Enhanced Marrow Recovery by Short Preincubation of Marrow Allografts With Human Recombinant Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor

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We studied an alternative method of using hematopoietic growth factors (HGFs) to enhance hematopoietic recovery in patients undergoing bone marrow transplantation (BMT), by short in vitro preincubation. Twenty consecutive patients with leukemia received T-cell–depleted allografts using Campath-1G. Two thirds of the marrow was infused on the scheduled day of transplant and one third of the marrow following preincubation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) on day 4. Engraftment parameters and duration of hospitalization were compared by actuarial analysis to those of 40 historical controls. Patients receiving the incubated boost had significantly faster platelet recovery ($P = .017$) and shorter hospitalization period ($P = .001$) when compared with the control subjects. Platelet count reached greater than 25 × 10^9/L on day 17 (median) in the study group and on day 23 in the controls. The median duration of hospitalization was 20 and 36 days, respectively. In the early posttransplantation follow-up, two of four patients in the study group died as a result of graft rejection, while all 13 deaths in the control group resulted from complications associated with marrow suppression. We suggest that pretransplant in vitro activation of bone marrow cells with IL-3 and GM-CSF may prove to be an efficient method for enhancing marrow recovery after BMT.

DESPITE THEIR ADVANTAGE in enhancing hematopoietic recovery in conditions associated with extreme bone marrow suppression,[1-4] hematopoietic growth factors (HGFs) such as recombinant human interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte CSF (G-CSF) in bone marrow transplantation (BMT) recipients have been used predominantly in some solid tumors and lymphoid malignancies.[5-8] The major concern stems from the possible stimulation of residual malignant cells and tumor progression,[9,10] such as has been implicated in phase I/II studies in patients with myelodysplastic syndromes.[11,12] However, there has been no suggestion of increased relapse or decreased tumor response in patients transplanted for a variety of nonhematologic malignancies. Additional obstacles that may be associated with prolonged in vivo administration of HGFs include possible systemic side effects attributed to the activation of other vasoactive lymphokines.[13] As a majority of the studies have been performed in patients undergoing autologous marrow transplantation, little is known as to the possible effects of these cytokines on allograft rejection or graft-versus-host (GVH) reaction. Overall, considering the above, prospective randomized placebo-controlled studies are still needed to clarify the full effect of HGFs on the long-term disease-free survival in patients undergoing BMT for a large variety of malignant diseases.

An alternative approach for the use of HGFs in BMT is to activate the bone marrow progenitor cells in vitro by short incubation with a single HGF or combination of HGFs before BMT. Numerous studies have clearly shown that a combination of growth factors can have additive and even synergistic effects in increasing the in vitro colony-forming ability of human progenitor cells.[14-19] The most reasonable combination seems to be of growth factors capable of stimulating the earlier marrow progenitors, such as IL-1, IL-3, or IL-6 with those acting on late and committed progenitors such as GM-CSF. We have previously shown that a combination of IL-3 and GM-CSF had a maximal enhancing effect on the number of colony-forming unit–granulocyte-macrophage (CFU-GM) in whole, T-cell–depleted, and mafosfamide (ASTA-Z)–purged marrow.[20]

The aim of the present study was to evaluate whether a short in vitro preincubation of bone marrow with IL-3 and GM-CSF before BMT can accelerate hematopoietic recovery in patients undergoing allogeneic marrow transplantation for malignant hematologic diseases.

MATERIALS AND METHODS

Patients. Twenty consecutive patients undergoing allogeneic BMT for malignant hematological diseases entered the study. All had an HLA-identical and mixed lymphocyte culture (MLC) nonresponding sibling donor. A group of 40 patients served as nonrandomized, nonpaired but nearly contemporaneous controls: 20 of them were transplanted just before the group under study, and 20 after the study. Patient characteristics and stratification into disease and treatment categories are detailed in Table 1.

