFU-GM are removed at the outset and because stromal cells, which inhibit CFU-GM production, are not present to any significant degree in the first 7 days of culture. We have used this assay to measure the frequency and proliferative capacity of these cells in PBPC collections.

Limiting dilution analysis has shown that the frequency of PΔ progenitor cells in PBPC collections is similar to that of normal bone marrow (ie, 1 to 5.3 v 2.5 to 10 per 10⁵ MNC, respectively), but their capacity to generate CFU-GM is markedly less. Comparison of the distribution of the numbers of CFU-GM produced by PΔ cells in PBPC with the distribution of the numbers produced by PΔ cells in normal bone marrow reveals a significant difference (P = .001, Wilcoxon signed rank test). The overall mean value of 4.5 ± 1.8 CFU-GM per PΔ progenitor cell that we have
measured for PBPC can be compared with a mean value for normal marrow \( n = 9 \) of 24 ± 14 (Gordon\textsuperscript{15} and unpublished data, 1995; \( P = 0.05 \), Mann-Whitney \( U \) test). This could reflect the presence of more mature PA progenitor cells in the PBPC harvests compared with bone marrow, which may only be capable of a small number of divisions before terminal differentiation. Alternatively, the PA cells from PBPC may undergo undetectable self-renewal divisions resulting in lower output of CFU-GM in the short time period used for the assay. In support of this suggestion, the cumulative distribution of CFU-GM production (Fig 2) is similar to that for P\( \Delta \) cells in human umbilical cord blood (HUCB), which have been shown to be capable of self-renewal.\textsuperscript{26} Sutherland et al\textsuperscript{27} have reported a similar phenomenon with regard to LTC-IC in PBPC collections, which produce fewer CFU-GM than LTC-IC in normal bone marrow.

The heterogeneity of CFU-GM production by individual progenitor cells is a common feature of several hematopoietic cell assay systems.\textsuperscript{1,27-28} One explanation is that the heterogeneous progenitor cell population represents a spectrum of cells with different degrees of maturity and ability to generate more mature progeny. Another is that cells undergo self-renewal or differentiation according to a stochastic distribution of probability. It is tempting to speculate that the probability of P\( \Delta \) progenitor cell self-renewal in PBPC and HUCB is greater than that of P\( \Delta \) cells in adult bone marrow. This would imply that PBPC collected after mobilization therapy contain a population of cells that are more capable of amplification than those found in a bone marrow harvest. It is relevant in this context that PBPC have been identified as ideal targets for retroviral gene transfer.\textsuperscript{14} It is difficult to demonstrate, as the majority of patients recover their counts within 16 days. Although there is no correlation evident between P\( \Delta \) cells and engraftment, or between LTC-IC and engraftment, there may be a threshold effect for primitive cells similar to that for the numbers of CFU-GM infused.\textsuperscript{24}

We conclude that the plastic-adherent progenitor cell assay is capable of measuring the quality and quantity of primitive cells that are ancestral to CFU-GM in the hematopoietic hierarchy.

ACKNOWLEDGMENT

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