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## Cryopreserved human haematopoietic stem cells retain engraftment potential after extended (5–14 years) cryostorage

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### Abstract

Harvesting of stem cells during the early phases of treatment with no immediate intention to perform a stem cell transplant is becoming an increasingly common practice. Such “insurance” harvests are often stored for many years before being needed for transplant in a subsequent relapse. The effect of long-term cryostorage (5–14 years) on the viability and functional capacity of haematopoietic stem cells (HSCs) was investigated in 40 bone marrow and peripheral blood harvests using standard *in vitro* methods, the colony forming unit-granulocyte/macrophage (CFU-GM) assay and a single platform viable CD34<sup>+</sup> cell absolute count by flow cytometry. Forty percent of harvests had CD34<sup>+</sup> HSC counts of at least  $0.7 \times 10^6$ /kg bodyweight and 85% had CFU-GM counts of at least  $1.0 \times 10^5$ /kg bodyweight, these values representing our institutional minimum requirements for safe transplantation. Based on these results, it appears that HSC collections can remain adequate for safe transplantation after up to 14 years of cryostorage. However, as deterioration of HSC quality and viability may occur, some precautions may be warranted, namely harvesting higher than normal numbers of HSCs in collections intended for long-term storage and repeating *in vitro* assays on harvests after long-term storage prior to transplantation. © 2002 Elsevier Science (USA). All rights reserved.

*Keywords:* Haematopoietic stem cells; Long-term storage; Cryopreservation; Engraftment potential; Time factors

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The use of autologous haematopoietic stem cell (HSC) transplantation in the treatment of a number of haematological and solid malignancies and some non-malignant conditions has become increasingly common. Current therapeutic strategies necessitate the cryopreservation and storage of virtually all autologous HSC-containing bone marrow and peripheral blood collections for a

variable, but generally brief, period of time between harvest and transplantation. However, several scenarios exist that necessitate extended periods of storage.

Long-term storage of HSCs is fundamental to the practice of performing ‘insurance’ or ‘rainy day’ HSC collections on patients in remission who are considered to be at a significant risk of either relapse or the development of a secondary malignancy and hence may require a transplant in the future. There have been reported instances of

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patients requiring transplantation with HSCs after up to 10 and 11 years of storage [1,23]. Without long-term storage, HSC transplantation may not be a feasible treatment option in such patients due to the difficulty in harvesting disease-free HSCs at a later stage when there is disseminated malignant relapse or HSC quality and function has been compromised by extensive treatment with alkylating agents and/or radiation therapy. In addition, with the advent of gene therapy and umbilical cord blood banks, the applications of long-term HSC storage are widening and assume a new level of importance and relevance.

Theoretically, HSCs stored at ultra-low temperatures should not deteriorate, however, in practice, this may not necessarily be the case. More than 30 years of research concerning the effect of long-term cryostorage on HSCs has produced equivocal results ranging from a total loss of HSC viability after several months of storage to successful transplantation years later [3,19,22,32]. The results of many studies using *in vitro* assay techniques have been ambiguous and difficult to interpret because of poorly standardised methodologies and failure to exclude the effects of confounding factors such as processing and freezing as a source of any observed changes [1,5]. Although HSCs have been successfully transplanted after up to 11 years of cryostorage, several studies have noted a gradual decline in HSC clonogenic capacity with increasing periods of storage [19,24,32]. Also, despite widespread acceptance of flow cytometrical CD34<sup>+</sup> cell counts as an effective *in vitro* measure of HSC engraftment potential, few previous studies have investigated viable CD34<sup>+</sup> cell recovery after cryopreservation. We are unaware of any previous studies using this assay method to assess the effects of long-term cryostorage on HSCs.

In this study, CFU-GM assays and viable CD34<sup>+</sup> cell counts were used to assess the viability and functional capacity of HSCs that had undergone long-term (5–14 years) cryostorage and results were compared to our present institutional minimum requirement to determine the adequacy for transplant by modern criteria.

