

Long-Term Preservation of Bone Marrow and Stem Cell Pool in Dogs

By Norbert Claude Gorin, Geoffrey Herzig, Malcolm I. Bull, and Robert G. Graw, Jr.

A canine model in vivo was established to assess the viability of stem cells frozen and stored for prolonged periods in liquid nitrogen. Forty-six foxhounds received total-body irradiation (1000 rads at 9 rads/min) followed by the infusion of autologous fresh bone marrow or frozen bone marrow stored for 2 or 5 mo in the vapor phase of liquid nitrogen (-140°C). The results demonstrated a direct relationship between the dose of bone marrow infused, the percentage of successful engraftments, and the kinetics of peripheral recovery. The minimum dose of fresh bone marrow for autologous engraftment was between

0.1 and 0.25×10^8 nucleated cells/kg. There was no difference between fresh bone marrow and bone marrow stored for 2 mo (100% recovery of frozen stem cells). Following a 5-mo storage period, the results suggested a slight but not statistically significant decrease in stem cell viability. If it could be demonstrated that this technique successfully preserves human bone marrow stem cells, these data would support the inclusion of frozen autologous bone marrow rescue in the management of patients with malignant diseases as an adjunct to ablative chemotherapy.

IN 1955, Barnes and Loutit¹ demonstrated that bone marrow could be successfully preserved by freezing, but the viability of the frozen stem cells in relation to the duration of the storage has been questioned. Studies in vitro performed in the past, using heterogeneous cell populations, have provided conflicting results concerning long-term cryopreservation.²⁻⁵ More recent techniques have shown good preservation of the pool of committed granulocytic stem cells¹⁷ and erythropoietin-responsive cells.⁷⁻⁸ However, none of these techniques assesses the ability of the frozen stem cells to repopulate an aplastic host. Studies in vivo evaluating the potential of frozen autologous or isologous bone marrow for engraftment in a variety of animal systems (mice,⁹⁻¹³ rabbits,¹⁴ monkeys,¹⁵⁻¹⁷ dogs¹⁸⁻²²) have confirmed the efficacy of short-term storage, but few long-term studies have been done.

The purpose of this study was to quantitate the stem cell pool in fresh and cryopreserved marrow as a function of storage duration by determining the minimal doses required to obtain an autograph and from the kinetics of peripheral blood recovery following engraftment. Parallel studies in vitro, including trypan blue permeability, optic and electronic microscopy, DNA synthesis, and chromosome studies, will be the subject of a separate report.³¹

From the Experimental Hematology Section, Pediatric Oncology Branch, National Cancer Institute, Bethesda, Md.

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Address for reprint requests: Dr N. C. Gorin, Service d'Hématologie (Pr G. Duhamel), Hôpital Saint-Antoine 184, Rue du Faubourg Saint-Antoine, 75012 Paris, France.

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MATERIALS AND METHODS

Animals

Forty-six closed-colony randomly bred male and female English American foxhounds were used. Prior to the experiment all dogs were immunized against hepatitis and distemper, dewormed, and housed in indoor runs or cages for a minimum of 3 wk before bone marrow aspiration.

Bone Marrow Aspiration

Following induction of satisfactory anesthesia, the animal was intubated, and bone marrow was aspirated from the central medullary canal of both femurs and humeri, put in a sterile Teflon beaker containing 50 ml of heparinized (500 U/ml) TC 199 medium (Flow Labs, Rockville, Md.), and constantly mixed with a magnetic stirrer. The bone marrow was first filtered through a double thickness of sterile gauze and then through a 60-mesh stainless steel wire screen. An average volume of 20 ml of bone marrow per kilogram was obtained. The nucleated cell count (usually 30–45,000/cu mm) was corrected for dilution with media and peripheral blood, using the assumption that the number of nucleated cells present in excess of the peripheral blood leukocyte count represented bone marrow cells. Fresh bone marrow was maintained at room temperature in a plastic transfer pack for no longer than 6 hr before being infused (TA4, Fenwal Laboratories, Morton Grove, Ill.)

