Circulating Stem Cells in Mice Treated With Cyclophosphamide

By C.F. Craddock, J.F. Apperley, E.G. Wright, L.E. Healy, C.A. Bennett, M. Evans, P.G. Grimsley, and M.Y. Gordon

Chemotherapy has been used clinically to mobilize hematopoietic progenitor cells into the peripheral blood so that they can be harvested for autologous transplantation. In humans, this is demonstrated by the presence of circulating granulocyte-macrophage colony-forming cells (CFU-GM) and CD34-positive cells, but it has not been possible to confirm the presence of marrow-repopulating stem cells. In this study, we treated mice with 200 mg/kg cyclophosphamide (CY) and measured the numbers of white blood cells, day 12 CFU-S (CFU-Si2), and CFU-GM in the peripheral blood. There was a peak in the numbers of CFU-Si2 and CFU-GM 8 days after treatment with cyclophosphamide. Peripheral blood cells taken at this time rescued lethally irradiated mice and engraftment of donor cells was confirmed after 140 days in sex mismatched recipients using a Y chromosome-specific probe. In vitro culture of the blood cells harvested after cyclophosphamide showed that they proliferated in suspension cultures for at least a year in the presence of interleukin-3. The cultured cells rapidly lost their abilities to rescue irradiated mice and to form colonies in vitro, but they did not become leukemic. Also, CY-treated mice were irradiated with a leukemogenic dose of x-rays to coincide with peak circulating cell numbers but these animals did not develop an excess of leukemias over mice given irradiation alone.

Two methods have been used clinically to mobilize hematopoietic cells for peripheral blood autografting.1 One, mobilization using hematopoietic growth factors (GM-colony-stimulating factor [GM-CSF] or G-CSF), has recently been investigated in a murine model by Molineux et al.3 Here, we have based our study on the second method, mobilization of cells after chemotherapy. This approach may avoid the risk of stimulating the leukemic cells in vivo which might occur with the use of growth factors.4 Also, because patients with leukemia are already treated with chemotherapy, it might avoid the introduction of a further agent into the treatment. The purpose of our study was to clarify, using a murine model, the existence, in the blood of mice treated with a mobilizing dose of cyclophosphamide, of cells capable of rescuing lethally irradiated recipients, and to define the temporal relationships between increases in circulating CFU-Si2 and CFU-GM.

MATERIALS AND METHODS

Mice. CBA/ca male and female mice, 10 to 14 weeks old at the beginning of the study, were provided with sterile food and water ad libitum. Male mice were treated with cyclophosphamide (CY) and their blood or marrow subsequently harvested or used as controls. CY (200 mg/kg) (Endoxana, Degussa Pharmaceuticals, Cambridge, UK) was injected intraperitoneally and blood was harvested by cardiac puncture 4, 8, or 12 days later. Female mice were used as recipients in transplantation experiments.

Cell counts and colony assays. The blood or bone marrow was first separated, according to the manufacturer’s recommendations, on Lymphoprep (Nyegaard, Oslo, Norway) to obtain the mononuclear cell fraction. White blood cell (WBC) and mononuclear cell counts were made by hemocytometry. Differential cell counts were made on cytospin (Shandon Runcorn, Cheshire, UK) preparations of mononuclear cells stained with May Grunwald-Giemsa. Colony formation by CFU-GM in the peripheral blood of individual mice was assayed on semisolid agar dissolved in a-medium supplemented with 15% fetal calf serum (GIBCO-BRL, Glasgow, Scotland, UK) and 15% WEHI-3b conditioned medium. Colonies of more than 50 cells were scored after 14 days of incubation at 37°C in 5% humidified CO2 in air. Recipients of cells for the spleen colony (CFU-S) assay (15 mice per group) were irradiated with 850 Gy x-rays from a Pantak H machine and injected with normal bone marrow cells or blood cells collected from CY-treated mice. With the exception of the preliminary titration experiments, individual peripheral blood recipients were injected with cells from individual donors. The mice were killed when they became moribund or sick.
Remainig well mice were killed on day 12. The spleens removed and fixed in Tellesniczky's medium and the numbers of colonies counted. Control mice which had been irradiated but not transplanted were included for each group. The level of endogenous colony formation in irradiated controls was zero.

