

Bone marrow processing and cryopreservation

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SUMMARY Three different closed procedures for concentrating bone marrow progenitor cells prior to cryopreservation have been compared. These were by a manual double centrifugation method, a Hemonetics 30 cell separator and an Aminco Celltrifuge. The best results were achieved using the paediatric pheresis set on the Hemonetics model 30. Marrow was frozen in 120 ml aliquots in a programmed freezer with rapid cooling of the freezing chamber during the phase change from the liquid to the solid state. After freeze-thawing the average nucleated cell recovery was approximately 50% and the progenitor cell recovery 80%.

The transfusion of autologous cryopreserved bone marrow cells is a well established procedure in the management of both haematological and non-haematological malignant diseases,¹⁻³ though its precise role has yet to be defined. Before freezing harvested bone marrow it is desirable to reduce the total volume and this is achieved by removing erythrocytes, plasma and fat, with minimal loss of stem cells. Manipulation of allogeneic bone marrow in an attempt to ameliorate graft versus host disease also requires similar volume reduction. In the present paper three "closed" methods of processing bulk marrow are compared and the myeloid colony-forming assay is used to estimate the stem cell content, before and after processing. The method of freezing and thawing is described and the nucleated cell and progenitor cell recoveries after freezing and thawing are reported.

Patients and methods

PATIENTS

Marrow processing by various techniques was compared in 21 consecutive patients who were either in remission from acute leukaemia or had small cell carcinoma of the bronchus. Nucleated cell and progenitor cell recoveries after freezing and thawing are reported in 12 cases.

MARROW HARVESTING

Marrow was aspirated from the posterior and anterior iliac crests and from the sternum by a standard technique.^{4,5} The marrow was injected

directly, without filtering, into a two-litre Fenwal transfer pack (4R2041) containing heparinised tissue culture fluid (TC 199 Gibco Europe). An average of 765 ml of marrow diluted with blood was harvested on each occasion and in addition to this was 200 ml TC199 with 8000 units of preservative-free heparin.

MARROW PROCESSING

The total marrow volume was then processed in its entirety on a cell separator or was divided into two equal aliquots by closed transfer to two one-litre Fenwal transfer packs (FPR2031). The two aliquots were then both processed by a double centrifugation technique or the centrifugation technique was compared directly with processing by the Hemonetics 30 intermittent cell separator or Aminco Celltrifuge continuous flow cell separator.

DOUBLE CENTRIFUGATION METHOD

The one-litre Fenwal bag containing an average of 470 ml of marrow plus TC199 was placed vents-down in a one-litre bucket of MSE Coolspin centrifuge and spun at 1000 rpm (250 g) for 10 min. The bag was kept upright by means of rubber spacers. The transfer bag was then carefully removed and suspended in the vent-down position so that the red cell-rich fraction could be run off via the filling tube. Approximately 25% of the total volume was removed, but this was very variable. If a buffy coat layer was clearly visible then a large volume of red cells could be removed, but no buffy coat layer was discernible in half the cases. After clipping off the filling tubing, the remaining marrow was mixed thoroughly and returned to the centrifuge with the vents uppermost and spun at 2000 rpm (800 g)

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for 15 min. The majority of the plasma was then removed via the filling tube by means of a Fenwal plasma extractor press.

HEMONETICS INTERMITTENT CELL SEPARATOR METHOD

The Hemonetics model 30 cell separator was set up using a paediatric pheresis pack (part No 9151) as for a conventional paediatric leukapheresis. As the marrow was already anticoagulated with heparin the anticoagulant line on the harness was clipped off. The draw needle adaptor was also clipped off and marrow entered the system through the saline reinfusion female connector via a conventional drip set (Baxter No FKC 2055). The filter in this drip set prevented small clots frequently present in the harvested marrow from entering the system. The Bowl was then filled at a rate of approximately 100 ml/min. When the buffy coat reached the top of the bowl the filling rate was reduced to 20 ml/min and the buffy coat redirected through the white cell collection port into a 600 ml Fenwal transfer pack (No FPR 2022). Collection was usually continued for two minutes. The aim of processing was to end with a predetermined volume of buffy coat (250 ml) in the transfer bag for freezing. Once all the marrow had been processed the drip set was transferred to the reinfusion bag port allowing the red cell and plasma discard to be reprocessed, extracting the last vestiges of buffy coat until the desired volume was obtained. For efficient processing of marrow using the intermittent flow cell separator the total volume of red cell-discard must fill the bowl (i.e. 100 ml) as this is used to displace the buffy coat.

