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Hematopoietic Recovery Following Autologous Bone Marrow Transplantation: Role of Cryopreservation, Number of Cells Infused and Nature of High-dose Chemotherapy

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Abstract—*Twenty-nine patients were treated with single or combined high-dose melphalan therapy followed by autologous bone marrow transplantation. Hematopoietic recovery from these treatments was studied. No correlation was found between the number of GM-CFC infused and the time required for hematopoietic recovery. It is suggested that this correlation is only demonstrable for low 'doses' of infused bone marrow cell and/or GM-CFCs. The role of bone marrow cell preservation techniques was examined and results were similar for fresh and cryopreserved bone marrow. The erythrocyte, lymphocyte and granulocyte levels of the patients reported here reached a normal or subnormal hematological steady state 3 months after autograft. Our results confirm the value of cryopreservation techniques. Hematopoietic recovery was short and of the same duration whether the patients were given single or combined high-dose melphalan before autologous bone marrow transplantation. These results also demonstrate the value of such transplantation in shortening the myelosuppression caused by high-dose chemotherapy.*

INTRODUCTION

HEMATOPOIETIC toxicity remains the major limit to the intensity of high-dose therapies used in malignancies. Total-body irradiation (TBI) combined with high-dose chemotherapy regularly produces irreversible myelosuppression which can be averted by autologous bone marrow transplantation (ABMT) [1-3]. In several high-dose chemotherapy protocols the ability of ABMT to shorten the myelosuppression has been demonstrated. For instance, Appelbaum *et al.* [4] showed a substantial reduction in the aplastic phase after high-dose chemotherapy with ABMT. Abrams *et al.* [5] found the same results with another high-dose regimen. McElwain *et al.* [6] demonstrated that ABMT had similar effects after high-dose melphalan treatment. However, the best technique of bone marrow preservation has not been completely established.

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The aims of the present study were (a) to study the relationship between the 'dose' of stem cells (GM-CFC) and hematopoietic recovery; (b) to compare the hematopoietic recovery that follows a high-dose melphalan regimen with the rescue obtained by transplantation of cryopreserved or non-cryopreserved bone marrow in order to assess the value of the preservation process; and (c) to compare the hematopoietic recovery occurring after single or combined high-dose melphalan therapy in order to demonstrate the benefit afforded by the ABMT.

MATERIALS AND METHODS

Patients

Thirty patients were treated by high-dose chemotherapy followed by ABMT. One died early of sepsis and was not evaluable for hematological recovery. The remaining patients survived at least 3 months after transplantation and are the subjects of this study.

Twenty patients were treated with one course of high-dose melphalan followed by ABMT. They included 17 children and three adults, all with metastic disease. There were 14 cases of neuroblastoma, three of carcinoma of the testis, two of Ewing's sarcoma and one of rhabdomyosarcoma. The diagnosis, age, sex and duration of therapy before bone marrow harvesting as well as the mode of bone marrow preservation are shown in Table 1. In addition, nine patients were treated with one course of combination chemotherapy containing high doses of melphalan followed by ABMT. They comprised five children and four adults, and their characteristics are shown in Table 2. All patients had received previous conventional therapy for 4–18 months (median 10 months) before high-dose chemotherapy and ABMT. During the aplastic period induced by high-dose chemotherapy, patients were kept in standard reverse isolation, without gut decontamination or prophylactic antibiotics. The bone marrow was never harvested at the onset of the disease but after several courses of chemotherapy, usually when the patient was considered to be in first remission. The periods elapsing between onset of therapy and bone marrow harvest are given in Tables 1 and 2.

Table 1. Single HDM with ABMT: patients

	No.	Diagnosis	Age/sex	Duration of previous therapy before harvesting (days)	Fresh BM	Cryo BM
Group 1	1	neuroblastoma	7.5/F	510	×	
	2	neuroblastoma	4/M	240	×	
	3	neuroblastoma	2.5/F	360	×	
	4	neuroblastoma	13/M	270	×	
	5	neuroblastoma	2/M	420	×	
	6	neuroblastoma	2.5/M	180	×	
	7	neuroblastoma	2.5/M	210	×	
	8	neuroblastoma	10/M	300	×	
	9	neuroblastoma	1.5/M	360	×	
	10	Ewing's sarcoma	7.5/M	150	×	
	11	Ewing's sarcoma	11.5/M	550	×	
	12	rhabdomyosarcoma	5/M	530	×	
	13	carcinoma of testis	35/M	300	×	
	14	carcinoma of testis	22/M	250	×	
	15	carcinoma of testis	28/M	275	×	
Group 2	16	neuroblastoma	2/M	180		×
	17	neuroblastoma	4.5/M	210		×
	18	neuroblastoma	3/M	270		×
	19	neuroblastoma	2/M	330		×
	20	neuroblastoma	1.5/M	270		×

