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Peripheral Blood Expansion of Early Progenitor Cells after High-dose Cyclophosphamide and rhGM-CSF

Corrado Tarella, Dario Ferrero, Marco Bregni, Salvatore Siena, Eugenio Gallo, Alessandro Pileri and Alessandro M. Gianni

In 20 patients with non-Hodgkin lymphoma or breast cancer, high-dose cyclophosphamide induced, during the post-nadir period of rapid leucocyte recovery, on median day 19 about a 30-fold increase in the peak concentration of granulocyte–macrophage (CFU-GM) and erythroid (BFU-E) colony-forming cells, and an even higher increase in the more immature pluripotent progenitors (CFU-Mix, 72-fold). After infusion of recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF), peak concentration was reached earlier (median day 15) and with further enhancements (159, 116 and 283-fold, respectively, in the number of CFU-GM, BFU-E and CFU-Mix). Most CFU-GM were immature, lacking the differentiation antigen CD15, and gave rise to large myeloid colonies, reflecting a high proliferative capacity of the founder cells. Very immature maphosphamide-resistant progenitors were detectable. The marked expansion in the circulating pool was predictable and reliable, allowing harvesting, after two or three leukaphereses, of sufficient haematopoietic progenitors for autologous bone-marrow reconstitution.
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INTRODUCTION

SEVERAL GROUPS have used autologous peripheral blood mononuclear cells following high-dose chemotherapy or chemo-radiotherapy for haematopoietic reconstitution [1, 2]. Compared with bone-marrow stem-cell autografting, the use of circulating progenitors has several advantages. The harvesting of circulating cells is simple, does not require general anaesthesia and can be readily repeated; the risk of graft contamination by tumour cells is likely to be less for leukapheresed cells than for bone-marrow cells; the rate of haematopoietic recovery is faster for neutrophils and, in some instances, for platelets; and restoration of immune functions is possibly accelerated [3–5].

The number of circulating progenitors is low in haematopoietic steady-state, and most patients who were successfully autografted with circulating stem cells received mononuclear cells that had been collected during recovery from previous myelosuppressive chemotherapy, when the circulating stem-cell pool is expanded [2]. However, cytotoxic agents have different effects on haematopoietic progenitors [6] and conventional combination chemotherapy might not be ideally suited to expanding the circulating pool of haematopoietic stem cells that are capable of complete and stable engraftment.

We have designed and tested, in a variety of human tumours, novel regimens that involve the sequential administration of high doses of anticancer drugs [7]. The start drug, cyclophosphamide, appears ideally suited to expanding the circulating stem-cell pool, as suggested in animal systems [8]. Indeed, we have observed that circulating progenitors harvested after high-dose cyclophosphamide can accelerate haemopoietic reconstitution in patients autografted with both bone marrow and peripheral blood precursors [9, 10]. Administration of recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) after high-dose cyclophosphamide can add to this effect [11].

We now report the pattern of release and the biological characteristics of the progenitor cells appearing in the peripheral blood after high-dose cyclophosphamide, with and without rhGM-CSF.

PATIENTS AND METHODS

Patients

Circulating haemopoietic progenitor cells were studied in 20 patients treated with high-dose cyclophosphamide (7 g/m^2) for advanced diffuse, large cell non-Hodgkin lymphoma (NHL) (12 patients) or high-risk breast cancer (8 patients). Only patients without evidence of bone marrow involvement by neoplastic cells were eligible. 13 patients (median age 40, range 20–54) received cyclophosphamide only. 7 (50, 21–56) were additionally treated with rhGM-CSF (mammalian glycosylated, Sandoz–Schering Plough) at 5.5 μg of protein per kg per day via continuous intravenous infusion for 14 consecutive days, starting the day after cyclophosphamide administration. 4 NHL patients (3 in the cyclophosphamide group, 1 in the cyclophosphamide plus rhGM-CSF group) had been treated with a combination including cyclophosphamide [12] 2 to 18 months previously. The other patients had never received chemotherapy or radiotherapy.

All treatment procedures were approved by the hospital's ethical committee and patients gave informed written consent before entry.