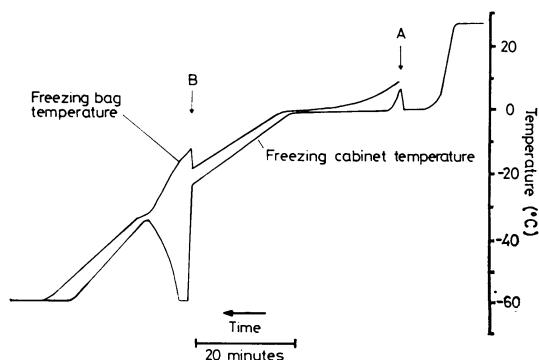
AMINCO CONTINUOUS FLOW CENTRIFUGE METHOD

The Aminco Celltrifuge (J4 6900 D) is a continuous flow cell separator with independently variable donor flow rate, centrifuge speed and nucleated cell collection rate. The processing of bone marrow was performed using our existing protocol for granulocyte harvesting, except that no sedimenting agent was used. The machine parameters used were centrifuge speed 750 rpm, donor flow rate 60 ml/min (in this instance the donor is the marrow sample in a closed circuit with the machine) and nucleated cell collection rate 5 ml/min.

MARROW FREEZING

The volume of the processed marrow was determined by weight and an equal volume of precooled 20% (vol/vol) dimethyl sulphoxide (DMSO Analar grade) in TC 199 was added via a Fenwal AE2 transfer set (FPC 2240) and three-way tap using a 50 ml disposable syringe. This addition was per-

formed under a 10 minute period, with good mixing to minimise osmotic shock. This procedure was performed on ice in a laminar flow sterile hood. Equal volumes (100-150 ml) of the processed marrow and cryopreservative were then transferred via a second AE2 transfer set attached to the three-way tap into four 700 ml Gambro Hemofreeze bags (DF-700-3). Ten to twenty millilitres were also placed in 2 ml freezing vials to enable repeated evaluation of the freezing and storage. Previous studies with peripheral blood leucocytes from patients with chronic granulocytic leukaemia showed that the progenitor cell recovery from both the 2 ml vial and the 120 ml bag were very similar when both were frozen at the same time. The four Hemofreeze bags were sandwiched between aluminium plates and placed with the vials in a Cryoson BV-4 biological freezer (Cryotech Ltd, UK). A thermocouple placed against one of the bags gave a continuous reading of the bag temperature and another thermocouple within the freezing cabinet gave a continuous reading of the cabinet temperature. The samples were allowed to equilibrate at 0°C for approximately 10 min and the cabinet was then cooled at 1.2°C per minute until the liquid to solid phase change. This was indicated by the rise in the bag temperature due to the release of the latent heat of fusion. At this stage the cabinet temperature was dropped rapidly by a manual override to -60°C to minimise the post-freezing plateau. When the latent heat release was over, the cabinet temperature was allowed to rise to -35°C and the cabinet was then cooled at 2°C per minute, during which time the freezing bag cooled at about 1.25°C per minute. This cooling rate was then maintained to -60°C at which stage



Cryopreservation of bone marrow—freezing curves.

Representative cabinet and freezing bag temperature recordings are shown.

A = freezing bags and 2 ml vials placed in precooled freezing cabinet.

B = phase change with latent heat release.

the bags and vials were transferred immediately into liquid nitrogen where they were stored until used (Figure 1). Similar freezing regimes have been used by others.^{6 7}

MARROW THAWING AND INFUSION

The bags of frozen marrows were transported to the bedside in a portable liquid nitrogen container. The bags were then rapidly thawed, one at a time, by immersion in a water bath (37°C). The marrow was then infused without washing through a blood-giving set into a large vein, each bag being infused over about ten minutes.

