Collection of Peripheral Blood Hematopoietic Progenitors (PBHP) From Patients With Severe Aplastic Anemia (SAA) After Prolonged Administration of Granulocyte Colony-Stimulating Factor


The aim of this study was to test whether prolonged administration of granulocyte colony-stimulating factor (G-CSF) would allow the collection by leukapheresis of PBHP in patients with SAA. For this purpose, nine SAA patients, 7 to 46 years old, six of whom were enrolled at diagnosis of their disease and three after previous immunosuppression had failed, were treated with antilymphocyte globulin (ALG) (day 1 to 5), cyclosporin A (5 mg/kg/d orally) (day 6 to 90) and G-CSF 5 μg/kg/d (day 6 to 90). A total of 40 leukaphereses were performed, (range 2 to 7 per patient), between days +10 and +186 from G-CSF treatment. White blood cell count at the time of harvest ranged from 1.2 to 18.1 × 10⁹/L. Results can be summarized as follows: the median number of cells collected per patient was 5.0 × 10⁹/kg (range 2.6 to 18.7), the median number of CD34⁺ cells was 1.8 × 10⁶/kg (range 0.27 to 3.9) and the median number of colony-forming units granulocyte-macrophage (CFU-GM) was 3.9 × 10⁴/kg (range 0 to 39). Twenty leukaphereses performed between days +33 and +77 of G-CSF treatment grew granulocyte macrophages and erythroid colonies in vitro. No colony growth was obtained from 20 leukaphereses performed before day +33 or after day +80. In six patients the total number of CFU-GM recovered were in the range described for autologous peripheral blood stem cell grafts. (2.6 to 39 × 10⁹/kg).

In conclusion, this study suggests that circulating hematopoietic progenitors can be recovered after ALG priming and after at least 1 month of G-CSF treatment in a proportion of patients with SAA. Whether these cells will be suitable for autologous transplantation remains to be determined.

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MATERIALS AND METHODS

Patients. Nine patients were admitted with the diagnosis of acquired aplastic anemia: main clinical data are outlined in Table 1. All nine were transfusion dependent at the time of G-CSF treatment according to an ongoing European Group for Bone Marrow Transplantation (EBMT) trial designed for patients presenting with less than 0.5 × 10⁹ polymorphonuclear neutrophil leukocytes (PMN)/L. This protocol includes horse ALG (Merieux, Lyon, France), 15 mg/kg/d on days 1 through 5; 6-methylprednisolone, 5 mg/kg/d intravenously (IV) on days 1 through 5, 2.5 mg/kg/d IV on days 6 through 10, then tapering the dose until discontinuation on day +30; CsA, 5 mg/kg/d orally from day +6 to day +90; and G-CSF 5 μg/kg/d (IV for 15 days, then subcutaneous) from day +6 to day +90. One patient (no. 2) was not eligible for this study (PMN 0.94 × 10⁹/L) and therefore received the same regimen without G-CSF. He underwent a leukapheresis immediately after ALG administration and, because of deteriorating peripheral blood (PB) counts, was started on G-CSF on day +51.

Leukapheresis. Each patient underwent two to seven leukaphereses using a continuous flow cell separator Fenwal CS 3000 (Deerfield, USA).