Correspondence to C. Tarella.
C. Tarella, D. Ferrero, E. Gallo and A. Pileri are at the Dipartimento di Medicina ed Oncologia Sperimentale, Cattedra di Ematologia–Ospedale Molinette, Università di Torino, Via Genova 3, 10126 Torino; M. Bregni, S. Siena and A.M. Gianni are at the Divisione di Oncologia Medica, Unità di Trapianto di Midollo Osseo "Cristina Gandini", Istituto Nazionale Tumori di Milano, Milano; A.M. Gianni is also at the Istituto di Scienze Mediche, Università di Milano, Milano, Italy.
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Haemopoietic progenitor assays

The amount of circulating haemopoietic progenitor cells was evaluated by daily collection of heparinised peripheral blood over the 22 days after cyclophosphamide administration. For standardisation, the assays were done by plating total leucocytes obtained after red blood cell sedimentation in the presence of 33% Emagel (Behring). Cells were then seeded between 2×10^4 and 10^5/ml in semisolid medium.

Granulocyte–monocyte colony-forming units (CFU-GM) were assayed in Iscove's modified Dulbecco's medium (IMDM) containing 0.3% agar, 20% fetal bovine serum (FBS) (Gibco) and 10% TPA-30-1 supernatant as a source of CSF [13]. Erythroid burst-forming units (BFU-E) and erythroid-mixed CFU (CFU-Mix) were preferentially assayed on 0.3% agar or, alternatively, in 0.9% methylcellulose, IMDM being supplemented with 30% FBS, 10% autologous plasma, 1 U/ml rh-erythropoietin (Amgen) and 60 U/ml rh-interleukin 3 (rhIL-3) (Genzyme). After 14 days of incubation at 37°C in 5% CO_2, colonies were scored with an inverted microscope. BFU-E and CFU-Mix were generally identified by their morphological appearance. Agar cultures were particularly suitable for CFU-Mix detection, due to a lower chance of overlapping of distinct myeloid and erythroid colonies. In some instances, single colonies were picked up from methylcellulose cultures, smeared on a slide and stained with Giemsa to assess accurately the kind of colonies. The amount of CFU-GM, BFU-E and CFU-Mix per ml of peripheral blood was determined by multiplying the concentration of circulating progenitors (number of colonies per number of plated cells) by the total number of leucocytes in 1 ml peripheral blood.

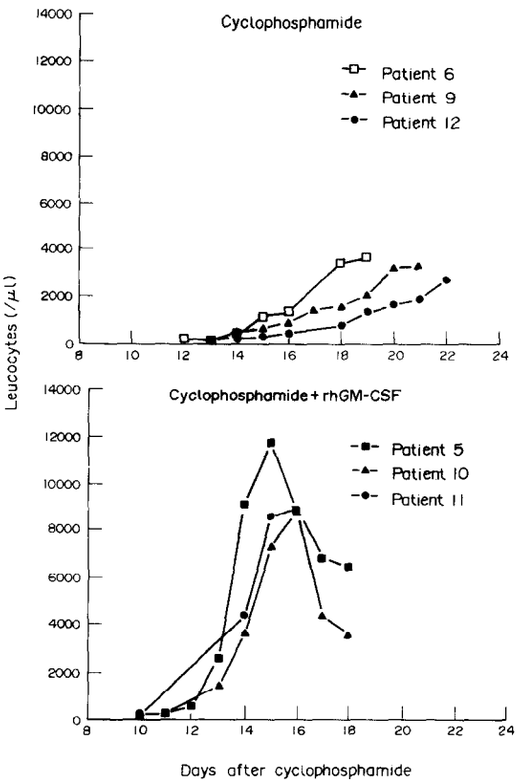

Fig. 1. Total leucocyte counts during recovery after high-dose cyclophosphamide and high-dose cyclophosphamide plus rhGM-CSF.

Phenotypic characterisation of circulating CFU-GM

Circulating myeloid progenitors were studied for their membrane reactivity to the DS1-1 monoclonal antibody (Mab). DS1-1 (provided by Dr G. Rovera, Wistar Institute, Philadelphia) is a cytotoxic IgM Mab, analogous to R1B-19, and reacts with a lacto-N-fucopentaose III antigen detected on granulocytes and their precursor cells [14]. This antigen has been classified as CD15 [15] and is expressed on the cell surface of granulopoietic cells from late CFU-GM to mature granulocytes, but it is absent on early myeloid progenitors [16]. Indeed, bone-marrow colonies appearing after 7–9 days of agar culture mainly derive from $CD15^+$ progenitors, while immature $CD15^-$ progenitors form colonies in 12–14 days of culture [16]. Immature $CD15^-$ CFU-GM are present both in bone marrow and peripheral blood, while differentiated $CD15^+$ CFU-GM are detectable in bone marrow only [17]. The reactivity of circulating CFU-GM appearing after cyclophosphamide and rhGM-CSF with CD15-DS1-1 Mab was evaluated by complement mediated cytotoxicity. Cells were incubated with DS1-1 hybridoma supernatant for 40 min at 4°C, then rabbit complement was added and the incubation continued for 90 min at 37°C [17]. After incubation, cells were washed and seeded in agar. Colonies were scored at days 7 and 14. The number of $CD15^+$ progenitors was calculated by subtracting the number of colonies grown at day 7 after DS1-1 and complement treatment from the total number of colonies grown in the control. Complement was previously tested for cytotoxic activity on normal bone-marrow cells.

