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Delayed Kinetics of Recovery of Haemopoiesis following Autologous Bone Marrow Transplantation. The Role of Excessively Rapid Marrow Freezing Rates after the Release of Fusion Heat*†

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Abstract—*Thirty-five patients were treated by intensive chemotherapy and/or whole-body irradiation followed by reinjection of cryopreserved autologous bone marrow. In 8 patients the kinetics of recovery of haemopoiesis was delayed (recovery to 10^9 leucocytes/litre beyond day 27 and recovery to 50×10^9 platelets/litre beyond day 25). This delay was directly responsible for the death of 3 patients and contributed to a fatal outcome in 2 others (mortality rate 9–14%). Retrospective analysis of these 8 cases revealed that failure of autologous transplantation was associated with poor recovery of CFU_c, which was in turn related to an excessively rapid freezing rate after the release of fusion heat. Recovery of CFU_c to 50% or more was achieved in 100% of cases when the freezing rate was less than 5°C/min, 45% for freezing rates between 5 and 10°C/min and 22% when the freezing rate exceeded 10°C/min ($n = 71$, $P < 0.001$). There was an inverse linear or logarithmic relationship between CFU_c recovery and freezing rate after the transition phase ($r = -0.46$, $r = -0.43$, $P < 0.001$). The quantity of nitrogen introduced into the freezing chamber to annul the fusion heat must therefore be calibrated with accuracy so that the desired shortening of the transition phase will not be accompanied by an overly marked increase in the freezing rate, which would result in the destruction of stem cells. To ensure an adequate freezing rate, it is crucial to monitor the temperature continuously in each sample of bone marrow during the freezing process. This study also suggested that other factors may have interfered with the kinetics of recovery after autologous bone-marrow transplantation. These factors include myelofibrosis, the presence of an Australia antigen and administration of compounds that are toxic for the bone marrow after reinjection of cryopreserved marrow. However, the responsibility of these factors cannot be stated with certainty.*

INTRODUCTION

AUTOLOGOUS bone marrow grafting is now used in many institutions as a means of haematological support following intensive chemotherapy and/or whole-body irradiation for the treatment of leukaemias [1, 2], non-Hodgkin's lymphomas of poor prognosis [3, 4] and some solid tumours [4, 5]. The reinjection of autologous cryopreserved stem cells virtually always leads to recovery of haemopoiesis within a short, well-defined period [4–6], thus substantially reducing the risk of infection and the mortality rate associated with high-dose chemotherapy and radiotherapy.

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From January 1976 to July 1981 we performed an autologous bone marrow transplantation in 35 patients following intensive cytoreductive therapy. The kinetics of haematological and immunological recovery, as well as the tumoricidal effect of the cytoreductive therapy, have been analysed and published elsewhere [4].

In the present paper we report the results of a study of 8 patients who failed to recover adequate haemopoietic function within the usual interval, defined statistically in a previous study of 23 patients [4]. The aim of the present study was to identify the factors responsible for abnormal perpetuation of bone marrow aplasia following infusion of cryopreserved marrow stem cells. It was shown that there was a close correlation between the rate of bone marrow freezing during the transition phase, immediately after the release of fusion heat, the recovery of CFU_c progenitors after thawing and the kinetics of the autologous graft. An overly rapid freezing rate following the transition phase carries a high risk of destroying marrow stem cells. We believe that this risk factor should be brought to the attention of groups working in the field of autologous cryopreserved bone marrow transplantation.

MATERIALS AND METHODS

Patients

From January 1976 to July 1981, 35 patients underwent autologous bone marrow grafting following treatment with the high-dose TACC chemotherapy regimen (25 patients), a different high-dose chemotherapy regimen (7 patients) or a combination of TACC and whole-body irradiation (3 patients). A further 7 patients were treated with TACC without bone marrow transplantation.

Details of the TACC regimen, the other chemotherapy combinations used, the method of bone marrow cryopreservation and the technique of autologous grafting have been described elsewhere [4].