Cell surface markers. PB cells recovered from leukapheresis were processed with a workstation Coulter Q-Prep (Coulter, Hialeah, FL), without further separation. Cell surface antigens were detected by a direct immunofluorescence using a panel of conjugated fluorescein isothiocyanate (FITC)/phosphatidylethanolamine (PE) MoAbs: Cyto-stat Coulter Clone CD3 (Coulter), CD4, CD8, for T lymphocytes; CD19 and HLA-DR for B lymphocytes; CD33 for myelomonocytic cells (Coulter); and CD34 HPCA-2 for progenitor cells (Becton Dickinson, Mountain View, CA). Fluorescence was analyzed with a Coulter EPICS Profile II (Coulter). Isotypically matched mouse Igs, directly conjugated to FITC or PE, were used as negative controls in all experiments.
In vitro colony growth. PB cells recovered from leukapheresis (10⁶) were plated unfraccionated in 1 mL of Iscove's modified-Dulbecco's medium (IMDM) containing 30% fetal calf serum (FCS), in 0.9% methylcellulose, for colony-forming unit granulocyte-macrophage (CFU-GM) growth with 100 ng/mL of recombinant human (rh)GM-CSF (Sandoz, Basel, Switzerland). In the attempt to improve colony formation, PB cells from three leukaphereses were kept in liquid culture (10⁶ cells/mL) for 5 days in the presence of SCF (Genzyme, Boston, MA), 100 ng/mL.

Cells from the first 18 leukaphereses were further separated as follows: after a Ficoll Hypaque gradient (ICN Flow, Costa Mesa, CA) (1,077 g/cm²) and plastic adherence (2 hours at 37°C), cells were kept in liquid culture and CD34-positive fractions were plated in 0.9% methylcellulose (Roth, Karlsruhe, Germany) for colony-forming unit granulocyte-macrophage (CFU-GEMM) growth with IMDM containing 30% FCS, 2/10⁻⁴ mol/L Hemin, beta mercaptoethanol, 1% bovine serum albumin (Fraction V; Sigma, St Louis, MO), in the presence of 2U/mL rh erythropoietin (rhEpo; Cilag, Milan, Italy). 100 ng of rhGM-CSF (Sandoz, Basel, Switzerland), and 100 ng/mL rh interleukin-3 (provided by Sandoz) with or without SCF (20 ng/mL) (Genzyme).

The cultures were incubated for 14 days in a 37°C, 5% CO₂ humidified incubator. Colonies were counted with an Olympus IM inverted microscope (Olympus, Lake Success, NY).

Cryopreservation. PB cells were cryopreserved in culture medium (TC 199) containing 10% dimethyl sulfoxide and 5% autologous heparinized plasma by means of a freezing flow rate unit (Planer 203). Cryopreserved samples were stored in gas phase of liquid nitrogen.

RESULTS

Patients. All patients completed the designed course of treatment (Table 1). Two patients (no. 2 and 3) died on day +150 and +200 of infection; we were unable to collect from these patients PB cells capable of colony formation in vitro. The other seven patients are surviving between day 100 and day 365 after G-CSF treatment; four are transfusion dependent and three are transfusion independent. The number of patients is too small to draw any conclusions on the effect of G-CSF administration on hematologic response.

Leukapheresis. A total of 40 leukaphereses were performed. There were no major problems in performing the procedures also with low leukocyte counts. Leukaphereses were performed, when possible, at intervals of 7 to 15 days. However, there were variations because of the fact that these were all referred patients, often not available in Genova for the procedure. The median number of cells collected per procedure was 5.0 × 10⁶/kg (range 2.6 to 18.7) (Table 2).

Cell phenotype. The surface phenotype of recovered cells was expressed as medians (range) as follows: CD3, 55% (13% to 80%); CD4, 30.5% (4% to 39%); CD8, 26% (17% to 62%); CD19, 13% (3.3% to 28%); DR, 12% (3.2% to 31%); CD33^+34^, 10% (0.3% to 30%); CD33^−34^, 0.2% (0% to 1%); CD33^+34^, 0.3% (0% to 4.4%).

We saw no major increment of circulating CD34^+ cells during treatment with G-CSF. The median number of CD34^+ cells collected per leukapheresis was 1.8 × 10⁶/kg (range 0.27 to 3.8) (Table 2).