Bone Marrow Freezing and Storage

To avoid potential inaccuracies in determining the dose of thawed bone marrow, the fresh marrow suspension was separated into samples containing 1, 0.5, 0.25, and 0.1×10^8 nucleated cells/kg (c/kg) prior to freezing in Hemoflex bags (Union Carbide, Chicago, Ill.). The freezing solution, consisting of 20% dimethylsulfoxide (DMSO) and 10% decomplexed fetal calf serum (Flow Labs) in TC 199 medium (Flow Labs), was prepared fresh and added to the bone marrow suspension in an equal volume immediately prior to freezing. Each bag (none containing more than 200 ml total volume) was compressed between two flat aluminum plates so that a preparation with uniform temperature distribution was obtained. The marrow was frozen in the vapor phase of liquid nitrogen in a Cryoson BV-4 automatic biologic freezing system (Cryoson Midden Beemster, Holland) modified to supercool at the beginning of the release of the heat of fusion. The freezing rate was constant at $-1^\circ\text{C}/\text{min}$ from room temperature to -50°C . The bags were then transferred to the gas phase of a liquid nitrogen freezer, where they were stored below -140°C for 2–5 mo.

Autologous Bone Marrow Grafts

Total-body irradiation, 1000 rads midplane absorbed dose at 9 rads/min, was administered to unanesthetized animals from two opposing ^{60}Co sources in a facility located at the Armed Forces Radiobiological Research Institute. This radiation dose had previously been shown to exceed the LD_{100} in dogs.²⁴

Bone marrow was administered by intravenous infusion, without filter, within 1–3 hr following total-body irradiation. Before the infusion of frozen bone marrow, diphenhydramine hydrochloride (25 mg intravenous push) was given to counteract the effects of expected histamine release associated with intravenous DMSO. Frozen marrow was quickly thawed in a waterbath at 37°C and infused immediately. No attempt was made to remove either DMSO or destroyed red cells prior to infusion. The time from the removal of the bone marrow from the nitrogen freezer to administration never exceeded 15 min.

Supportive Care

All dogs received the same supportive regimen: prochlorperazine dimaleate, 32 mg orally per day; lactated Ringer's solution, 1000 ml daily by clysis, starting on day 0; ampicillin, 100 mg/kg daily subcutaneously, starting on day 2; and gentamicin, 5 mg/kg daily subcutaneously starting on day 4. Hemoglobin, white cell count, and platelet count were measured daily. Hemoglobin was maintained above 9.5 g/dl. Platelet transfusions were given prophylactically for counts below 30,000/cu mm. All blood products were irradiated with 2500 rads prior to infusion. Blood cultures

were drawn daily during the period of granulocytopenia and twice weekly thereafter. Liver function tests were obtained weekly.

Criteria for Engraftment

Each animal was evaluated for total white cell, neutrophil, and platelet recovery. Using total leukocyte counts, two points were chosen to define engraftment: the day when the white cell count reached 1000/cu mm, and the estimated day when leukocyte recovery began, as determined from the kinetic curves. For platelets, criteria for engraftment were more difficult to define since dogs were transfused with platelets during the period of thrombocytopenia. However, it appeared that a count greater than 50,000/cu mm unmaintained by transfusion was never followed by a significant drop, and this point was chosen arbitrarily as a criterion of platelet engraftment.

RESULTS

Six control animals, irradiated at 1000 rads midplane absorbed dose at 9 rads/min and not given bone marrow, died on days 6, 9, 9, 10, 12, and 12 (median 9.5 days). Blood cultures and autopsies of these animals consistently demonstrated gram-negative bacterial sepsis associated with hemorrhagic pneumonitis and bone marrow aplasia. Eighteen dogs received fresh autologous bone marrow. Seventeen dogs received frozen bone marrow that had been stored for 2 mo, and 11 dogs received bone marrow stored for 5 mo. Table 1 shows the percentage of engraftment obtained in each group.

Ten dogs given fresh bone marrow in a dose equal or greater than 0.5×10^8 c/kg were engrafted. Two of four dogs given 0.25×10^8 c/kg were fully grafted. One additional animal, grafted as determined by white cell count, died on day 18 from massive bleeding associated with thrombocytopenia, and one animal died on day 10 without evidence of engraftment at autopsy. One of four dogs given 0.1×10^8 c/kg was grafted, two died without evidence of engraftment (on days 12 and 22), and one survived with prolonged aplasia until day 33, when he slowly recovered.

Bone marrow stored for 2 mo was equally as effective as fresh marrow. Six dogs receiving a dose equal or greater than 0.5×10^8 c/kg were successfully grafted, as were six of eight dogs receiving 0.25×10^8 c/kg. Two died (on days 9 and 15), with autopsy evidence of engraftment in one. Three dogs received 0.1×10^8 c/kg and two were grafted; the other one died on day 17 without evidence of engraftment.