Patients were conditioned for transplant with a protocol consisting of total body irradiation (TBI) 1,200 cGy in six fractions over 3 days, followed by cyclophosphamide 60 mg/kg day −5, melphalan 60 mg/m^2 day −4, etoposide 1,500 mg/m^2 day −3 (protocol 1); or with a protocol consisting of busulfan 16 mg/kg over 4 days and cyclophosphamide 200 mg/kg over 4 days (protocol 2). In addition, all patients received total lymphoid irradiation (TLI), four doses of 150 cGy in protocol 1 or five doses of 200 cGy in protocol 2 for further immunosuppression.

Supportive care. Patients were treated in single rooms in limited reversed isolation. *Pneumocystis carinii* prophylaxis consisting of oral cotrimoxazole was administered to all patients pretransplant and twice weekly (trimethoprim dose 10/kg) after sustained engraftment was attained. Acyclovir (200 mg three times a day orally) was administered to all patients who had detectable IgG titer for herpes simplex virus (HSV). Broad-spectrum antibiotics

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Table 1. Clinical Data of 20 Patients Receiving a Boost of Bone Marrow Cells Preincubated With rhIL-3 and rhGM-CSF on Day 4 After BMT in Comparison With 40 Controls

	Study Group	Control Group
	No. (%)	No. (%)
Sex		
Female	8 (40)	18 (45)
Male	12 (60)	22 (55)
Age		
Median	28	22
Range	5-42	3-46
Acute leukemia		
1 CR	6 (30)	15 (37)
≥2 CR	8 (40)	11 (27)
ANLL	11 (55)	11 (27)
ALL	3 (15)	17 (42)
CML		
Chronic phase	2 (10)	3 (7)
Accelerated/blastic	1 (5)	7 (17)
Myelodysplastic syndrome	2 (10)	2 (5)
Other	1 (5)	—
Total	20	40

Abbreviation: CR, complete remission.

consisted of gentamicin, mezlocillin, and cefazolin were initiated at the first peak of fever greater than 38°C during granulocytopenia. Amphotericin B was added and antibiotics changed if fever persisted. All of the patients received total parenteral nutrition and irradiated blood products. No posttransplant immunosuppression for prevention of graft-versus-host disease (GVHD) was given.

BMT and T-cell depletion. On the day of the transplantation, 2×10^8/kg or greater bone marrow cells were T-cell–depleted and transfused to secure engraftment with a satisfactory inoculum. T-cell depletion was performed by adding Campath-1G antibody (provided by H. Waldman, Cambridge, UK) (0.3 μg/10^6 cells) directly into the marrow bag. Following a 30-minute incubation at room temperature and gentle agitation, marrow was infused via a Hickman catheter. In ABO mismatched transplants, red blood cells (RBC) were removed and Campath-1G was added similarly into the buffy coat. Patients who did not develop signs of GVHD by day 28, received donor's peripheral blood T lymphocytes for induction of graft-versus-leukemia activity.

Bone marrow incubation. Marrow cells (1×10^8/kg) were put aside for in vitro incubation. RBC were removed by sedimentation with Hespan (hydroxyethyl starch, DuPont) for 30 minutes at room temperature. Cells were then washed and resuspended at 2.5×10^6 cells/mL in 150-cm^2 flasks (Costar, Cambridge, MA) in RPMI medium (GIBCO, Grand Island, NY) containing L-glutamine, penicillin 10,000 U/mL, streptomycin 10 mg/mL, and 7% to 10% pooled inactivated human AB serum obtained from three healthy donors. IL-3 and GM-CSF were added at 0.1 μg/mL each.[20] Following 4 days of incubation in a fully humid incubator with 5% CO_2 in air at 37°C, the cells were harvested from the cultures and washed three times to remove excess GM-CSF or IL-3. Campath-1G was similarly added to the marrow before infusion.