Table 2. Combined HDM with ABMT: patients (group 3)

No.	Diagnosis	Age/sex	Duration of previous therapy before harvesting (days)	Cryopreserved BM
21	Ewing's sarcoma	10/M	450	×
22	Ewing's sarcoma	2.5/M	450	×
23	Ewing's sarcoma	6/F	240	×
24	rhabdomyosarcoma	4.5/F	250	×
25	rhabdomyosarcoma	1.5/F	180	×
26	oat cell carcinoma of the lung	38/F	360	×
27	oat cell carcinoma of the lung	42/F	350	×
28	oat cell carcinoma of the lung	35/M	120	×
29	oat cell carcinoma of the lung	40/M	270	×

METHODS

Bone marrow procedures. At the time of harvesting, the bone marrow was always considered as cytologically normal. Bone marrow was harvested under general anesthesia by anterior and posterior iliac crest punctures. The process used for aspiration and filtration is that described by Thomas and Storb [7].

Bone marrow preservation. Preservation of fresh marrow: after harvesting, bone marrow was kept in a polyethylene plastic bag for 8 or 24 hr in a cold room at 4°C.

At this temperature there was no loss of cells or GM-CFCs, whether the storage period was 8 or 24 hr, which is in agreement with the results of other studies [8, 9]. In the present work non-cryopreserved bone marrow is bone marrow which has been kept at 4°C for 8 or 24 hr.

Cryopreservation: technical details are reported elsewhere [10]. Briefly, a solution of 20% DMSO in TC-199 with 10% human serum was mixed with an equal volume of bone marrow suspension (final DMSO concentration: 10%). The mixture was frozen at a controlled rate of -1°C/min. The bone marrow was kept in liquid nitrogen for 1-3 weeks. The frozen bone marrow was thawed by immersion in a water bath at 40°C, washed by slow dilution with a mixture of 25% plasma and 75% isotonic sodium chloride, centrifuged and resuspended in the same medium.

Colony-forming cell controls. Granulomonocytic colony-forming cells (GM-CFC) were assayed in each marrow sample. For each patient, the marrow was tested twice: immediately after harvesting and immediately before infusion. Details of culture techniques are described elsewhere [10]. The granulomonocytic content of the colonies was confirmed by microscope examination of the cells pipetted from the plates and stained with May-Grunewald Giemsa. The macrophagic colonies were not counted.

Hematological recovery measurements. Red and white blood cells, platelets and blood differential were counted three times a week from the beginning of high-dose chemotherapy until complete reconstitution was obtained. Hematological recovery was assessed by three major parameters: the number of days necessary to reach blood levels of 0.5×10^9 neutrophils/l, 1×10^9 neutrophils/l and 50×10^9 platelets/l.

For red blood cells (RBC) recovery during the aplastic phase was not measurable since patients were transfused when their hemoglobin level was below 4.5 mmol/l. The number of patients requiring RBC transfusion after day 30 post-ABMT was noted in order to find out how many had reached the steady state.

For lymphocytes recovery during the aplastic phase was difficult to assess, since differences between monocytes and lymphocytes were not studied in leukopenic patients. The absolute number of lymphocytes was counted at days 30, 60 and 90 post-ABMT for rough estimation of their recovery, and to evaluate their condition in relation to the steady state.

High-dose chemotherapy. Melphalan, either alone or combined with other drugs, was administered as a bolus at doses of 140-180 mg/m^2 under hyperhydration. The chemotherapy combination was the following: day 1: 300 mg/m^2 BCNU; days 1, 2, 3 and 4: 400 mg/m^2 procarbazine; days 2, 3 and 4: 200 mg/m^2 VP16; and day 5: 140-180 mg/m^2 melphalan.