Maphosphamide assay

Cyclophosphamide derivatives, such as 4-hydroperoxicyclophosphamide and maphosphamide, have a potent toxic effect on committed progenitors, including CFU-Mix [18], without affecting the viability and the renewal capacity of pluripotent stem cells. Maphosphamide resistant cells can generate committed progenitors after a few days of liquid culture [19]. Maphosphamide treatment followed by liquid culture (maphosphamide assay) was therefore used in 3 patients to evaluate the presence of circulating immature pluripotent progenitors. To reduce red blood cell contamination, which would hamper the cytotoxic effect of maphosphamide, nucleated cells obtained after Emagel sedimentation were further purified by Ficoll gradient centrifugation. Cells were then resuspended in IMDM at $1–3 \times 10^6$ /ml in the presence of maphosphamide at 100 μg/ml and incubated for 30 min at 37°C. After incubation, cells were washed, seeded in liquid culture at 10^6/ml in IMDM containing 20% FBS and rhIL-3 at 60 U/ml. At days 0 and 10 of liquid culture 0.2 ml cell suspension was plated in agar to evaluate CFU-GM numbers.

RESULTS

Kinetics of CFU-GM reappearance

In all patients peripheral blood CFU-GM were undetectable immediately after cyclophosphamide infusion. In patients treated with cyclophosphamide only, circulating CFU-GM reappeared after 11–16 days. The concentration of CFU-GM progressively increased and was paralleled by a rise of white blood cells. In patients treated with cyclophosphamide and rhGM-CSF, both CFU-GM and leucocyte reappearance was anticipated. These patients had a more homogeneous pattern, with CFU-GM first detectable between days 8 and 10. Both CFU-GM and leucocytes rapidly increased. However, after 5–8 days from their reappearance in the peripheral blood, the number of CFU-GM declined towards baseline in both groups of patients. Figs 1 and 2 show the changes of leucocytes and CFU-GM between days 10 and 22 in 3 patients from each group.

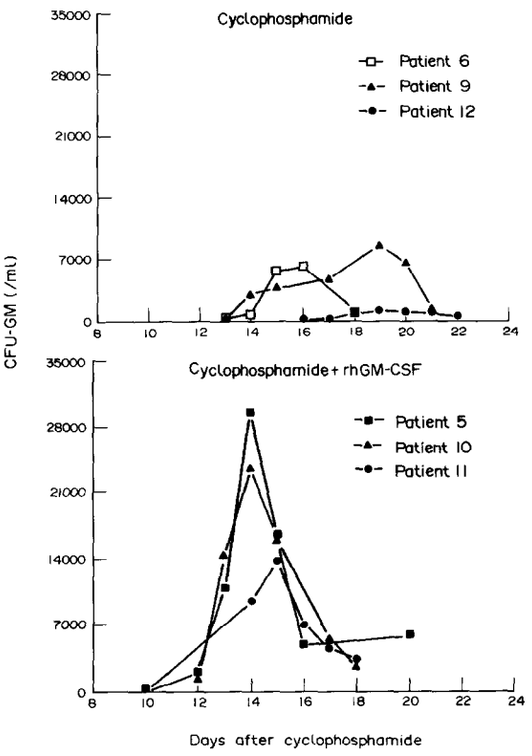

Fig. 2. Circulating CFU-GM during recovery after high-dose cyclophosphamide and high-dose cyclophosphamide plus rhGM-CSF.

Peak values of CFU-GM, BFU-E, CFU-Mix

Circulating CFU-GM evaluated in all 13 patients treated with cyclophosphamide peaked between days 15 and 21. The highest concentration of CFU-GM ranged between 1200 and 10,123/ml (Table 1). Values of CFU-GM, BFU-E and CFU-Mix in normal untreated subjects are also shown. BFU-E and CFU-Mix, evaluated in 4 patients treated with cyclophosphamide, also had a pronounced increase, matching the CFU-GM peak.