Table 1. Clinical Data of Patients Studied

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Cause</th>
<th>Interval Between Diagnosis and G-CSF Treatment</th>
<th>PMN at Treatment</th>
<th>RBC</th>
<th>Plt</th>
<th>Present Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>46</td>
<td>palp</td>
<td>30 d</td>
<td>0.28 × 10⁹/L</td>
<td>5</td>
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<td>Yes</td>
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<tr>
<td>2</td>
<td>M</td>
<td>11</td>
<td>idio</td>
<td>60 d</td>
<td>0.38 × 10⁹/L</td>
<td>8</td>
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<td>Yes</td>
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<tr>
<td>3</td>
<td>F</td>
<td>12</td>
<td>idio</td>
<td>1 yr</td>
<td>0.40 × 10⁹/L</td>
<td>12</td>
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<td>Yes</td>
</tr>
<tr>
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<td>M</td>
<td>17</td>
<td>idio</td>
<td>4 yrs</td>
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<td>15</td>
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<td>Yes</td>
</tr>
<tr>
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<td>M</td>
<td>45</td>
<td>idio</td>
<td>120 d</td>
<td>0.03 × 10⁹/L</td>
<td>8</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>18</td>
<td>idio</td>
<td>60 d</td>
<td>0.03 × 10⁹/L</td>
<td>15</td>
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<tr>
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<tr>
<td>9</td>
<td>M</td>
<td>10</td>
<td>idio</td>
<td>15 d</td>
<td>0.30 × 10⁹/L</td>
<td>10</td>
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</tr>
</tbody>
</table>

Abbreviations: d, days; Plt, platelets; AW, alive and well; Tx-D, transfusion dependent; Tx-I, transfusion independent.

Table 2. Summary of Collected PB Cells, CFU-GM Growth, and CD34 Positivity in Nine Patients Undergoing Leukaphereses After G-CSF Treatment

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of Cells × 10⁶/kg</th>
<th>No. of Colonies × 10⁹/kg</th>
<th>No. CD34^+ Cells × 10⁹/kg</th>
<th>Autologous Hematologic Recovery</th>
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<tr>
<td>1</td>
<td>5.0</td>
<td>39.1</td>
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<td>1.34</td>
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</tr>
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<td>3.1</td>
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<td>No</td>
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<td>0.93</td>
<td>0.27</td>
<td>No</td>
</tr>
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<td>13.8</td>
<td>26.2</td>
<td>nt</td>
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</tr>
<tr>
<td>9</td>
<td>18.7</td>
<td>17.1</td>
<td>nt</td>
<td>No</td>
</tr>
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</table>

Median: 5.0, range (2.6-18.7).
Colony formation in vitro. Twenty leukaphereses performed before day +30 or after day +80 grew no colonies (Fig 1). Twenty leukaphereses performed between day +33 and day +77 yielded a large number of GM and erythroid colonies (Table 2; Figs 1 and 2). PB cells from two aphereses (patient no. 1) also grew large numbers of burst-forming units erythroid (BFU-E): 17 and $37 \times 10^4$/kg (Fig 2). In the last two patients (no. 8 and 9) who underwent 6 to 7 leukaphereses in the suggested appropriate "window" at 7 to 10-day intervals, the number of CFU-GM recovered was very high ($17 \text{ and } 26 \times 10^4$/kg (Table 2). Samples from four leukaphereses were thawed and plated in vitro for colony formation: CFU-GM recovery was 36% when compared with top colony formation before cryopreservation.

T-cell depletion and colony formation. T-cell depletion (TCD) did not seem to enrich for hematopoietic progenitors, and indeed had a detrimental effect on three leukaphereses from which large number of colonies had been grown from unseparated cells. Two of these cell populations had been treated with CAMPATH-1 M and one with E-rosetting. There was one exception: the last leukapheresis from patient no. 1 grew no colonies before or after TCD but did grow 35 mixed colonies (CFU-GEMM) from $10^5$ CD34+ positively selected cells.