**Long-term reconstititution of irradiated mice.** Groups of 20 mice were irradiated with 850 cGy x-rays and injected intravenously (IV) with the mononuclear cell fraction of 1.0 mL blood from CY-treated mice taken 0, 4, and 8 days after treatment. An untransplanted control group and a control group transplanted with 5 x 10^5 normal marrow cells were included. Mice were killed if they became moribund or sick, and this is recorded as the day of death.

**Analysis of the origin of hematopoiesis in female recipients of male cells.** One hundred and forty days after transplantation of male day 8 post-CY blood cells, the bone marrow and spleens of the female recipients and the spleens of male controls were removed and single cell suspensions were prepared. Containing erythrocytes were removed by lysis in 0.168 mol/L ammonium chloride for 10 minutes at room temperature. Spleen cells (2 x 10^7) were labeled with either Thy 1 monoclonal antibody (MoAb) (MAS108, Sera-Lab, Crawley Down, Sussex, UK) or B220 (hybridoma 14.8, American Type Culture Collection; ATCC, Rockville, MD) for 30 minutes on ice. After washing, sheep anti-rat IgG-coated Dynabeads (Dynal, Norway) were added to the cells and they were incubated on ice for 10 minutes. The cells attached to the beads were isolated using a magnetic particle concentrator (Dynal) and washed three times. Unattached cells were analyzed by immunofluorescence (see below) to confirm depletion of Thy-1 and B220-positive cells. The DNA from the positively selected cell populations and from unseparated bone marrow and spleen cell suspensions was extracted using standard techniques.

Ten micrograms of DNA was digested with *Hind*III and separated on a 0.8% agarose gel, denatured, neutralized, and blotted onto a nitrocellulose filter (Scheicher and Schuell, Germany). Probe pY353 was 3P random oligonucleotide-labeled using a commercial kit (GIBCO-BRL, UK) and hybridized to the blot in a solution of 50% formamide, 10 mmol/L EDTA (pH 8), 5X standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), and 5X Denhardt's at 42°C for 16 hours. The blot was washed once in 2X SSC/0.1% SDS at 42°C for 45 minutes and twice at 0.1X SSC/0.1% SDS for 45 minutes. The filter was then exposed to Kodak XAR5 for 2 hours with intensifying screens at ~70°C. To compare the amounts of DNA loaded into each lane, the blots were reprobed using a murine CD34 probe (G. May, unpublished data, December 1991). The densities of the CD34 and Y bands were measured with a Chromoscan 3 to control for DNA loading and to quantitate chimerism.

**Immunofluorescence.** Washed cells were resuspended in 20% immune globulin (Cutter Biological, Elkhill, IN) in phosphate-buffered saline (PBS) at a concentration of 1 x 10^6 cells per mL. The following MoAbs were used to detect cell surface antigens: Mac 1 (M1/70.15) and Thy 1 (MAS 108) were obtained from Sera Lab; the Ly 5 (B220) hybridoma (RA3-3A1) was obtained from the ATCC; F4/80 was a generous gift from Dr S. Gordon (University of Oxford, Oxford, UK); BP1 and BP3 were kindly donated by Dr K. McNagny (University of Alabama, Birmingham). Cells were stained as directed by the suppliers of the antibodies, using standard techniques, and bound antibody was detected using fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse or goat-anti-rat antibodies (Southern Biotechnology Associates, Birmingham, AL). Fluorescein-labeled goat-anti-mouse IgM (Southern Biotechnology) was used to identify cell surface Ig.

**In vitro liquid cultures of mobilized cells.** Peripheral blood was collected by cardiac puncture from mice treated 8 days earlier with CY. The mononuclear cells were separated using Lymphoprep. The cells were diluted to 2 x 10^6 per mL in Fischer's medium (GIBCO-BRL) supplemented with 20% horse serum (GIBCO-BRL) and 15% WEHI 3b conditioned medium.

**Assessment of leukemogenic potential of mobilized cells.** In some experiments, mice were exposed to a potentially leukemogenic dose of 300 cGy x-rays 8 days after treatment with 200 mg/kg CY and monitored for the development of myeloid leukemia. In other experiments, mice were sublethally irradiated (650 cGy x-rays) and injected IV with 10^7 cells that had been cultured for 7 months or longer.

**RESULTS**

**Effects of CY treatment on WBC count, circulating CFU-S12, and circulating CFU-GM.** Treatment with CY had no effect on the peripheral WBC count but resulted in a 90-fold increase in day 12 CFU-S numbers per milliliter of blood and a 530-fold increase in CFU-GM numbers (Table 1). There was also a 50-fold increase in the platelet count. The progenitor cells were present in increased numbers the eighth day after administration of CY but were not raised on days 4 or 12 post-CY (Table 1).