CELL COUNTING AND CULTURE STUDIES

Nucleated cell counts were made on appropriately diluted samples using a Coulter S Plus. Viability was determined by trypan blue exclusion. Differential counts were performed after staining films with May-Grünwald-Giemsa stain. Myeloid progenitor cells (CFU-GM) were assayed in a double nutrient agar system⁸ using optimal concentrations of placental conditioned medium as a source of colony-stimulating activity.⁹ Nucleated cells (10⁵/dish) were plated in the overlay; a minimum of three dishes were set up for each sample. Cell aggregates greater than 40 cells were scored as colonies after 12-14 days in culture. Thawed marrow samples, which contained 10% (vol/vol) DMSO, were diluted tenfold in tissue culture medium with 40% (vol/vol) serum (human AB or fetal calf

over 5-10 minutes at 37°C to minimise cell toxicity attributable to the DMSO. No washing steps were performed. DMSO contamination up to 0.5% (vol/vol) in the final culture medium has no effect on CFU-GM growth (DC Linch, unpublished observations).

Results

MARROW PROCESSING

The average cell recoveries after processing by double centrifugation and by the Hemonetics cell separator are shown in Table 1. There is no significant difference between the means of the cell populations processed by the two methods, but the ranges of the volume and red cell recoveries are considerably wider in those samples processed by double centrifugation. In both instances the percentage recovery of CFU-GM is greater than that of total nucleated cells.

In six patients a direct comparison of the two methods of processing was made and the detailed results are shown in Table 2. In these six experiments, the final volume was significantly less in the Hemonetics processed samples ($p < 0.05$) as was the red cell recovery ($p < 0.01$). The total nucleated cell yields and the CFU-GM recovery were similar by both methods. Review of the differential counts of the processed samples showed preferential loss of more mature myeloid and erythroid cells in only some cases, and this was always minor. No difference

Table 1 Cell recovery (percentage \pm SD) after processing by double centrifugation or by Hemonetics cell separator

Method of processing	Final volume percentage of original	Total nucleated cells recovered %	Remaining red cells %	CFU-GM recovered %
Double centrifugation	33 \pm 15 (15-61)	81 \pm 8 (66-94)	27 \pm 8 (15-44)	98 \pm 29 (63-154)
Hemonetics Model 30	25 \pm 9 (14-37)	75 \pm 5 (70-86)	25 \pm 2 (23-28)	88 \pm 17 (60-121)

Range is indicated in parentheses.

Table 2 Comparison of two methods of processing

Patients	Centrifuge yields				Hemonetics yields			
	Volume %	Nucleated cells %	Red cells %	CFU-GM %	Volume %	Nucleated cells %	Red cells %	CFU-GM %
1	31	76	46	82	24	77	14	91
2	36	94	33	81	28	70	25	81
3	33	83	61	68	23	75	23	99
4	24	71	24	86	26	72	25	60
5	44	92	59	99	23	73	25	83
6	29	66	—	69	25	70	—	80
Mean \pm SD	33 \pm 7	80 \pm 11	45 \pm 16	81 \pm 12	25 \pm 2	73 \pm 3	22 \pm 5	82 \pm 13

Statistics by *t* test of difference between small sample means:

Volume $p < 0.05$.
 Nucleated cells NS.
 Red blood cells $p < 0.01$.
 CFU-GM NS.

in differential counts between the two methods was noted.

Marrow processing on the Aminco continuous flow cell separator was attempted on three occasions in direct comparison with manual double centrifugation. On one occasion a very satisfactory yield was obtained (29% volume; 82% nucleated cells; 82% CFU-GM), but on two subsequent occasions the yield of nucleated cells was so poor that the marrow had to be reprocessed by manual double centrifugation.

CELL RECOVERIES AFTER FREEZING AND THAWING

After freezing and thawing the average viable nucleated cell recovery was 48% (Table 3). Granulocytes and metamyelocytes were particularly sensitive to the freezing and thawing procedure. The progenitor cell recovery was significantly greater at 79%. The method of marrow processing had no effect on the cell recoveries after freezing and thawing. Similar progenitor cell recoveries were found in the freezing bag (assessed at reinfusion) and in the freezing vials and no deterioration during storage was detected.

Discussion

The minimal number of marrow cells and CFU-GM required for autologous marrow engraftment is unknown, but is probably less than for allogeneic transplantation.^{10,11} Optimal processing of bone marrow prior to freezing is important, however, because this will enable smaller volumes to be harvested and will enable serial ablative chemotherapy to be performed¹² by using only part of the cryopreserved marrow for each rescue procedure. It is possible to store whole, unprocessed bone

marrow, but the cost of storage in liquid nitrogen is prohibitive, and the volume of DMSO used is so large that it is probably necessary to wash the cells prior to reinfusion, a process that increases the risk of microbial contamination and also increases the tendency for the nucleated cells to clump. It is our practice to infuse thawed marrow straight from the freezing bag immediately after thawing. Clumping has not occurred and the only problems attributable to the DMSO infused were that of its unsavoury smell and one minor febrile reaction. Furthermore, by removing most of the red cells prior to freezing and thawing, less free haemoglobin is transfused.