The thawed bone marrow was infused 24 hr after the end of chemotherapy.

Statistical analysis. The Wilcoxon statistic test was used for all comparisons. Since the number of GM-CFCs and duration of aplasia had a log-normal distribution, according to different criteria, correlations of their log values were computed.

RESULTS

Relationship between the numbers of GM-CFC and hematopoietic recovery

High-dose melphalan monochemotherapy (HDM). After one course of HDM, 15 out of the 20 patients had a non-cryopreserved ABMT and five had a cryopreserved ABMT (Table 1). The numbers of nucleated marrow cells and GM-CFCs infused per kg and the time required for hematopoietic recovery are given in Table 3 for patients grafted with fresh marrow (group 1) and in Table 4 for those grafted with cryopreserved marrow (group 2).

For the patients grafted with non-cryopreserved marrow there was no correlation between the number of GM-CFCs infused and any of the three parameters of hematopoietic recovery measured. No significant correlations were found between the number of GM-CFCs infused on the one hand and the respective periods required to reach levels of 0.5×10^9 neutrophils/l ($R = -0.06$, $P > 0.05$), 1×10^9 neutrophils/l ($R = +0.23$, $P > 0.05$) and 50×10^9 platelets/l ($R = +0.23$ $P > 0.05$) on the other (Table 5).

Although far fewer patients were grafted with cryopreserved bone marrow, there were no significant correlations between these same parameters, whose respective correlation factors were: ($R = -0.45$, $P > 0.05$), ($R = -0.68$, $P > 0.05$) and ($R = -0.51$, $P > 0.05$) (Table 4).

Combined HDM polychemotherapy. Nine patients had combined chemotherapy followed by a cryopreserved ABMT (Table 2). The numbers of marrow cells and GM-CFCs infused, and the

times elapsing until hematopoietic recovery are given in Table 5. Here, again, there were no significant correlations between the three parameters studied (respective correlation factors: $R = -0.65$, $P > 0.05$; $R = -0.69$, $P > 0.05$; and $R = -0.73$, $P > 0.05$).

Comparison between cryopreserved and non-cryopreserved bone marrow grafts

The number of marrow cells infused is higher for fresh than for cryopreserved bone marrow, and the same applies to the numbers of GM-CFCs. This difference is due to cell losses during freezing and thawing procedures, and is statistically significant ($P < 0.05$ and $P = 0.002$ for marrow cells and GM-CFCs respectively).

Figures 1-3 show that there was no difference between the times required to reach the three recovery levels measured whether the marrow was fresh or cryopreserved or whether HDM was single or combined: 0.5×10^9 neutrophils/l: $w = 42$, $P = 0.20$; 1×10^9 neutrophils/l: $w = 45$,

Table 3. Single HD melphalan: non-cryopreserved BM nucleated marrow cells, GM-CFCs and hematological recovery (duration)

No.	BM cells $\times 10^8$/kg	GM-CFCs infused $\times 10^4$/kg	Hematological recovery: Duration of neutropenia (days) $<0.5 \times 10^9$/l	Duration of neutropenia (days) $<1 \times 10^9$/l	Duration of thrombopenia (days) $<50 \times 10^9$/l
1	3.2	13.0	23	28	200
2	6.2	45.0	6	17	18
3	4.9	19.0	17	19	27
4	3.4	4.8	9	12	21
5	4.8	14.3	12	14	14
6	5.2	13.8	15	17	22
7	4.1	14.8	16	24	19
8	3.0	3.3	10	20	17
9	5.3	11.4	24	27	30
10	2.3	5.6	23	25	19
11	2.9	18.8	13	15	19
12	3.0	12.7	6	25	19
13	3.6	8.6	12	15	14
14	3.2	-	15	24	27
15	3.2	3.0	10	11	9

Table 4. Single HD melphalan: cryopreserved BM nucleated marrow cells, GM-CFCs and hematological recovery (duration)