## Materials and methods

### *Samples*

Forty autologous HSC samples (23 bone marrow [BM], 17 peripheral blood stem cells

[PBSC]) which had undergone 5–14 years cryostorage (median 9.5 years) were studied. Patients were harvested between 1985 and 1994, inclusive, from 36 patients with various haematological and solid malignancies who gave informed consent at the time of collection. All samples were independent of one another, having been collected, processed and frozen separately. All samples were cryopreserved in 10% dimethylsulphoxide (Me<sub>2</sub>SO<sub>4</sub>) prior to storage in the liquid phase of liquid nitrogen. BM or PBSC were mixed with an equal volume of 20% Me<sub>2</sub>SO<sub>4</sub> in RPMI 1640 medium containing 200 IU/ml sodium heparin. Harvests and reference vials were frozen in a controlled rate freezer using the following cooling curve: Step 1 = 4 to –12 °C at –1 °C/min; Step 2 = –12 °C to –20 °C at –4 °C/min; Step 3 = hold at –20 °C for 5 min; Step 4 = –20 °C to –40 °C at –1 °C/min; Step 5 = –40 °C to –80 °C at –3 °C/min; Step 6 = –80 °C to –120 °C at –5 °C/min.

### *Viable CD34<sup>+</sup> cell count*

A modified single platform flow cytometry method based on International Society for Hematology and Graft Engineering (ISHAGE) [18] was used to obtain an absolute viable CD34<sup>+</sup> cell count. Briefly, a reference vial was rapidly thawed at 37 °C and diluted with 5% dextran saline in order to reduce granulocyte clumping and cell loss [31]. The dilution was prepared so as to obtain a volume < 100 µL containing 8 × 10<sup>5</sup> cells, which was added to each of 4 microfuge tubes and incubated with 10 µL of a phycoerythrin-conjugated monoclonal anti-CD34 (HPCA-2, Becton Dickinson, San Jose CA), 10 µL of a fluorescein isothiocyanate-conjugated monoclonal anti-CD45 (HLe-1, Becton Dickinson, San Jose CA) and 10 µL of the viability dye 7-aminoactinomycin D (7-AAD, Sigma, St. Louis MO) (5 µg/ml). A negative control tube was prepared identically but contained a phycoerythrin-conjugated isotype control IgG<sub>1</sub> (Becton Dickinson, San Jose, CA) instead of anti-CD34. Immediately prior to analysis, a known concentration of FlowCount fluorospheres (Beckman Coulter, Miami, FL) was added to each tube. This established a ratio of fluorospheres to the original volume of the sample, enabling calculations of the absolute CD34<sup>+</sup> cell count. Samples were analysed with a FAC-Scan flow cytometer and the CellQuest software package (Becton Dickinson, San Jose, CA), using a modified ISHAGE gating strategy [18]. In accordance with the ISHAGE protocol for

enumeration of CD34<sup>+</sup> cells, the total number of CD34<sup>+</sup> cells was determined rather than subset analysis [18].

#### *CFU-GM assay*

Cells were rapidly thawed in a 37°C waterbath and viable nucleated cells enumerated using a haemocytometer and the exclusion dye trypan blue. CFU-GM assays were plated at densities of  $1 \times 10^5$  and  $2 \times 10^5$  viable nucleated cells per 35 mm plate using Methocult GFH4534 (StemCell Technologies, Vancouver, BC) and incubated for 14 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Colonies, defined as clusters of  $\geq 30$  cells, were scored using an inverted microscope and recorded as the mean of quadruplicate counts. The assay was repeated if plates were contaminated or  $> 50$  colonies were counted, since such high levels have an inhibitory effect on CFU-GM [12]. In the latter case, the number of viable nucleated cells plated was adjusted as appropriate in order to obtain an interpretable result.

#### *Evaluation of graft adequacy*

Our current minimum institutional requirements for safe HSC transplantation are a CD34<sup>+</sup> HSC count of at least  $0.7 \times 10^6/\text{kg}$  bodyweight and/or a CFU-GM content of at least  $1 \times 10^5/\text{kg}$  bodyweight. Harvests meeting or exceeding the minimum institutional requirements for each criterion are deemed to be adequate for safe transplantation.

#### *Statistical analysis*

The  $\chi$ -squared test was used to assess the relationships between quantitative results with the criterion for significance being a probability value,  $P < 0.05$ .