The optimal method of removing red cells and mature granulocytic cells from marrow progenitor cells is probably by centrifuging marrow layered over Ficoll-hypaque in 50 ml tubes.⁷ Wells has achieved 90% CFU-GM recoveries by this method with a total nucleated cell recovery of 18% with very little red cell contamination. This method requires considerable open manipulation of the bone marrow, however, and it is our contention that any frequent routine procedure should be simple and involve "closed processing" as far as possible. Our method of bone marrow harvesting and the three processing procedures compared all comply with these criteria.

Both the double centrifugation technique and the Hemonetics separation procedures gave comparable nucleated cell and CFU-GM yield, but the red cell elimination was far more variable with the double centrifugation technique. This was because of the individual variation in the appearance of a clear buffy coat which would enable most of the red cells to be sedimented off after the first centrifugation step. In many instances, there was no precise boundary visible between cells and plasma. Weiner *et al*¹³ obtained an average 59% nucleated cell yield using a Hemonetics cell separator, which is considerably less than our figure of 75% obtained using a paediatric pheresis bowl. They stated that the differential count changed significantly after such processing with a rise in mononuclear cell count; no CFU-GM data was reported. We did not observe a significant change in the differential count, but the average CFU-GM yield (88%) was greater than the total nucleated cell yield. The final volume after processing was also more variable, with double centrifugation. With the Hemonetics method, processing was continued to a predetermined volume. This was not always possible after the second manual centrifugation step, because leucocyte aggregates would sometimes form in the creases of the centrifuged transfer pack, and if these broke free during the plasma extraction, then this process had to be

Table 3 Cell recoveries after freezing and thawing of bone marrow (cell yields expressed as a percentage of the cells frozen)

	Viable nucleated cells %	CFU-GM %
1	68	92
2	57	73
3	53	35
4	33	68
5	70	105
6	47	101
7	79	158
8	60	62
9	24	80
10	31	30
11	51	42
12	61	103
Mean ± SD	48 ± 22*	79 ± 36*

*Significantly different $p < 0.02$.

halted. Most of the cells in these aggregates were platelets, but some other cells were also present, and as CFU-GM are among the most buoyant of all marrow cells it was thought inadvisable to lose any large aggregates. The variable final volume after processing is a considerable problem with the manual centrifugation method, because all the freezing bags must contain the same volume to ensure equal cooling rates in the programmed freezer and if precise predictable cooling rates are to be achieved then the same freezing bag volumes must be used with each freezing procedure. We have aimed at a final marrow volume of 250 ml. When mixed with an equal volume of 20% (vol/vol) DMSO in TC 199, this allows four 120 ml aliquots to be put into freezing bags, and leaves sufficient marrow for cell culture studies and for freezing in 2 ml aliquots. These 2 ml aliquots can be used as guides to the freezing and storage efficiency.

The Aminco Celltrifuge gave disappointing results, due to drifting of the white cell layer. A deviation of the cell plasma interface away from the collection port by just one millimetre was sufficient to reduce the nucleated count in the collection port from $100 \times 10^9/l$ to $18 \times 10^9/l$. Manual adjustment of the red cell and plasma pump rates brought the interface back into position, but no clear buffy coat was visible on marrow separation making this difficult. The buffy coat was not readily discernible because, unlike the procedure for collection of granulocytes, no sedimenting agent was used. We have not used sedimenting agents as preliminary experiments suggested that cell clumping increased after thawing if a sedimenting agent was present.

The average progenitor cell recovery of 79% after freezing and thawing compares favourably with that reported by Wells.⁷ The nucleated cell recovery, however, was less than that seen by Wells (approximately 85%), and this is because they had previously removed the thermally fragile granulocytic cells on a Ficoll-hypaque gradient. The lowest CFU-GM recovery seen was 30% which still leaves a large safety margin in the autologous bone marrow transplant situation.

In conclusion, the Hemonetics model 30 cell separator with a paediatric pheresis bowl was found to be the optimal method of processing bone marrow prior to cryopreservation. Programmed freezing

in four 120 ml aliquots is simple, reliable and good progenitor cell recoveries are obtained.

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