CFU-GM. Samples of fresh and incubated bone marrow were tested for their in vitro formation of CFU-GM colonies in agar, as previously described.[20] Briefly, triplicates of 2×10^5 cells/mL in enriched McCoy 5A medium (GIBCO) supplemented with L-glutamine, essential amino acids, and 10% prescreened heat-inactivated fetal calf serum (FCS) (GIBCO) with 0.3% agar (Bacto, Difco, Detroit, MI) were incubated at 37°C in a fully humidified incubator with 5% CO_2 in air. Bladder carcinoma conditioned medium was used as source of CSF. Colonies of greater than 50 cells were scored on day 10.

Recombinant human HGFs. Recombinant human GM-CSF and IL-3 were kindly provided by Behringwerke, Marburg, Germany, in a lyophilized form. GM-CSF expressed in *Escherichia coli* had a specific activity of 5×10^7 colony-forming units-culture (CFU-C)/mg protein. IL-3 expressed in yeast had similar (1 to 5×10^7) biologic activity. Both were reconstituted with 0.9% normal saline with 0.5% to 1% human albumin and kept frozen until use. A single lot of both factors was used throughout the study. Fresh aliquots were thawed and further diluted in RPMI before each transplant.

Statistical evaluation. The lengths of time between transplantation and attainment of each engraftment criterion in the study group were compared with the corresponding data for the control group using a log-rank test[21]; the same test was applied to the respective lengths of hospitalization. The association between the increase in CFU-GM following marrow incubation and the length of time until engraftment was assessed with a Spearman rank correlation.[22] Relative frequencies of deaths due to different causes were compared with a Fisher exact test.[22] Cumulative distributions shown in Fig 1 were calculated using the Kaplan-Meier procedure.[23]

RESULTS

The clinical data of the 20 patients included in the study and 40 controls are given in Table 1. The two groups were comparable with respect to age, female to male ratio, and percent patients with acute leukemia. There were more patients with advance-stage acute leukemia in the study group than in the control group (40% and 27.5%, respectively), but there were fewer patients with advance-stage chronic myelogenous leukemia (CML). More than 80% of patients in both groups were conditioned with TBI and chemotherapy (protocol 1) (Table 2). Overall, the number of marrow cells infused was similar in the two groups: 4.6 and 4.1×10^8 nucleated cells/kg. In the study group, the total amount was composed of two fractions: a mean of 3.8×10^8 cells/kg in the fresh inoculum on day 0 and additional 0.87×10^8/kg of bone marrow cells precultured with IL-3 and GM-CSF 3 days later.

Engraftment parameters were defined as an increase of absolute neutrophil count (ANC) greater than 0.5×10^9/L for 3 consecutive days and unsupported platelets greater than 25×10^9/L. Cumulative distributions of the length of time to attain neutrophil and platelet counts are shown in Fig 1A and B, respectively. The corresponding distribution of hospitalization durations are shown in Fig 1C. There was no significant acceleration of the neutrophil recovery in the group given the incubated marrow boost ($P > .20$, log-rank test), but there was a significantly ($P = .017$) earlier increase in platelets. The unsupported platelet level reached 25×10^9/L or greater within 34 days in all the patients receiving the preincubated marrow fraction, while in the control group seven of 40 patients had slow platelet recovery (41 to 244 days) and one patient remained platelet-dependent. The median day to reach unsupported platelet count of 25×10^9/L was 17 days for patients in the study group, and 23 days for the controls.