No.	BM cells infused $\times 10^8$/kg	GM-CFCs infused $\times 10^4$/kg	Hematological recovery: Duration of neutropenia (days) $<0.5 \times 10^9$/l	Duration of neutropenia (days) $<1 \times 10^9$/l	Duration of thrombopenia (days) $<50 \times 10^9$/l
16	3.8	3.3	9	12	22
17	3.5	4.4	15	16	15
18	3.7	8.2	16	31	25
19	3.8	1.7	10	16	13
20	3.2	3.6	8	20	21

Table 5. Combined HDM: cryopreserved BM nucleated marrow cells, GM-CFCs and hematological recovery (duration)

No.	BM cells infused $\times 10^8$/kg	GM-CFCs infused $\times 10^4$/kg	Hematological recovery: Duration of neutropenia (days) $<0.5 \times 10^9$/l	Duration of neutropenia (days) $<1 \times 10^9$/l	Duration of thrombopenia (days) $<50 \times 10^9$/l
21	1.9	1.0	14	21	15
22	2.4	9.0	9	10	13
23	2.7	12.5	13	17	50
24	2.2	4.5	10	13	21
25	1.65	0.5	25	35	>100
26	2.1	1.3	7	11	20
27	1.9	1.7	6	14	16
28	1.9	15.4	6	9	12
29	3.1	4.9	10	13	21

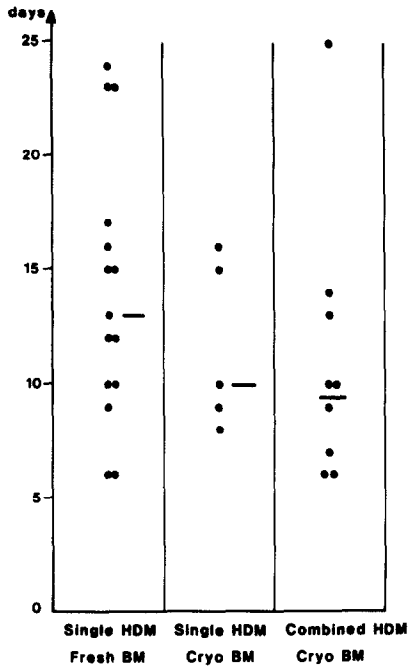

Fig. 1. Time required to reach 0.5×10^9 neutrophils/l.

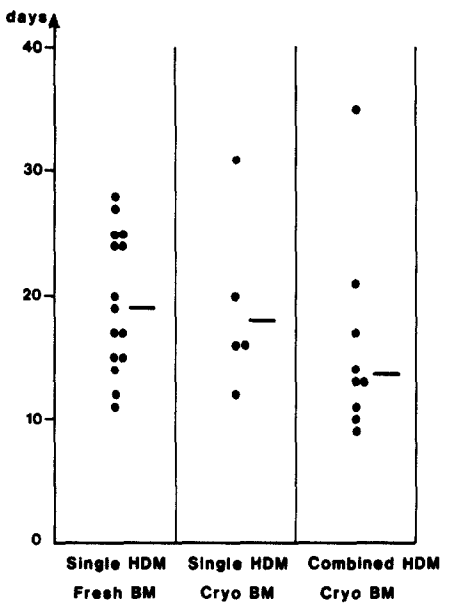

Fig. 2. Time required to reach 1×10^9 neutrophils/l.

$P = 0.46$; and 50×10^9 platelets/l: $w = 39$, $P = 0.42$.

Comparison between single HDM and combined HDM

Figures 1-3 show no significant difference in the values for the three hematopoietic recovery parameters between combined HDM followed by cryopreserved ABMT and single HDM followed by either cryopreserved or fresh ABMT (0.5×10^9 neutrophils/l: $w = 41$, $P = 0.21$; 1×10^9 neutrophils/l. $w = 49$, $P = 0.46$; and 50×10^9 platelets/l: $w = 50$, $P = 0.5$).