## **Results**

#### *Clinical outcomes*

Within the 36 patients studied, 12 had been autologously transplanted between 1 and 44 months after collection with 5 patients receiving BM autografts and 7 patients receiving PBSC derived stem cells. Of 11 patients who survived long enough (i.e.,  $>$ day 20 post-transplant), 10

achieved tri-lineage engraftment. The patient who did not achieve megakaryocytic engraftment died at day 69 post-transplant of recurrent disease following a BM autograft.

#### *CD34<sup>+</sup> stem cell counts after long term cryostorage*

The results of the CD34<sup>+</sup> cell count are shown in Fig. 1. Samples were numbered from 1–40 according to their cryostorage period, with sample number 1 having the shortest duration of storage (5 years), and sample number 40 the longest (14 years). The line transecting at CD34<sup>+</sup> cells/kg body weight =  $0.7 \times 10^6$  represents our institutional minimum requirement for safe transplantation for both bone marrow and peripheral blood harvests, hence points lying on or above this line indicate samples fulfilling the CD34<sup>+</sup> cell count criterion for safe transplantation. Of the samples analysed, 16/40 were assessed as containing adequate CD34<sup>+</sup> stem cells for transplantation. The majority of the samples with adequate CD34<sup>+</sup> cell counts (10 of 16) were PBSC collections. In contrast, most samples with inadequate CD34<sup>+</sup> cell counts (17 of 24) were BM harvests. However, although the median CD34<sup>+</sup> HSC content was lower in bone marrow samples than in peripheral blood samples ( $0.50$  and  $1.0 \times 10^6/\text{kg}$ , respectively) the difference did not reach statistical significance ( $P = 0.06$ ). Pre-storage CD34 counts were not available as the test was not performed prior to 1995.

#### *CFU-GM colony assays after long term cryostorage*

The current CFU-GM assay results are shown in Fig. 2. Samples were numbered from 1 to 40 according to their cryostorage period, with sample number 1 having the shortest duration of storage (5 years), and sample number 40 the longest (14 years). The line transecting at CFU-GM/kg body weight =  $1 \times 10^5$  represents our current institutional minimum requirement for safe transplantation, hence points lying on or above this line indicate samples fulfilling the CFU-GM assay criterion for safe transplantation. Results were assayable in only 39 samples since the single available reference vial for one sample, a BM harvest stored 14 years, was bacterially contaminated and failed to yield results.

Of the 39 assayable samples, 33 (85%) had an adequate CFU-GM content, including samples stored up to 14 years. The median CFU-GM/kg