Using bone marrow frozen for 5 mo, four of four dogs were successfully grafted with 0.5×10^8 c/kg. Seven dogs received 0.25×10^8 c/kg; four died

Table 1. Canine Autologous Bone Marrow Transplantation Using Fresh and Frozen Bone Marrow: Relation of Engraftment* to Dose and Storage Duration

Dose (nucleated cells/kg) $\times 10^8$	Frozen		
	Fresh	2 mo	5 mo
> 1.0	7/7† (100)‡	2/2 (100)	
0.5	3/3 (100)	4/4 (100)	4/4 (100)
0.25	3/4 (75)	6/8 (75)	3/7 (40)
0.1	1/4 (25)	2/3 (66)	

*WBC > 1000/cu mm and platelet count > 50,000/cu mm.

†Number engrafted per number given each dose.

‡Number in parentheses indicates percentage of dogs successfully grafted.

between days 9 and 12, and three grafted. There was no statistically significant difference in engraftment percentage for marrow stored for 5 mo compared to fresh marrow or marrow stored for 2 mo. In each group of dogs, the rate of engraftment declined when the dose of bone marrow was lowered. Combining the results for fresh bone marrow and bone marrow stored for 2 mo, the percentage of engraftment for 0.1×10^8 c/kg was 40% (3/7), as compared to 75% (9/12) for 0.25×10^8 c/kg and 100% (16/16) for doses of 0.5×10^8 c/kg or greater. The difference between 0.1×10^8 c/kg and the higher doses was statistically significant ($p < 0.01$), whereas the apparent difference between 0.25×10^8 and the higher doses was not significant. It thus appeared that 0.25×10^8 c/kg approximated the minimum effective marrow dose for autologous engraftment with fresh bone marrow or cryopreserved marrow stored for 2 mo. Figure 1 shows the kinetics of recovery of peripheral leukocytes for all dogs given bone marrow stored for 2 mo. When the kinetics for the mean leukocyte recovery in each dose group using marrow stored for 2 mo were considered, a direct relationship to dose of marrow infused appeared (Fig. 2). Following total-body irradiation, the nadir of leukopenia was reached on day 5 or 6, recovery started on day 7 for 1×10^8 c/kg, on day 8.5 for 0.5×10^8 c/kg, on day 10 for 0.25×10^8 c/kg and on day 14 for 0.1×10^8 c/kg. The non-parametric test of Kruskal and Wallis comparing the white cell counts in each group, showed a statistically significant difference ($p < 0.5$) on days 12, 15, and 20.

The kinetics of recovery appeared to be similar for fresh bone marrow and bone marrow stored for 2 or 5 mo (Fig. 3), and there were no statistical differences between the median day of engraftment, as previously defined, for leuko-

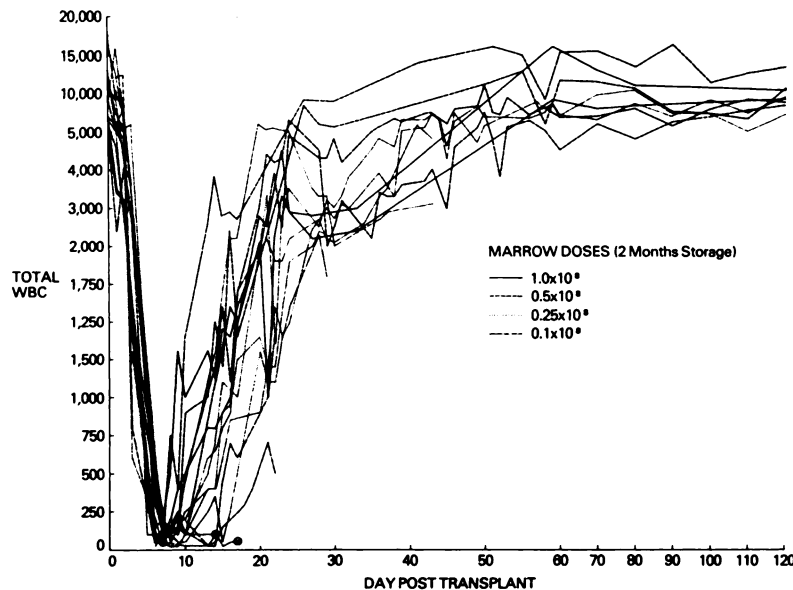


Fig. 1. Composite of total leukocyte counts for all animals given various doses of frozen autologous marrow stored for 2 mo.