The mononuclear fraction of blood from mice treated with CY 8 days beforehand had a noticeably elevated platelet count and increased numbers of macrophages and immature myeloid cells (myeloblasts, promyelocytes, and myelocytes) compared with controls (Table 2). The increase in cells of the monocye-macrophage series was confirmed on analysis of the immunophenotype which also showed an elevation in cells with B-cell markers (Table 3).

**Transplantation potential of blood from CY-treated mice.** Preliminary dose-response experiments in 30-day survival studies showed that transplantation of 0.25 mL of blood taken 8 days after CY failed to rescue irradiated (850 cGy) recipients, 0.5 mL rescued 25%, and 1.0 mL rescued 95%. Transplantation of the mononuclear cells from 1.0 mL of peripheral blood from mice treated with CY 4 or 12 days beforehand failed to rescue lethally irradiated recipients (Fig 1) and the survival curves were identical to those obtained for irradiated nontransplanted controls and for recipients of 1.0 mL blood from untreated donors (data not shown). In contrast, 90% of the recipients of mononuclear cells from 1.0 mL of peripheral blood taken 8 days after CY survived and showed the same survival curve as recipients of 5 x 10^6 normal bone marrow cells. All mice that survived for 30 days also survived long-term (for more than 1 year).

**Table 1. Numbers of WBCs, CFU-S Day 12, and CFU-GM per Milliliter of Blood Before and After CY**

<table>
<thead>
<tr>
<th>Days After KY</th>
<th>WBCs/mL Blood x 10^8</th>
<th>CFU-S12/mL Blood</th>
<th>CFU-GM/mL Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.7 ± 2.1</td>
<td>0.5 ± 0.4</td>
<td>3.6 ± 1.9</td>
</tr>
<tr>
<td>4</td>
<td>2.0 ± 0.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>6.9 ± 2.6</td>
<td>44.8 ± 2.0</td>
<td>2.094 ± 1.145</td>
</tr>
<tr>
<td>12</td>
<td>4.9 ± 1.9</td>
<td>0.5 ± 0.4</td>
<td>1 ± 0.5</td>
</tr>
</tbody>
</table>

Blood cells from 15 mice per time point were counted and assayed individually for CFU-S12; blood from groups of five mice was pooled and assayed for CFU-GM.

*Mean ± SD.
Table 2. Differential Mononuclear WBC Count of Control Mice and Mice Administered CY Eight Days Earlier

<table>
<thead>
<tr>
<th></th>
<th>% Monocyte-Macrophages</th>
<th>% Immature Myeloid Cells</th>
<th>% Remaining Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control blood*</td>
<td>2.0 ± 0.8</td>
<td>1.3 ± 1.2</td>
<td>97.6 ± 2.5</td>
</tr>
<tr>
<td>Post-CY blood*</td>
<td>20.6 ± 4.0</td>
<td>13.0 ± 1.6</td>
<td>66 ± 4.6</td>
</tr>
</tbody>
</table>

Differential count on 500 cells (mean ± SD).

*Blood from three individual control and three individual CY-treated mice.

Donor origin of hematopoiesis in transplanted mice. Southern analysis was used to demonstrate the presence of male cells in irradiated recipients 140 days after transplantation of day 8 post-CY male blood. Representative results from one of the animals are shown in Fig 2. The probe hybridized to DNA from bone marrow cells and from B and T cells of the transplanted female. There was no hybridization of the probe to DNA from the marrow of female control mice but it hybridized to DNA from male control spleen cells. The presence of Y chromosome–specific sequences 140 days after transplantation demonstrates that CY mobilizes cells capable of radiation rescue into the peripheral blood and that these cells can repopulate the myeloid, B-lymphoid, and T-lymphoid compartments. The degree of chimerism, evaluated by comparing the intensities of the CD34 and Y bands, exceeded 90% in all three lineages. Four long-term survivors have been investigated and all show greater 90% chimerism.