The kinetics of recovery of haemopoiesis in bone marrow and peripheral blood has been studied and defined in an initial group of 23 patients and has been published elsewhere [4]. In brief, in the peripheral blood recovery to 10^9 leucocytes, 5×10^9 reticulocytes and 50×10^9 platelets/litre was observed after a median interval of 13 days (range 10–19 days), 13 days (range 8–25 days) and 14 days (range 9–28 days) respectively. Recovery to the same numbers of leucocytes and platelets in patients treated by the TACC combination without bone-marrow grafting occurred beyond days 28 and 32 respectively [4].

The 8 patients under consideration in the present study received an autologous bone marrow transplant following TACC chemotherapy (5 cases), a different intensive chemotherapy combination (1 case) or combined TACC and whole-body irradiation (2 cases). These 8 patients were selected because they exhibited unusually slow recovery of haemopoiesis as compared to the kinetic patterns previously established and described above. In these 8 patients platelet recovery took longer than 25 days and in 6 of the patients leucocyte recovery occurred beyond day 27. On the basis of the clinical context and environmental circumstances, these patients were considered by the medical team as graft failures, partial takes or delayed takes.

Analysis of variables

Each patient's history was scrutinized for any factors that may have accounted for a less than optimal result of autologous grafting. Particular attention was focused on the following points: the date of bone marrow harvesting, the duration of conservation and the date of grafting; the bone-marrow freezing rate from room temperature, the duration of thermal elevation associated with the release of fusion heat and the freezing rate beyond this point; recovery of CFU_c from bags of frozen marrow; the quantity of marrow injected (in terms of both nucleated cells/kg and CFU_c/kg); and the patient's status at the time of autologous bone-marrow grafting and subsequent administration of toxic drugs liable to affect the recovery of haemopoiesis.

Study of temperature curves during the freezing process

The procedure we use for harvesting and freezing bone marrow has not varied substantially since 1975 [6, 7]. The rate of freezing was -1°C/min to fusion. It was then increased to counteract the heat of fusion down to -60°C, at which temperature the bags were transferred to the gas phase of a liquid nitrogen freezer. The bags were stored at a temperature below -190°C (Fig. 1).

Both the temperature of the bone marrow sample and the temperature inside the freezing chamber were continually recorded during each freezing operation.

All the recorded freezing curves were conserved and kept available for this study. A review of these freezing curves revealed that whereas the freezing rate was uniformly constant before the transition phase, substantial variations did occur from patient to patient and from one bone marrow sample to another following the release of fusion heat. The cooling rate ($\Delta\theta = \theta/t$, Fig. 1) was therefore calculated with great accuracy for each

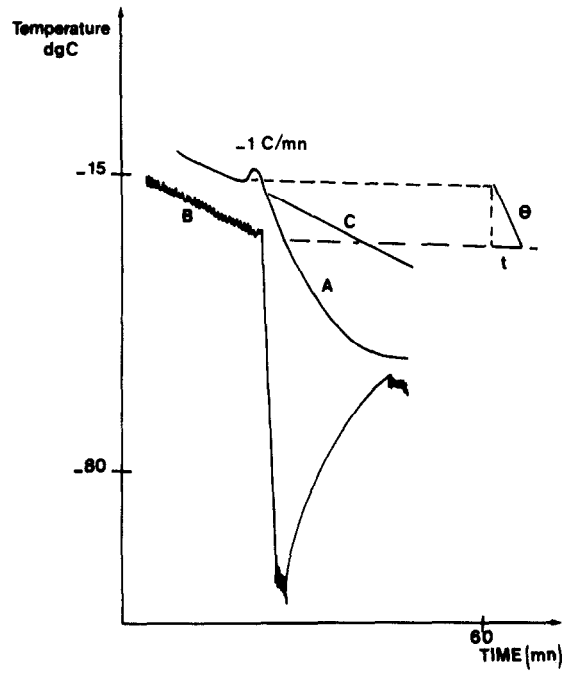

Fig. 1. Diagram of the freezing curves. (A) Temperature of the sample of bone marrow; (B) temperature inside the freezing chamber; (C) ideal bone marrow freezing curve. The cooling rate after the release of fusion heat ($\Delta\theta = \theta/t$) was calculated in the space defined by a horizontal line through the point where the release of fusion heat begins and a second horizontal line 13.5°C lower.

freezing curve within the same graphic space, defined by a horizontal line through the beginning of release of fusion heat and another horizontal line 13.5°C below.