Similarly, duration of hospitalization was significantly shorter in the study group when compared with the control

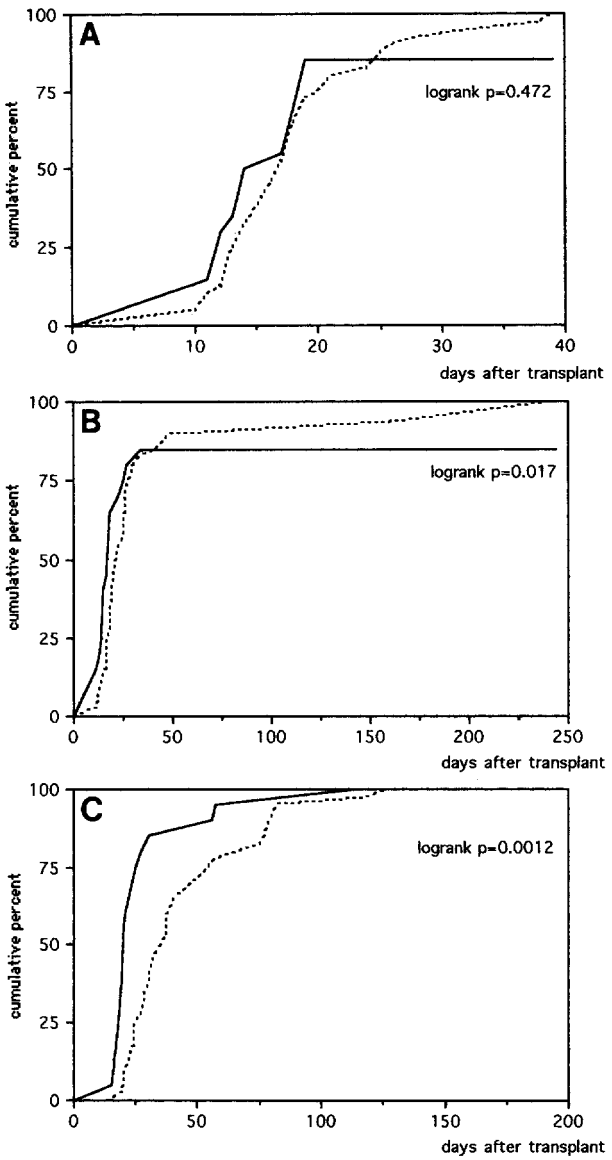

Fig 1. Kaplan-Meier analysis of marrow engraftment and duration of hospitalization in 20 patients receiving GM-CSF/IL-3 incubated marrow boost (—) and 40 controls (---). (A) Cumulative proportion of patients attaining ANC > 0.5 × 10^9/L. (B) Cumulative proportion of patients attaining platelet count > 25 × 10^9/L. (C) Length of hospitalization.

group ($P = .001$). The median hospitalization period for the study group was 20 days, and for the controls, 36 days.

In 15 patients, results of CFU-GM performed on both fresh and cultured marrow samples were obtained. The increase in the number of CFU-GM following 3 days incubation with IL-3 and GM-CSF correlated with the rapid platelet increase in only 12 patients with durable engraftment ($\rho = -0.59, P = .02$).

Table 3 summarizes the immediate post-BMT outcome of both groups. Two patients in the study group had an initial increase in peripheral blood white blood cells with subsequent rejection. Both died following an attempted second transplant. A third patient, a 32-year-old woman with CML, had a questionable engraftment and received autologous cryopreserved marrow rescue. She developed acute GVHD later, but at the same time had recurrence of Philadelphia chromosomes; hence, it was impossible to distinguish between late engraftment causing GVHD or GVHD following autologous marrow reconstitution. Of the remaining 17 patients who had full engraftment, two died, one of intracerebral hemorrhage and one of cytomegalovirus (CMV) infection. Sixteen patients were discharged from the hospital with Karnofsky scores greater than 80.

None of the patients in the control group rejected, but four had a delayed engraftment. One of these four received a second boost marrow, two recovered slowly, and the fourth died of bleeding related to thrombocytopenia. Of the remaining 36 patients, 12 died, eight of whom were never actually discharged from the hospital. The major cause of death was infection, in three patients associated with GVHD.