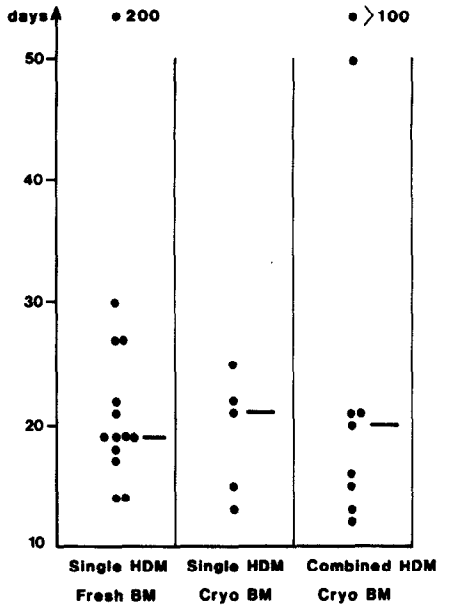

Fig. 3. Time required to reach 50×10^9 platelets/l *recovery.*

Red blood cell recovery

Five out of 29 patients needed RBC transfusions during the second month post ABMT. Three out of five had one transfusion, one had two transfusions and one had three transfusions. After the second month none of the patients needed transfusions except in cases of relapse and renewed therapy.

Lymphocyte recovery

As shown in Fig. 4, lymphocyte recovery was slow. At day 30 post-ABMT the median value of the lymphocytes was 0.95×10^9/l for these patients. At day 60 post-ABMT the median was 1.4×10^9/l and at day 90 post-ABMT, 1.95×10^9/l. After the third month post-ABMT the absolute number of lymphocytes for the surviving patients remained stable at between 1.5 and 4×10^9/l.

DISCUSSION

Relationship between number of GM-CFC grafted and hematopoietic recovery

In animal models the minimal dose of cells necessary to obtain engraftment has been extensively studied. In mice the minimal dose required for 50% engraftment has been defined for both fresh and cryopreserved bone marrow [11]. In rats more than 1×10^8 bone marrow cells/kg seem necessary to shorten myelosuppression [12]. In dogs 1×10^5 GM-CFCs/kg are usually necessary to procure engraftment [13, 14]. Using high doses of cells, Thomas *et al.* [15] obtained very rapid hematopoietic recovery. With the same animal model, Gorin *et al.* [16] showed that 0.25×10^8 cells/kg was approximately the minimum effective marrow dose for autologous engraftment.

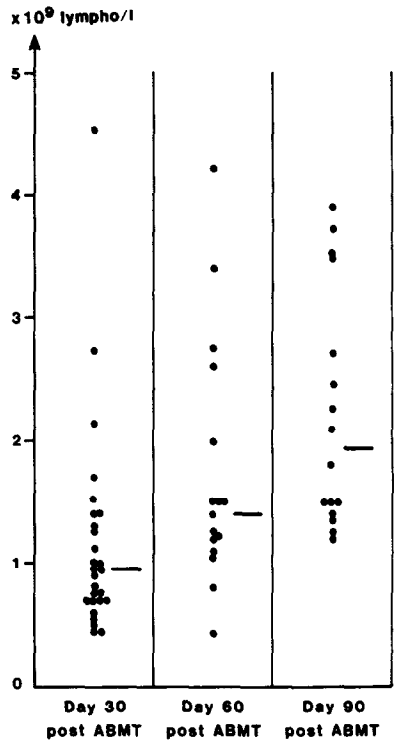

Fig. 4. Lymphocyte recovery at 30, 60 and 90 days post-ABMT.

They demonstrated that the engraftment rate declined when the marrow dose was reduced. In monkeys the minimal dose necessary to obtain an allograft has been calculated [17].

In humans the results are much less clear. It has been shown that a minimum of 3×10^8 nucleated donor cells per kg of recipient's body weight is required for an allograft [18]. In both autologous [19] and allogenic [20] grafts 10^5 GM-CFCs/kg are recommended to insure engraftment, but successful grafts have been described with lower doses of GM-CFCs [21, 22].

Ekert *et al.* [23] were unable to demonstrate any significant relationship between the number of bone marrow cells and/or GM-CFCs infused and the time elapsing until hematopoietic recovery. Spitzer *et al.* [24], on the contrary, showed a good correlation between the number of GM-CFCs infused and the neutrophil recovery in leukemia and solid tumor patients.