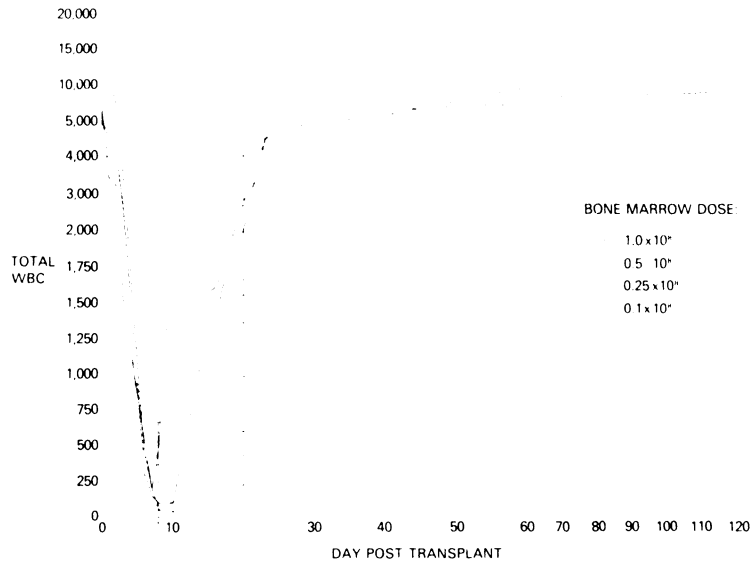


Fig. 2. Relationship between the kinetics of engraftment and bone marrow dose for marrows stored for 2 mo. Mean ± SEM (vertical bars).

cytes and for platelets (Table 2). However, following a 5-mo storage period, all three dogs given 0.25×10^8 c/kg who engrafted failed to reach a leukocyte count of 1000/cu mm prior to day 20 and a 50,000/cu mm platelet count before day 40. These data suggested a slight reduction of the stem cell pool that might have become more evident after longer periods of storage.

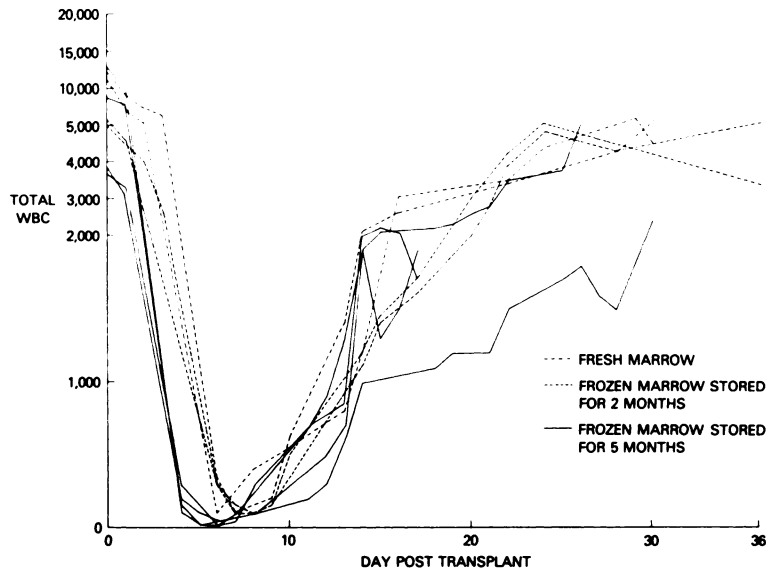


Fig. 3. Comparison of the kinetics of leukocyte recovery of fresh and frozen bone marrow given at a dose of 0.5×10^8 c/kg. (Counts on single dogs.)

Table 2. Median Day to Recovery of Peripheral Leukocytes* and Platelets† Relation to Dose and Storage Duration (46 Dogs)

Dose (nucleated cells/kg) × 10 ⁸	Frozen					
	Fresh		2 mo		5 mo	
	WBC*	Platelets†	WBC*	Platelets†	WBC*	Platelets†
≥ 1	9 (8–12)‡		12 (9–15)	21		
0.5	13.5 (13–14)	30 (28–32)	13§ (10–14)	32.5 (27–43)	14 (13–14)	34 (32–48)
0.25	16 (15–20)	29 (28–30)	16.5§ (13–21)	34 (27–43)	20 (19–20)	43 (40–65)
0.1	21	40	22 (21–23)	35.5 (35–36)		

*WBC > 1000/cu mm.

†Platelets > 50,000/cu mm.

‡Range.

§These two values are statistically different ($p < 0.05$ rank sum test)

DISCUSSION

The success of marrow grafting is dependent on the repopulating potential of the marrow. When dealing with bone marrow preservation, the major question is whether the stem cell pool has survived damage and what relationship exists to the duration of storage.