Peak values of CFU-GM in the 7 patients treated wtih cyclophosphamide and GM-CSF were higher (Table 1). Again, BFU-E and CFU-Mix, measured in 5 patients, paralleled the course of CFU-GM, with peak values several fold higher than those observed in controls. In the rhGM-CSF group, peak values were reached at day 14 in 1 patient, at day 15 in 4 patients and at day 18 in the remaining 2 patients.

Colony morphology

Most CFU-GM collected either after cyclophosphamide alone or cyclophosphamide plus rhGM-CSF gave rise to large myeloid colonies by day 14 of agar culture, containing at least 300–400 cells. Several colonies were huge (over 1000 cells) and visible to the naked eye, about 0.5–1 mm in diameter. An example of such colonies, which were more frequently seen in patients treated with rhGM-CSF, is shown in Fig. 3 (upper). Erythroid colonies were also large, generally containing between 4 and 10 subclones. Mixed colonies, stained with Giemsa, were mostly erythroid cells and eosinophils (Fig. 3, lower). Some of these colonies also contained macrophages and large cells with the appearance of megakaryocytes.

Reactivity to CD15 Mab

When cells were plated during peak value days at cell concentrations higher than 5×10^4 per dish, large colonies were detectable by day 7 of incubation. To discriminate the stage of differentiation of these progenitors, the reactivity to the CD15-DS1-1 Mab was examined in 3 patients. $CD15^+$ progenitors ranged between 0 and 75 per 5×10^4 plated cells, compared with 208–390 colonies derived from $CD15^-$ progenitors (Table 2). Indeed, the proportion of late CFU-GM never reached values higher than 20% of the total amount of circulating myeloid progenitor cells.

Detection of maphosphamide-resistant cells

Table 3 shows results obtained with peripheral blood cells from 1 patient treated with cyclophosphamide and 2 patients treated with cyclophosphamide and rhGM-CSF. Immediately after maphosphamide exposure, none or very few CFU-GM survived. However, many myeloid committed progenitors were generated after 10 days of liquid culture. Indeed, the number of CFU-GM in 1 ml of liquid culture ranged between 600 and 5260. Similar experiments were done with peripheral blood cells from 3 normal volunteers. Cells surviving after maphosphamide treatment gave rise to CFU-GM after 10 days of liquid culture. However, the generation of committed progenitors was lower than that in patients recovering from cyclophosphamide. The number of CFU-GM in 1 ml of liquid culture varied between 3 and 115. The increase in the number of CFU-GM after *in vitro* suspension culture can be explained by either a higher blood

Table 1. Peak values of circulating haemopoietic progenitors after cyclophosphamide or cyclophosphamide plus rhGM-CSF

	Day of peak	CFU-GM (ml)	BFU-E (ml)	CFU-Mix (ml)	CFU-GM/CFU-Mix ratio
Cyclophosphamide	19 (15–21)*	5000 (1200–10 123, $n = 13$)	3535 (879–6713, $n = 4$)	364 (103–539, $n = 4$)	13.7 : 1
Cyclophosphamide + rhGMCSF	15 (14–18)	23 767 (13 862–85 942, $n = 7$)	15 933, (8054–35 898, $n = 5$)	1413 (663–5069, $n = 5$)	16.8 : 1
Normal volunteers ($n = 8$)	–	149 (39–329)	137 (40–237)	5 (1–13)	29.8:1

*Median (range).