Our results are similar to those of Ekert *et al.* and different from those of Spitzer *et al.* Careful examination of both sets of data may explain these discrepancies. In our study, as in Ekert *et al.*'s investigations, the number of bone marrow cells infused was never less than 1.5×10^8/kg and the number of GM-CFCs never less than 1×10^4/kg. In contrast, in Spitzer *et al.*'s study, 7/24 patients were infused with less than 1.5×10^8 marrow cells/kg and 16/24 with less than 1×10^4 GM-CFCs/kg. It is possible that a relationship exists between GM-CFCs and hematopoietic recovery for low doses of infused cells, but that this relationship is no longer demonstrable when higher doses are infused. In that case the relation between the dose of GM-CFCs and the recovery time would follow a linear curve for the low doses and reach a plateau for doses exceeding 1.5×10^8 cells/kg or 1×10^4 GM-CFCs/kg. This relationship between the number of GM-CFCs grafted and the number of circulating granulocytes is certainly complex, since several factors are involved in regulating the number of these granulocytes. In the bone marrow the growth rate and differentiation process depend on several cellular and humoral factors. The part played by the stromal cells and by several humoral factors such as colony-stimulating factors or erythropoietin is not yet well understood but is currently under study *in vivo*. In the bloodstream the ratio of circulating to marginated granulocytes is dependent on several factors such as infection or stress.

Comparison between fresh and cryopreserved bone marrow

Studies of several animal systems have confirmed the potential of frozen autologous or isologous bone marrow for engraftment. They indicated that cryopreserved murine marrow and fresh marrow appeared to have similar transplantation efficiency [11, 12, 25, 26]. In dogs the value of cryopreserved marrow for transplantation has been demonstrated [27-29], and similar engraftment with fresh or cryopreserved marrow was obtained [16]. The same results have been described in monkeys [17].

In humans most autologous marrow transplantations have been performed with cryopreserved bone marrow. There are few reports of transplantation with autologous non-cryopreserved bone marrow [6, 8, 30]. Since several different regimens have been employed to treat malignancies prior to ABMT, comparisons between hematological recoveries are difficult.

In this study the only difference between the two groups of patients (1-15 and 16-20, Table 1) lies in the use of cryopreserved or fresh bone marrow. All these patients were given the same treatment: high doses of melphalan, at roughly the same intervals after diagnosis. Since no difference was found in the hematological recovery of these two groups, we may assume that, for these patients, the effects of fresh and cryopreserved bone marrow transplantation were similar.

Comparison between single and combined HDM

In the medical literature relationships between ablative therapy and hematological recovery are difficult to assess since each publication deals

with different therapies, numbers of cells infused and bone marrow preservation techniques. In this study groups 2 and 3 are comparable for the period elapsing between marrow harvesting and diagnosis, previous conventional chemotherapies, numbers of marrow cells and GM-CFCs infused, and techniques and periods of marrow preservation (cryopreservation). All the patients in these two groups received high doses of melphalan, the difference being that for group 3 patients HDM was combined with other myelotoxic drugs. Despite the small numbers of patients studied, it seems interesting to emphasize the absence of differences in the hematological recovery of these two groups. We may therefore assume that, in these cases, hematological recovery does not depend on the ablative therapy but on the efficacy of bone marrow transplantation.

Erythrocyte and lymphocyte recovery

For the patients, the duration of erythropoietic recovery is not perfectly known since the long half-life of the transfused RBC made it difficult to measure. Nevertheless the very limited need for transfusions after day 30 post-ABMT and the subsequent stability of hemoglobin levels are certainly due to the normal steady state obtained for erythropoiesis after grafting. Similarly, the data for lymphocytes showed slow recovery but, quantitatively speaking, a normal steady state was obtained after a period of 2 or 3 months. From this quantitative viewpoint, complete recovery was apparently obtained for all the bone marrow cell lines in the entire series of patients 2 or 3 months after high-dose chemotherapy and ABMT.

This study demonstrates the safety of the technique used to preserve bone marrow for transplantation. Whether the marrow was fresh or cryopreserved, the duration of myelosuppression following high-dose chemotherapy and ABMT was short and did not depend on the ablative therapy.

In view of these results, it is probably not necessary to infuse more than 1.5×10^8 marrow cells/kg or 1×10^4 GM-CFCs/kg since these amounts apparently ensure normal myeloid and lymphoid recovery. These findings permitted us to limit the quantity of marrow harvested for one transplantation. The demonstration that our marrow storage methods were completely reliable allows us to go on to another step in marrow manipulation, and work on 'purging' bone marrow to remove any contaminating tumor cells is now in progress.

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