In vitro proliferation by blood cells from mice 8 days after CY treatment. Peripheral blood cells were harvested from mice 8 days after treatment with CY and cultured in liquid culture with interleukin-3 (IL-3). The growth curves for cells in IL-3 are shown in Fig 3. Cultures were set up on seven separate occasions and each time the same pattern was observed. After 1 to 2 days, the viability was very low (0.1%), but thereafter there was a phase of rapid proliferation followed by slower sustained expansion. Cumulative cell counts were determined for the first 90 days and periodically thereafter to check the doubling time of the population. The doubling time of 14 days was maintained for the first 250 days but proliferation then accelerated to give a doubling time of 3 days. The dependence of the cells on IL-3 was checked on days 30, 82, 145, 199, 268, and 278 and on each occasion withdrawal of the cytokine resulted in 100% cell death after 48 to 72 hours.

Table 3. Mononuclear Cell Phenotype of Normal Blood Cells and Blood Cells Taken Eight Days After CY

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Normal Blood % Positive</th>
<th>Post-CY Blood % Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>M1/70</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td>F4/80</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>Thy-1</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>BP3</td>
<td>13</td>
<td>30</td>
</tr>
</tbody>
</table>

Major antibody specificities: B220: pre B cells, B cells; M1/70: monocytes, macrophages (Mac 1); F4/80: mature macrophages (p160); Thy-1: T cells; BP-1: pre B cells; BP-3: B cells and mature myeloid cells; slgM: cell surface immunoglobulin.

Fig 1. Thirty-day survival of mice irradiated with 850 cGy and transplanted with normal bone marrow cells or 1 mL blood from mice treated with CY 4 days, 8 days, or 12 days earlier.

Fig 2. Hybridization of probe pY353 to DNA from the bone marrow, T cells, and B cells of female mice transplanted 140 days earlier with blood cells from day 8 post-CY males and to DNA from male and female controls. Arrows indicate bacteriophage λ/HindIII size markers (kb).
Morphologically (Fig 4) and phenotypically (Table 4) cells resembled factor-dependent cell lines after 5 months. The cells were assayed for CFU-S~ after 3, 4, and 8 weeks in culture and at no time were any spleen colony-forming cells detected, although mice were injected with $10^6$ cells each. Neither did the cells produce colonies when cultured in semisolid medium with WEHI-3b conditioned medium. Sublethally irradiated ($650$ cGy) mice injected with $10^7$ cells that had been cultured for 240 days have already survived for 5 months without developing leukemia.

**Susceptibility of circulating stem cells to leukemogenic X irradiation.** Twenty percent of mice irradiated with 300 cGy x-rays develop myeloid leukemia starting 6 months after irradiation. Seventy percent of mice treated with CY 8 days before irradiation survived the combined treatment. These survivors did not show any increased incidence or rate of development of leukemia compared with 100 control mice given 300 cGy x-rays alone. However, the influence of CY treatment on radiation toxicity is clear from the survival curve in Fig 5 because 100% of the animals in the irradiation alone group survived at 100 days.

**DISCUSSION**

It has been known for some time that the numbers of WBCs and CFU-GM in the peripheral blood increase after clinical chemotherapy and that these cells have been collected by leukapheresis for use in autologous transplantation. It has not been possible to study the effect of chemotherapy on human long-term repopulating cells for two reasons. First, the available progenitor assays do not measure cells that fulfill the required stem cell criteria and, second, methods that can be used to demonstrate engraftment of allogeneic cells are not applicable to recipients of autologous peripheral blood cells. Sustained hematopoiesis after peripheral blood cell transplantation has been adduced as evidence for stem cell engraftment. However, it is possible that the peripheral blood graft supports hematopoiesis transiently, but long enough to allow recovery of endogenous marrow function. The possibility of transient support followed by endogenous recovery is supported by the occurrence of transient cytopenias about 6 weeks after peripheral blood transplantation. In murine models, in contrast, it is possible to assay stem cells by transplantation and repopulation and to demonstrate engraftment in syngeneic sex-mismatched donor-recipient pairs.

The data that we have presented here are the first evidence that CY mobilizes long-term marrow repopulating cells into the peripheral blood of mice. Moreover, the increases in CFU-S and CFU-GM occur at the same time as the blood can be used to rescue irradiated mice. The results obtained from recipients of day 8 post-CY blood show that long-term repopulating cells are present as well as CFU-S$^{12}$ and CFU-GM. This conclusion is based on the detection of donor-derived hematopoiesis on day 140 posttransplantation, which is unlikely to have been derived from progenitors at the CFU-S$^{12}$ level. This information supports the suggestion that cells mobilized into the peripheral blood can be used as a source of stem cells for transplantation.
Moreover, if the relationship between CFU-GM and stem cell circulation applies to humans, the kinetics of the CFU-GM response might be useful in predicting the most appropriate time for harvesting blood for transplantation.