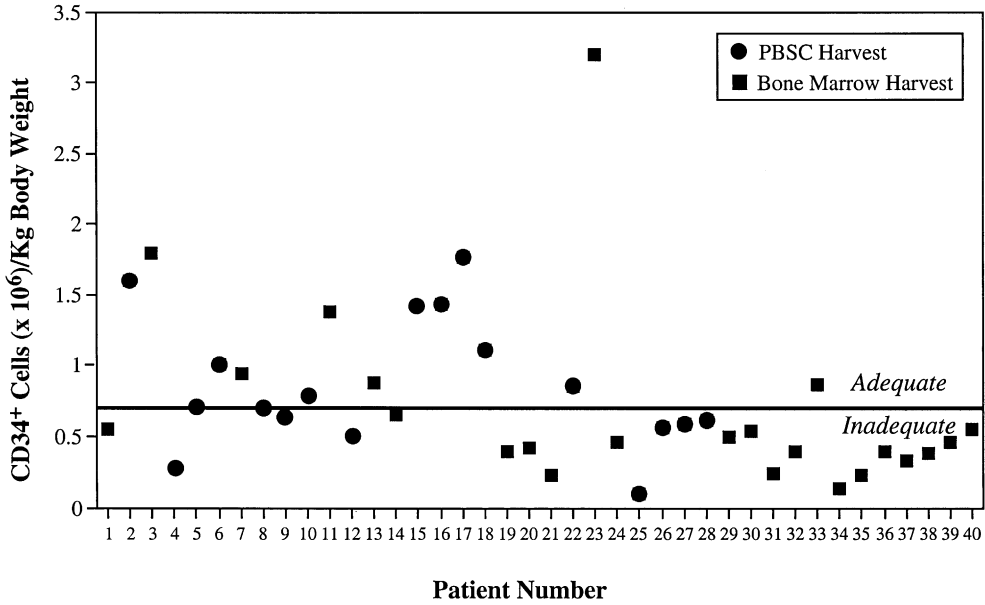


Fig. 1. Number of CD34<sup>+</sup> HSC recovered after long-term cryostorage. The absolute number of viable CD34<sup>+</sup> HSC was determined by single platform flow cytometry and the total number of these cells in the cryopreserved full harvest subsequently calculated. Samples are numbered from 1 to 40 in order of ascending duration of storage. The line transecting CD34<sup>+</sup>/kg = 0.7 represents our institutional minimum criterion for safe transplantation, hence samples falling on or above this line are considered to contain adequate numbers of CD34<sup>+</sup> HSC.

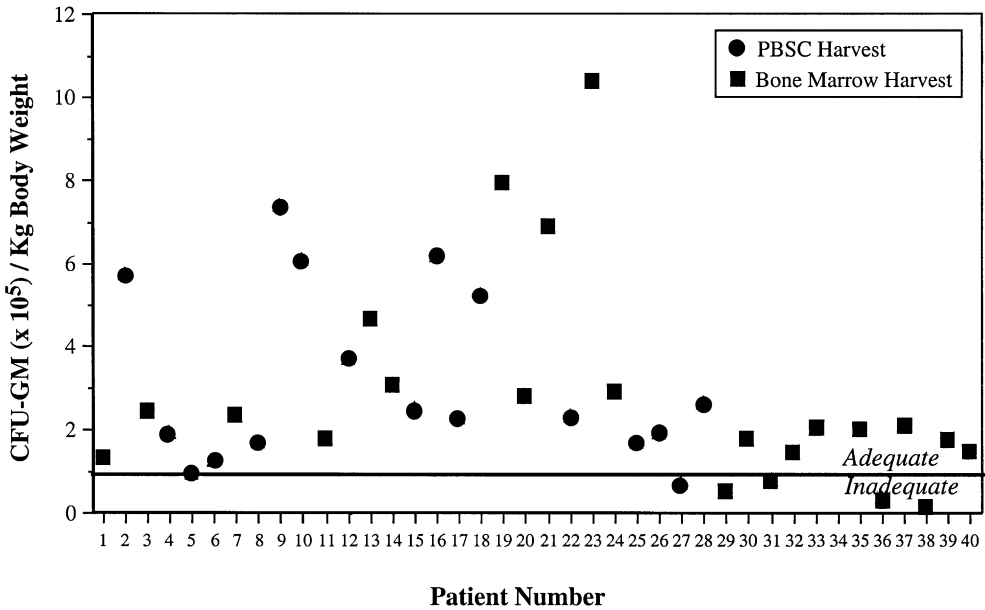


Fig. 2. Number of CFU-GM units recovered after long-term cryostorage. The number of CFU-GM units was determined by culture in semi-solid medium and the total number of these cells in the cryopreserved full harvest subsequently calculated. Samples are numbered from 1 to 40 in order of ascending duration of storage. The line transecting CFU-GM ( $\times 10^5$ )/kg = 1.0 represents our institutional minimum criterion for safe transplantation, hence samples falling on or above this line are considered to contain adequate numbers of CFU-GM.

body weight was 1.93 (range: 0.16–10.4). The samples with the highest and lowest CFU-GM counts corresponded to those with the highest and lowest numbers of CD34<sup>+</sup> stem cells, respectively. The six samples with inadequate CFU-GM numbers comprised four BM and two PBSC collections. The median CFU-GM count did not differ between BM and PBSC samples. Eighteen of the 22 assayable BM samples were assessed to have sufficient CFU-GM content, as did 15 of the 17 PBSC collections.