Of the many studies in vivo, very few have assessed the long-term viability of frozen marrow. In mice, Davies et al.¹¹ used bone marrow frozen in DMSO and stored at -79°C ; they found a 90% reduction in stem cell viability by survival and a 50% decrease in colony-forming units (CFU), as determined by the CFU spleen assay, following 1 wk of storage. This dramatic alteration was related to the freezing and thawing procedures, and no further decrease in viability was observed following storage periods up to 6 mo. Others have reported much better results: Phan Te Tran et al.³³ demonstrated good survival of stem cells stored for periods up to 126 wk with survival as the test. Lewis and Trobaugh⁹ reported a minimal dose of 25,000 cells necessary to obtain a 50% rate of engraftment in mice and, in contrast to the previous study, found 100% preservation of the stem cell pool by survival and 74% preservation by CFU spleen assay after short-term storage; glycerol appeared to be more effective than DMSO. However, Schaefer et al.,¹⁷ who used slow stepwise dilution following thawing, reported the same result with DMSO. Finally, O'Grady and Lewis¹³ found 80% preservation following freezing in glycerol and 2 yr of storage in liquid nitrogen.

In monkeys, the minimal dose necessary to obtain an allograft has been shown to be identical for fresh and recently frozen marrow, implying no loss of stem cell function.¹⁷

In dogs, autologous bone marrow stored for periods up to 14 mo has been found to be effective in protecting against radiation injury.³² Despite extensive further experimentation,^{18–23} however, no quantitative data exist. Engraftment was usually achieved with doses that were actually 2–10-fold higher than the threshold so that a quantitative evaluation of the viability of the stem cells after storage was not possible.²⁴ Such huge doses probably accounted for more rapid

recovery in blood leukocytes and platelets. Using doses of bone marrow greater than 2×10^8 c/kg, the nadir for leukocytes rarely dropped below 1000/cu mm; platelet recovery began as soon as day 12 and a count of 50,000/cu mm was achieved by day 15.²⁴ These data are in agreement with previous experiments in our laboratory and suggest a direct relation of the rapidity of peripheral blood recovery to the dose of bone marrow infused.

The present study has demonstrated that the same relationship between marrow dose and kinetics of engraftment holds for low marrow doses ($0.1-1 \times 10^8$ c/kg). Since the major aim of this study was to establish the effect of storage duration on stem cell viability, a single freezing technique was used based on the best available methods. Several features of this "optimum" technique deserve emphasis. A lag in the cooling curve or a significant rise in temperature produced by the heat of fusion is known to favor extracellular ice crystal growth that can be deleterious.²⁵ For this reason, a modification of the freezing system was made in order to supercool at the beginning of the release of the heat of fusion. Lysosomes are very sensitive to cryoinjury, and, although DMSO is protective,²⁹ it does not prevent lysosome release resulting from freezing or thawing.²⁶ Infusions of leukocyte suspensions have been shown to produce a rapid rise in plasma histamine levels in dogs²⁷ and in humans.²⁸ Diphenhydramine hydrochloride was therefore administered to dogs prior to the infusion of bone marrow with the purpose of preventing a histamine-induced increase in capillary permeability, which may improve engraftment rate. In other experiments diphenhydramine has been omitted without apparent ill effects. Finally, recent studies on platelet cryopreservation have shown a better recovery with Hemoflex bags³⁰ as compared to other plastic containers. It has been postulated that this improvement might result from the fact that Hemoflex bags (made of polyolefin) contain no lypophilic plasticizer which may be responsible for cell membrane damage. Despite its potential benefit, the slow stepwise dilution technique¹⁷ was not used in this study to avoid an increase in the amount of fluid to infuse or, alternatively, the potentially damaging spinning procedures. Whether these technical modifications account for any improvement in the preservation of the frozen stem cells cannot be stated.

Several conclusions can be drawn from this study. The minimal dose of bone marrow required for reproducible autologous engraftment in dogs is about 0.25×10^8 c/kg. Based on the rate of engraftment and the kinetics of recovery of peripheral counts, there is no difference between fresh bone marrow and bone marrow stored for 2 mo. After 5 mo of storage, there is still no statistically significant difference, but the data suggest that the minimal dose may be higher ($0.25-0.5 \times 10^8$ c/kg) and that recovery may be delayed slightly at any given dose. Such differences might become more obvious with prolonged storage. These results indicate that frozen autologous bone marrow rescue after hematopoietic destruction is feasible in dogs. A similar conclusion for patients with malignancies submitted to ablative chemotherapy would, unfortunately, be premature.

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