DISCUSSION

In this study, we have shown that prolonged administration of G-CSF and CsA to patients with aplastic anemia, following treatment with ALG, can result in mobilization of large numbers of hematopoietic progenitors. We believe this finding confirms that some patients with SAA still have a stem cell reservoir in spite of pancytopenia. This is supported by previous work from Torok Storb who first showed an increased number of BFU-E in the PB after ALG therapy. Our finding is also in keeping with data obtained in animals showing that treatment with G-CSF alone mobilized pluripotent stem cells in the PB which could then be successfully used for autografts. Indeed, in six patients the total number of CFU-GM recovered from the PB was in the range reported to allow hematopoietic reconstitution if infused after ablative chemoradiotherapy. This may also suggest that the "response" of SAA patients to ALG with or without G-CSF may be dependent on circulation in the PB and re-seeding of hematopoietic progenitors: in patients in whom the accessory cell network has been modified by so-called immunosuppression, this leads to hematologic recovery. In patients in whom the marrow microenvironment is still abnormal, re-seeding of hematopoietic progenitors fails. It is in the latter patients that harvesting and cryopreserving hematopoietic progenitors from PB can offer an additional chance of treatment if the underlying disease can be cured, for example, by high-dose cyclophosphamide.

However, there are still several open questions: the timing of cell collection, the quality of cells collected, and their ability to allow sustained hematologic recovery if used for autologous transplantation. As to the first question, our data would indicate that circulation of hematopoietic progenitors in PB occurs in the second and part of the third month of G-CSF treatment. As more patients are enrolled in the study we may find colony growth also beyond the third month, but for the time being we would advise performing leukapheresis every 10 days between day +30 and day +80 of G-CSF treatment.

As to the quality of cells collected we know they can form granulocyte-macrophage and erythroid colonies in vitro in large numbers. We also know that CFU-GM growth is a functional test predicting engraftment after autologous PB cell transplants, although information concerning the association between CFU-GM growth and engraftment has been obtained in patients with malignancy and a functioning bone marrow such as lymphomas, myelomas, and solid tumors. An exception is the work by Haas et al, who "mobilized" PBHP from patients with chemoradiotherapy-induced cytopenia and used them successfully for autologous grafts.

Finally, will these cells be capable of sustained hematologic recovery if infused after high-dose cyclophosphamide.

**Fig 1. Numbers of CFU-GM colonies \( \times 10^4$/kg grown for single leukaphereses, plotted against time (days) of G-CSF treatment. There is no colony formation before day +30 or after day +80. Considerable numbers of CFU-GM are collected between days +33 and +80. Each asterisk represents one leukapheresis.**
There are several in vitro studies suggesting that hematopoietic progenitors from SAA patients are abnormal: in particular Marsh et al. recently showed with long-term cultures that they have a reduced capacity of proliferation and survival also when grown on normal stromal layers: we do not know if this is caused by a functional and possibly reversible defect or an intrinsic abnormality. The finding of a high proportion of female patients with clonal hematopoiesis would argue in favor of the latter hypothesis. However, a recent review has pointed out that abnormalities of hematopoietic progenitors from SAA patients, and particularly clonality, may not necessarily indicate an intrinsic defect, or a preleukemic disorder. In keeping with this view is a recent contribution showing the lack of point mutation of N-ras oncogene in marrow cells from SAA patients. We favor this view, and regard the high incidence of leukemia and myelodysplasia seen in long-term survivors after ALG as the expression of long-lasting stressed hematopoiesis. If we were capable of modifying the accessory cell circuits, as known to occur after high-dose cyclophosphamide, and if we then reinfused autologous hematopoietic progenitors, this might allow better hematologic recovery.
than seen with ALG alone, and thus reduce the risk of late clonal disease.

Although this is only a working hypothesis, we believe our present findings warrant further investigations in this area.

REFERENCES


Collection of peripheral blood hematopoietic progenitors (PBHP) from patients with severe aplastic anemia (SAA) after prolonged administration of granulocyte colony-stimulating factor

A Bacigalupo, G Piaggio, M Podesta, MT Van Lint, M Valbonesi, G Lercari, PG Mori, M Pasino, E Franchini and L Rivabella