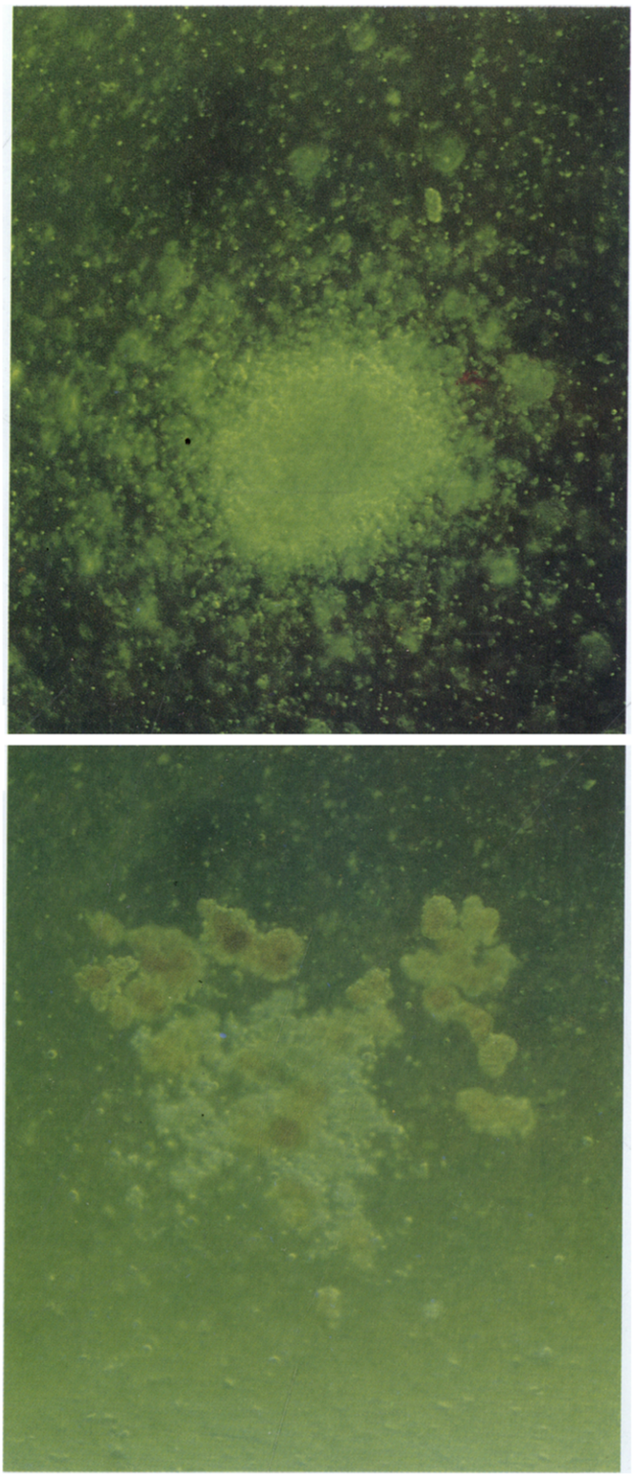

Fig. 3. Microscopic appearance of representative colonies derived from circulating CFU-GM (upper) and CFU-Mix (lower) in patients treated with high-dose cyclophosphamide and rhGM-CSF (original magnification × 40).

concentration of progenitors for CFU-GM or a higher proliferative potential of the same number of progenitor cells or, more likely, by both mechanisms. A higher than normal proliferative capacity of circulating $CD15^-$ cells was suggested by their ability to give rise to both day 7 and day 14 CFU-GM—i.e. to mature to large, morphologically identifiable colonies after only 7 days of incubation (Table 2).

Table 2. Surface antigen CD15 on circulating myeloid progenitors after cyclophosphamide and rhGM-CSF infusion

Experiment*	Colonies per 5×10^4 plated cells: $CD15^+$†	Colonies per 5×10^4 plated cells: $CD15^-$‡	$CD15^+$ progenitors (%)
1	0	390	0
2	75	340	18
3	22	208	9.5

*3 different patients.
†Day 7 and ‡day 14 (see methods).

DISCUSSION

Haematopoietic stem cells circulate in the blood of several mammalian species, including man. The progenitor cells are usually assayed by their colony-forming ability in semisolid media or, more recently, identified through expression of characteristic surface antigens [20–22]. The number of circulating precursors increases several fold under conditions of haematopoietic stress, especially during the period of rapid leucocyte recovery after myelotoxic chemotherapy [1]. The ability to expand this circulating pool reproducibly is expected to influence profoundly cancer treatments that use high doses of drugs and/or radiation. Circulating progenitors have been used to achieve a haematopoietic reconstitution of patients after myeloablative chemo-radiotherapy [2]. Yet their routine use cannot be advised, mainly because of wide fluctuations in their number and potency in promoting haemotopoietic reconstitution [2].