Other studies have shown that murine progenitor cells can be mobilized by a variety of treatments including administration of bacterial compounds, antibiotics, phenylhydrazine, and hematopoietic growth factors as well as chemotherapy. Goris et al. reported that peak numbers of circulating CFU-S coincided with peak numbers of CFU-GM in antibiotic-treated mice, suggesting that this relationship may be a general one. However, the timing of the peak may vary according to the mobilizing agent used and occurred 4 days after thiamphenicol treatment, 2 days after G-CSF, and, as we have shown, 8 days after CY. The variety of agents that can be used to mobilize progenitor cells suggests that mobilization might be a generalized response to perturbation of hematopoiesis. The physiological significance of this phenomenon is unknown but the ephemeral nature of the circulating cell population suggests that the cells are en route to another region of hematopoietic tissue. Indeed, in the mouse where the spleen normally is a hematopoietic organ, results indicate that stem and progenitor cells migrate from marrow to spleen.

The altered distribution of stem cells in mice treated to mobilize stem cells, suggests that the mechanisms normally assumed to localize stem cells in hematopoietic tissue are disrupted at least temporarily. Also, within the bone marrow, alterations in the radial distribution of stem cells in the femur have been noted in mice treated with G-CSF. The distribution of leukemic stem cells is grossly abnormal, possibly as the result of defective expression of cell adhesion mechanisms, and we found that stem cells mobilized by CY did not bind to cultured stroma (data not shown) whereas repopulating stem cells in bone marrow do bind to cultured stromal layers. Because the adhesive properties of the circulating stem cells were reminiscent of leukemia, we speculated that exposure in vivo to a leukemogenic dose of x-rays at the time of peak circulating stem cell numbers might result in an increased incidence or reduced latency of radiation-induced leukemia. No increase in leukemias was found, although the combination of CY plus radiotherapy was clearly toxic and resulted in fatalities spread over the subsequent 80 to 100 days.

The observation that mobilized stem cells did not bind to cultured stroma led to an evaluation of their requirements for growth in vitro. The cells proliferated for many months.

| Table 4. Phenotype of Cultured Cells After 1 Month and After 5 Months Compared With the Phenotype of the FDCP-mix Factor-dependent Cell Line |
|-----------------|-------------|-------------|-------------------|
| Antibody        | 1 mo in Culture | 5 mo in Culture | FDCP-mix Cell Line |
| Ly5 (B220)      | < 5          | > 90         | > 90              |
| C3bi (M1/70)    | > 90         | > 90         | > 90              |
| F4/80           | > 90         | > 90         | > 90              |
| Thy-1           | > 90         | > 90         | > 90              |
| BP1             | < 5          | < 5          | < 5               |
| BP3*            | > 90         | > 90         | > 90              |
| sigM            | < 5          | < 5          | < 5               |

See Table 3 for antibody specificities.

*Earlier passages of FDCP-mix were BP3 negative.

Fig 4. Morphology of cells derived from the blood of CY-treated mice and cultured with IL-3 for 5 months.

Fig 5. Survival curve for mice treated with CY followed by 300 cGy irradiation 8 days later.
in the absence of stroma but remained dependent on IL-3. The very slow growth rate of cells precluded karyotyping and cloning of a cell line. The growth factor dependence, phenotype, and morphology of the population derived from the blood of CY-treated mice resembled factor-dependent cell lines, although the post-CY cells were generated without intentional manipulation in vitro. The similarities between the post-CY cells and the multipotential murine cell line FDCP-mix,\(^3\) indicate that the former developed from a primitive stem cell population, although the cultured cells rapidly lost their ability to rescue irradiated mice. However, the cells did not transform in vitro and no leukemias were found in any recipients of large numbers of cultured cells. A proper understanding of the nature of the cultured cell populations and their physiological implications requires further detailed investigation.

In summary, our study has shown that transplantation of blood from CY-treated mice, like transplantation of blood from G-CSF–treated mice,\(^2\) can rescue and repopulate irradiated recipients. However, the mobilized blood cells do not behave like normal blood or bone marrow cells in vitro. The significance of these in vitro observations for autologous peripheral blood transplantation is not known, but our results encourage further studies on the properties of human stem cells mobilized for harvesting and transplantation.

**ACKNOWLEDGMENT**

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