Thawing and study of CFU_c recovery in vitro

In all, 71 bags of cryopreserved bone marrow from 22 different patients were studied. In most cases the concentration of CFU_c was evaluated directly on the bone marrow reinjected to the patient for autografting and the recovery rate was calculated by comparison with the CFU_c concentration in freshly harvested bone marrow before freezing. The bags were thawed rapidly, in about one minute, by immersion in a water-bath at 37°C and specimens were immediately drawn for *in vitro* assays of the number and proliferating potential of preserved CFU_c. The entire procedure was carried out at room temperature. The sample was immediately diluted in 9 vol. of Hank's balanced salt solution (HBSS) ×10 (Gibco Biocult) to reduce the concentration of DMSO to 1%. Additional procedures, such as the washing of cells immediately after thawing, were avoided since they may have caused a substantial loss of granulocyte precursors. The proliferating potential of the preserved CFU_c was evaluated by culturing 2×10^5 cells. The absolute number of CFU_c was determined with precision by culturing a constant volume (1 ml) of bone marrow.

CFU_c were cultured using the double-layer agar technique described by Pike and Robinson. CSF was provided by an underlayer containing 10^6 peripheral leukocytes from two normal donors.

For evaluation of the proliferative potential of the cryopreserved CFU_c, cultures were performed simultaneously in four Petri dishes, each one receiving 2×10^5 adherent and non-adherent mononuclear marrow cells per ml of medium.

For purely quantitative evaluation, all the cells harvested from 1 ml of bone marrow were put into culture. The cells were distributed into as many Petri dishes as required to achieve $2\text{–}3 \times 10^5$ cells per dish.

The results were expressed as the number of colonies per 2×10^5 cells (arithmetic mean of four Petri dishes) with the aggregate/colony ratio, and also as the total number of colonies per ml of bone marrow. The recovery rate, expressed as a percentage, was obtained by dividing the total number of CFU_c per ml of frozen and thawed marrow by the total number of CFU_c previously counted in freshly harvested marrow.

Statistical analysis

CFU_c recovery was evaluated as a function of freezing rate after the release of fusion heat. CFU_c recovery rates defined as adequate (>50%) or poor (<50%) were compared in patients with normal and delayed haemopoietic kinetics by the χ^2 test.

The best-fitting curves (straight line, exponential, logarithmic and power) were determined by the least-squares method using a statistical program of the HP41C calculator. The correlation coefficients were obtained by the same method.

RESULTS

Of the 8 patients in this study (Table 1) 3 recovered normal haemopoiesis, although with a delay. Two of these three patients are alive 2 and 3 yr after intensive chemotherapy and reinjection of cryopreserved autologous bone marrow. The third patient died on day 130 of tumor progression.

The 5 other patients died between days 19 and 100. In 3 of these patients failure to recover haemopoietic function was the direct cause of death (cases 2, 7 and 8), and was a contributing factor in the other two patients (cases 4 and 6). Three patients had terminal, chemoresistant acute lymphoblastic leukemia and in two of these patients prolonged aplasia coincided with the persistence of blasts. The two other patients were in a blast crisis of chronic granulocytic leukaemia. The first patient died on day 35 of a servere infection at which time bone marrow aspirates began to show early signs of regenera-

Table 1. Autologous bone marrow transplantation. Analysis of unsuccessful or delayed engraftments