There was no significant difference in the incidence of acute GVHD: overall, eight of 20 in the study group developed acute GVHD, as did 18 of 40 in the control group, although the incidence of advanced (grade ≥ III)

Table 2. Pretransplant Conditioning and BMT of Patients Receiving Preincubated Boost and Control Subjects

	Study Group No. (%)	Control Group No. (%)
Conditioning protocol		
Chemotherapy with TBI (protocol 1)*	16 (80)	34 (85)
Busulfan and cyclophosphamide (Protocol 2)†	4 (20)	6 (15)
Cell infused × 10^8/kg		
Fresh marrow (day 0) (mean ± SD)‡	3.8 ± 1.6	4.14 ± 0.64
Incubated marrow (day 4) (mean ± SD)§	0.87 ± 0.29	—

*Total body irradiation 1,200 cGy, cyclophosphamide 60 mg/kg, melphalan 60 mg/m², etoposide 1,500 mg/m².

†Busulfan 4 mg/kg × 4 days, cyclophosphamide 50 mg/kg × 4 days.

‡Marrow cells were treated with Campath-1G 0.3 μg/10^6 cells before infusion.

§Bone marrow was incubated with IL-3 and GM-CSF (0.1 μg/mL each) for 3 days, washed, treated with Campath-1G, and reinfused.

Table 3. Four-Month Outcome of 20 Patients Transplanted With Bone Marrow Cells Pretreated With IL-3 and GM-CSF in Comparison to Controls

Outcome	Study Group (20 patients) (%)	Control Group (40 patients) (%)	P
Alive	16 (80)	27 (67.5)	>.20
Dead	4 (20)	13 (32.5)	
Graft failure	2	0	.04
Other cause	2	13	
Acute GVHD*	8 (40)	18 (45)	
Rescue marrow	1 (5)	1 (2.5)	

*Acute GVHD developed following administration of donors' peripheral blood lymphocytes in patients that received no anti-GVHD prophylaxis.

acute GVHD was higher among the controls than the study group (10/18 *v* 4/8, respectively).

DISCUSSION

The present study has shown that a supplement of bone marrow preincubated in vitro with IL-3 and GM-CSF may enhance platelet recovery and improve the short-term outcome in allogeneic BMT recipients.

In the past several years, numerous studies performed in experimental animals,[24-26] as well as phase I/II studies in man, have shown the beneficial effect of human recombinant GM-CSF and, more recently, IL-3 in enhancing hematopoietic recovery following BMT[5-8,27] and in other conditions associated with marrow suppression.[28-32] In general, the response in vivo has been within the expected range of activity of both growth factors in vitro, but there are still some controversies as to the extent of response to GM-CSF: some investigators report a multilineage response with a parallel increase in both lymphocytes and platelets,[11,28,29] while most observed an increase in the myeloid cells only.

Concomitantly with the numerous in vivo trials, data demonstrating the synergistic effect of various HGFs in vitro are accumulating. We have previously shown that a combination of IL-3 and GM-CSF results in a significant enhancement of the number of CFU-GM[20] in T-cell–depleted marrow and marrow purged with ASTA-Z. Hence, similar experimental conditions were chosen for the present study.

The overall outcome of the group of patients that received the preincubated bone marrow fraction was better than their controls, despite the fact that the two or possibly three patients who rejected their grafts were in that group. There was no appreciable difference in recovery of the neutrophils, but there was a significant acceleration in the platelet recovery and shorter duration of hospitalization in the study group when compared with the controls. While 70% of patients in the study group reached platelet counts greater than 25×10^9 before day 21, only 47.5% of patients in the control group were platelet transfusion–independent before day 21. Seven patients in the control group had slow engraftment and prolonged cytopenia, which could be attributed to CMV infection in only one.