Early studies in dogs, and more recent experience in man have shown that cyclophosphamide induces a predictable and reliable expansion of circulating CFU-GM numbers and that these cells are several times more potent in fostering haemotopoietic recovery after total body irradiation [8–10, 23, 24]. These observations prompted our study. After high-dose cyclophosphamide administration, an approximately 30-fold expansion of circulating CFU-GM numbers was observed. This increase was further magnified, to over 100 times control values, when

Table 3. CFU-GM reappearance in liquid cultures after maphosphamide treatment of circulating mononuclear cells from healthy donors and from patients treated with cyclophosphamide

Peripheral blood	No. of colonies from 1 ml of maphosphamide treated cells: Day 0	Day 10
Normal	0	3 (3)
Normal	2 (1)*	115 (18)
Normal	1 (1)	30 (6)
After cyclophosphamide	0	906 (30)
After cyclophosphamide + rhGM-CSF	0	600 (240)
After cyclophosphamide + rhGM-CSF	20 (12)	5260 (310)

*Mean (S.D.) of triplicate cultures.

patients received rhGM-CSF as a continuous infusion to accelerate post-cyclophosphamide haematopoietic recovery. Under these conditions peak values as high as 85 000 CFU-GM per ml blood, representing about a 1000-fold increase over steady-state, were recorded.

These precursors were both increased in number and enriched in the more immature forms. The precursors appeared in the peripheral blood and gave rise to macroscopic colonies over 1 mm in diameter, containing several thousand cells. These types of colonies, which reflect a high proliferative potential of the founder cell [25, 26], were less frequently observed by us in the peripheral blood of normal individuals, and were even more expanded by rhGM-CSF infusion. The CFU-GM/CFU-Mix ratio was reduced from approximately 30:1 (normal value under steady-state) to about 15:1. This shift suggests a preferential expansion of the more immature CFU-Mix compartment over CFU-GM. Most of the colony-forming cells lacked the surface antigen CD15 expressed by late CFU-GM and granulocytes. The number of circulating progenitors resistant to maphosphamide increased about 50 fold over steady-state. These resistant cells are part of an earlier compartment than the CFU-Mix stage and, when bone-marrow derived, they include cells capable of self-renewal [27] and of establishing enduring grafts [28]. In addition, we have seen in peripheral blood very large numbers of $CD34^+/CD33^-$ myeloid progenitors [29] which include the most immature haematopoietic progenitors, including stem cells [29, 30].

Our data thus show that high-dose single-agent cyclophosphamide treatment induces a dramatic expansion of circulating haematopoietic precursors with a preferential release of the more immature forms. The effect is further magnified by rhGM-CSF continuous infusion starting from the day after cyclophosphamide. Because rhGM-CSF also induces an increase of circulating progenitors that exhibit a minor *in vitro* proliferative response to rhGM-CSF, the dramatic expansion of the circulating pool is likely to reflect the ability of this cytokine to affect the mechanism(s) underlying progenitor cell egression from the bone marrow.

Previous experiences with autografting with circulating progenitors collected during early remission of acute myelogenous leukaemia suggested that a CFU-GM dose of 30×10^4/kg or more is required for complete and durable engraftment [31]. This cell dose was easily collected from all the patients in the present study. Ongoing studies confirm that two or three leukaphereses are adequate for the collection of circulating CFU-GM in vast excess of this threshold [11].

The ability of these progenitors to accelerate haematopoietic recovery after myeloablative chemo-radiotherapy has been documented in several patients autografted with both bone-marrow and circulating precursors [9–11]. The presence within this population of marrow-repopulating stem cells has been documented in 2 patients with multiple myeloma [32]. As a consequence of massive bone-marrow involvement in the absence of morphologically and immunophenotypically identifiable circulating myeloma cells, these patients received, as a source of stem cells, autologous mononuclear cells collected solely from the peripheral blood during the post-chemotherapy recovery phase hastened by rhGM-CSF continuous infusion. After total body irradiation plus melphalan, both patients recovered rapidly and completely. The sustained haematopoietic reconstitution observed confirms that the progenitor cell pool appearing in the peripheral blood after high-dose chemotherapy and rhGM-CSF does include marrow-repopulating stem cells and can be used as a source of haematopoietic progenitors.

While the cyclophosphamide treatment might prove ideally suited to harvest very large numbers of early progenitors, experiments in progress indicate that the effect is by no means unique. A similar expansion has been observed by us after high-dose etoposide (2 g/m^2) and rhGM-CSF treatment and by Antman *et al.* [33] with doxorubicin, 5-fluorouracil and methotrexate plus rhGM-CSF in breast cancer patients. Thus the infusion of rhGM-CSF after severely myelosuppressive but stem-cell-sparing chemotherapy, promises to widen the applicability of autologous transplantation. Moreover, the ready availability of large numbers of early haematopoietic progenitors, including stem cells, offers the opportunity for biological studies and gene-transplantation experiments that have been previously precluded by their limited numbers.

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