Case No.	Diagnosis* (age and sex)	High-dose therapy	Engraftment†	Survival (days)	Storage duration (months)	Freezing rate‡ (°C/min)	Dose of marrow infused§ Nucleated cells (10^8/kg)	Dose of marrow infused§ CFU_c (10^3/kg)	Putative responsibility Patient	Putative responsibility Frozen marrow
1	NHL (54/F)	TACC	D (platelets)	1260+	1	9	1.6	—	Double dose of CCNU Marrow infused 48 hr only after CCNU	High freezing rate
2	ALL (26/M)	TACC	F	32	4	9.6	0.33	0.5	Ag Australia+ methotrexate post-transplantation	Dose of marrow inadequate High freezing rate
3	Malignant melanoma (31/M)	PACCE‖	D (neutrophils and platelets)	1080+	1	9.6	0.39	1	Drug combination (PACC (PACCE) different from TACC with vindesine Double dose of CCNU	Dose of marrow inadequate High freezing rate
4	ALL (26/F)	TBI + TACC (14 days later)	F	100	1.5	6.7–16.5	Day 1, 0.76 Day 18, 0.76	2.2 2.3	Conditioning regimen including TBI Myelofibrosis Persistence of blast cells	High freezing rate
5	Burkitt's lymphoma with leukaemic infiltration (24/M)	TACC + vindesine	F	130	4	5.6–8.4	0.3	0.1	TACC + vindesine	Dose of marrow inadequate High freezing rate
6	ALL (48/F)	TACC	F	19	9	11–13.5	0.56	0.58	Persistence of blast cells	Dose of marrow inadequate High freezing rate
7	CML Blast crisis (24/F)	TACC	F	35	11	13	2.4	2.6	Administration of ketoconazole on day 10	Minimal recovery of CFUc (2%) High freezing rate
8	CML Blast crisis (35/F)	TACC + TBI	P (Persisting thrombocytopenia)	95	12	4.2–11.2	4	3.5	Splenectomy Conditioning regimen combining TACC + TBI	BM harvested 1 week after busulfan was discontinued High freezing rate Poor recovery of CFU_c (10%)

*NHL: Non-Hodgkin's lymphoma; ALL: acute lymphocytic leukaemia; CML: chronic myelocytic leukaemia; M = male; F = female.
†F: Failure; D: delayed engraftment; P: partial engraftment.
‡Freezing rate (°C/min) following the heat of fusion.
§Nucleated cells/kg and CFU_c/kg calculated after thawing.
‖Procarbazine–actinomycin D–cyclophosphamide–CCNU–vindesine.

tion. The second patient died on day 95 of a cerebral haemorrage related to persistent thrombocytopenia, whereas she had recovered an adequate number of erythrocytes and leucocytes. In addition, a chromosome study revealed that the transformed clone (47 Phi + C+) and the Philadelphia chromosome-bearing clone (46 Phi+) had disappeared, all the residual cells being 46 Phi-. The case history of this patient has been published elsewhere [9].

A careful study of these 8 cases of delayed recovery (Table 1) revealed the following findings: reinjection of cryopreserved autologous bone marrow was performed over a certain lapse of time, in a similar fashion to other autologous transplants which resulted in a normal kinetic pattern of recovery. This time distribution factor does not appear, therefore, to have influenced the therapeutic outcome.

Six of the eight patients had their marrow cryopreserved during the same seven-month period (November 1979–May 1980), at which time a slight modification was introduced into the freezing technique, namely an increase in the quantity of liquid nitrogen allowed to enter the freezing chamber when the release of fusion heat occurred, in order to shorten the transition phase. This resulted in a faster freezing rate after the release of fusion heat (4.2–16.5°C/min). Analysis of the freezing curves of the two bone marrows cryopreserved before November 1979 also revealed that the freezing rates had been inordinately fast (9.0 and 9.6°C/min).

The study of CFU_c recovery in 71 bags of cryopreserved marrow showed that there was an inverse and statistically significant relationship with the rate of freezing after the release of fusion heat (Table 2, Fig. 2). Adequate CFU_c recovery ($\geqslant 50\%$) was observed in 100% of cases when the freezing rate was slower than 5°C/min, as compared to only 22% of cases when the freezing rate was faster than 10°C/min ($P < 0.001$).