Two or perhaps three patients in the study group did not engraft. All three received conditioning with TBI and chemotherapy and none had marrow fibrosis before transplant. They all received a sufficient number of fresh unmanipulated marrow cells, 3×10^8/kg, 2.2×10^8/kg, and 4.3×10^8/kg, respectively, on day 0; thus, inadequate marrow inoculum cannot be blamed for nonengraftment. All received an additional fraction of 1.2×10^8/kg, 1×10^8/kg, and 0.6×10^8/kg marrow cells precultured with IL-3 and GM-CSF on day 4. It is unlikely that the ex vivo incubation of part of a donor's marrow should affect the host's immune mechanism and predispose to graft rejection. Although we do not have an explanation as to the occurrence of rejection in this group, it is of major concern. A prospective randomized study will be needed to clarify whether rejection was random, associated with T-cell depletion, or related to the in vitro–treated boost.

The remaining 17 of 20 patients in the study group had a prompt engraftment (the latest day to reach ANC $> 0.5 \times 10^9$/L was 19, and to reach platelets $> 25 \times 10^9$/L was 34) and relatively uncomplicated course, with a median duration of hospitalization of 20 days. At 21 months after the last patient in this group had a transplant, 50% of the patients are alive and 45% are free of leukemia. In the control group, all of the patients engrafted, but 14 had a stormy course. The median hospitalization period was 36 days, with a range of 19 to 127 days. Ten months after the last transplant was performed in that group, 14 patients (42.5%) are alive and free of disease. Two patients in the study group and seven in the control group relapsed, despite the fact that the proportion of patients transplanted in advanced disease was similar in both groups.

Two patients died as a result of graft rejection, both belonging to the study group. Fifteen patients died of causes related to marrow suppression, and 13 of these (86.7%) belonged to the control group ($P = .04$).

Since the present study was not done prospectively, it can only be assumed without firm conclusion that the faster marrow recovery and the relatively uncomplicated and short posttransplant course in the hospital may be attributed to the addition of marrow preincubated with GM-CSF and IL-3. However, the exact mechanism by which a short preincubation of part of the marrow may accelerate the marrow recovery is not clear. There is no evidence in experimental animal models of increased self-renewal capacity or increased number of stem cells following marrow incubation in the presence of GM-CSF or IL-3, but rather of enhanced expression of homing receptors or improved seeding efficiency, which may subsequently lead to faster proliferation of progenitor cells.[33] Nevertheless, acceleration of differentiation and maturation of a large proportion of the infused marrow cells may result in an earlier appearance of circulating neutrophils and platelets, which can be a clinically important and meaningful factor.

The biologic activity of GM-CSF is complex and extends beyond its direct proliferative effect on the marrow progenitor. It also enhances the function and activity of the mature myeloid-monocyte/macrophage cells and thus has a dual beneficial effect in augmenting both antibacterial and antitumor activity.[3,34-37] We have recently shown that GM-CSF also enhanced the response of murine splenocytes to mitogens and alloantigens, and thus further contributes to a faster immune recovery following BMT (submitted for publication). Interestingly though, patients treated with GM-CSF following allogeneic BMT did not show increased incidence of GVHD[26] nor did our patients, despite the probable activation of monocytes and possibly lymphocytes during the in vitro marrow incubation with IL-3 and GM-CSF.

The better outcome of the patients treated with a supplement of bone marrow cells precultured with IL-3 and GM-CSF should be further cautiously analyzed. Our own study was not randomized and therefore no firm conclu-

sions can be drawn, except to suggest that the procedure may be safe, particularly since a sufficient amount of bone marrow cells was given unmanipulated, and the outcome may be potentially beneficial. The general outcome may reflect many other factors not necessarily attributed to the in vitro procedure, such as modification of GVHD. Nevertheless, it seems that short bone marrow preincubation with GM-CSF and IL-3 and possibly other cytokines may enhance hematopoietic reconstitution and shorten the post-transplant pancytopenia. This method is economic and safe, and may significantly reduce the need for prolonged and expensive in vivo administration of GM-CSF and IL-3 in patients after BMT without increasing the rate of relapse.

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