Table 2. CFU_c recovery in relation to freezing rate (following the heat of fusion)

Freezing rate (°C/min)	$\Delta < 5$	$5 < \Delta < 10$	$\Delta > 10$
CFU-C Recovery:			
$\geqslant 50\%$	100% (11/11)	45% (19/42)	22% (4/18)
$< 50\%$	0%	55%	78%

These values are statistically significant with $P < 0.001$ (chi squared test).

Linear or logarithmic regression lines can be plotted for these data. For the linear regression line CFU_c recovery (%) = $85.3 - 4.8\Delta\theta$ (Fig. 2) and for the logarithmic equation CFU_c recovery (%) = $112.4 - 33\ln\Delta\theta$ (not shown), where $\Delta\theta$ is the freezing rate after the release of fusion heat.

These lines are statistically significant with correlation coefficients (r) of -0.40 and -0.43 respectively ($P < 0.001$). The two lines are identical beyond 5°C/min.

CFU_c recovery greater than 50% correlated with successful autologous bone marrow grafting ($P < 0.025$ by the χ^2 test).

Of the 8 patients studied, 4 received a low dose of bone marrow, equal to or less than 10^3 CFU_c/kg after thawing, and for the two patients with chronic granulocytic leukemia, who received 2.6 and 3.5×10^3 CFU_c/kg, the CFU_c recovery rate was very low (2 and 10% respectively).

Among the other factors that may have delayed haemopoietic recovery, we noted (Table 1) the administration of a double dose of CCNU (500 mg/m^2) (cases 1 and 3), the use of vindesine (cases 3 and 5), the persistence of blast cells (cases 4

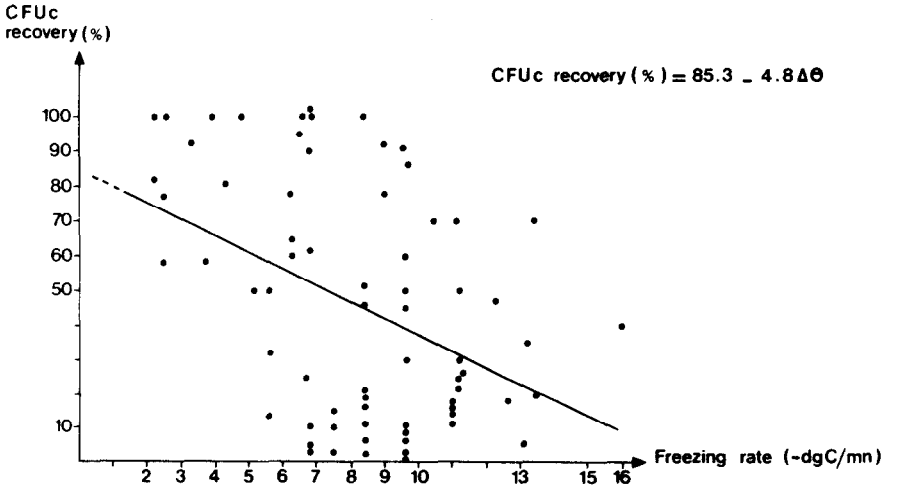

Fig. 2. CFU_C recoveries in relation to freezing rates following the heat of fusion.

and 6), the presence of an Australia antigen and the administration of methotrexate only a few days after the injection of autologous bone marrow (case 2), the presence of myelofibrosis (case 4) and prior alteration of the harvested bone marrow by busulfan (case 8). However, none of these factors was observed in more than two cases. The only factor common to all patients was a rapid freezing rate after the release of fusion heat. In almost all cases (6/8) the dose of CFU_c administered was low, or the rate of recovery of CFU_c exceedingly low. There was an inverse correlation between the rate of CFU_c recovery and the freezing rate after the release of fusion heat.

DISCUSSION

Forty-two patients from our department were treated by intensive chemotherapy and/or whole-body irradiation. Thirty-five of these patients received cryopreserved autologous bone marrow stem cells shortly after completion of cytotoxic therapy. Abnormal kinetics of haemopoietic recovery was observed in 8 patients. This delayed recovery was directly responsible for the death of three patients and contributed to the death of two others. Thus in our experience autologous bone marrow transplantation was a failure in 20% of cases (8/35) and was associated with a mortality rate somewhere between 9 (3/35) and 14% (5/35). This mortality rate is far from negligible, particularly if one considers that it must be added to the mortality due to the toxicity of the cytotoxic drugs and whole-body irradiation, evaluated at 5% (2/42) in our series.

The investigation reported here was conducted with a view to identifying the causes of autologous transplantation failures. Multiple causes may have contributed to such failures and, indeed, several factors were singled out in each patient that may have accounted for the delayed kinetics of haemopoietic recovery. Some of these factors initially appeared to be determinant and came to the immediate attention of the medical staff at the time the patients received treatment (administration of methotrexate after injection of autologous bone marrow in patient No. 2; myelofibrosis in case No. 4). In addition, 2 patients had CML, a disease which seems to be associated with an unusually high incidence of allogeneic marrow engraftment failures. From our own experience a similar concern applies to the autologous bone marrow transplantation setting.

Retrospectively, however, it appeared that the only factor common to all these patients was the inordinately high freezing rate after the release of fusion heat. Concomitantly, for 6 of these 8 patients CFU_c recovery was very low after thawing.

The preservation of viability of stem cells by freezing can be achieved only if the cryopreservation procedure complies with certain rules: in the presence of a cryopreserving agent, which reduces the solution effects, the initial freezing rate must be slow to avoid intracellular crystallization [10]; although it may still be debated, most investigators believe that the fusion heat released by the transformation of liquid to solid bone marrow during the transition phase must be annulled to avoid a prolonged thermal plateau during which recrystallization phenomena may cause cell destruction [10].

The freezing program we used meets these requirements and the fusion heat was annulled by massive introduction of liquid nitrogen into the freezing chamber at the precise moment when the continuous temperature recording within the bag of bone marrow began to show a rise in temperature.

Our *in vitro* study showed that preservation of viability of stem cells also depends on the freezing rate after the transition phase. Although occasionally excellent cell preservation is achieved after rapid freezing, statistically reliable preservation cannot be expected unless the freezing rate is slow (less than 5°C/min, and ideally between 1 and 2°C/min), that is, a freezing rate similar to that used in the early phase of the freezing program (Fig. 1).

The quantity of liquid nitrogen introduced into the freezing chamber to counteract the fusion heat must therefore be calibrated with accuracy so that the desired shortening of the transition phase produced by the introduction of liquid nitrogen is not accompanied by an excessive increase in the freezing rate, which would result in the destruction of stem cells (Fig. 1).

Our study has confirmed the preliminary observations made by Abrams *et al.* of 3 autologous transplantation failures in a group of 11 patients with Ewing's sarcoma and the *in vitro* findings of these authors who investigated the recovery of CFU_c progenitors in 8 bags of frozen bone marrow [11].

In brief, the results of our study may be formulated as follows: in addition to previously described factors, CFU_c recovery following cryopreservation is also related to the freezing rate after the release of fusion heat; CFU_c recovery of less than 50% and delayed recovery of haemopoiesis following infusion of cryopreserved autologous bone marrow are correlated with a freezing rate in excess of 5°C/min after the release of fusion heat. This result indicates that the pluripotent stem cell underlying the recovery of

autologous transplants and the more mature CFU_c behave similarly to cryopreservation and exhibit similar sensitivity to changes in the freezing procedure.

We are therefore of the opinion that careful monitoring of the cooling rate is mandatory for each individual bag of marrow and that particularly close attention should be paid during the transition phase, when the introduction of additional liquid nitrogen into the freezing chamber may produce an untoward increase in the cooling rate.

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