#### *Adequacy of HSCs for transplantation after long-term cryostorage*

Using the current results of CD34<sup>+</sup> cell counts and CFU-GM, samples were allocated qualitatively into one of four categories on the basis of whether they fulfilled our current institutional minimum requirements for safe transplantation in each assay (Fig. 3). A  $\chi$ -squared test revealed a statistically significant relationship between the qualitative results of the CD34<sup>+</sup> cell count and CFU-GM assay ( $P=0.02$ ). This was supported by the strong positive correlation found to exist between these parameters ( $r^2 = 0.76$ ,  $P \leq 0.0001$ ).

Of the 39 assayable samples, 16 were assessed to be adequate using both assays and would def-

initely be considered suitable for transplantation if required. In contrast, six samples had inadequate progenitor content using both assays, and definitely would not be suitable for transplantation. The remaining 17 samples had adequate CFU-GM's but an inadequate CD34<sup>+</sup> content. These collections would be considered marginally adequate overall and, depending upon the prevailing clinical circumstances, could be used for transplantation.

#### **Discussion**

This project aimed to determine whether HSCs retained adequate engraftment potential for transplantation after long-term (5–14 years) cryostorage.

The methods used in this study to evaluate the status of HSCs in samples were a flow cytometer-based single platform absolute viable CD34<sup>+</sup> cell count, and the CFU-GM cell culture assay, both of which are widely accepted as being useful predictors of engraftment potential and kinetics [2,4,13,16]. These assessments are routinely performed on HSC collections and are standardised, reproducible and complementary, together providing information regarding both the content and viability of primitive and more committed HSCs in collections [18,21]. This is particularly important given that more mature progenitors are believed responsible for the early phase of engraftment, but primitive progenitors are necessary for long-term haematopoietic reconstitution [30]. Despite reports of successful engraftment [29], transplantation of material that has no demonstrable clonogenic activity is not considered safe or routine practice.

There has been criticism of studies where the results from tested reference vials were assumed to be representative of the bulk of the collection stored in bags [17,34] as some results obtained from material stored in vials tended to be worse than for material in bags [35,11,33]. However other studies have failed to detect any discrepancy [14,27] and, of particular relevance to our study, Humpe et al. [15] clearly demonstrated that provided processing, freezing and storage conditions are identical, as in the present study, the use of reference vials was not an issue.

The number of viable CD34<sup>+</sup> HSC recovered post-storage was disappointingly low, with only half of samples exceeding our institutional minimum requirement, and only 1/40 exceeding our

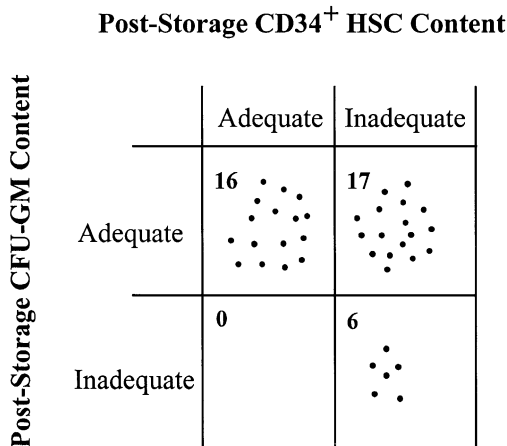


Fig. 3. Adequacy for safe transplantation of all harvests after long-term cryostorage. Using the results of both the current (post long-term storage) CD34<sup>+</sup> HSC enumeration and CFU-GM assays, harvests were allocated qualitatively into one of four categories on the basis of whether they fulfilled current institutional minimum requirements for safe transplantation. Adequate was defined as CD34<sup>+</sup> HSC  $\geq 0.7 \times 10^6/\text{kg}$  bodyweight and CFU-GM  $\geq 1.0 \times 10^5/\text{kg}$ .

preferred dosage of  $2 \times 10^6$ /kg. Granulocyte clumping in thawed specimens has been noted by others to cause loss of CD34<sup>+</sup> HSC [6], however this was not observed in any samples utilised in the present study. The use of a dextran based diluting solution [31] completely prevented clumping of thawed samples in this study. It is impossible to assess loss or deterioration of these cells as a result of extended cryostorage because a standardised flow cytometry assay was not in routine use at our institution until 1996 and only one patient in this study had a CD34 count on the original harvest. Also, minimum dosages of CD34<sup>+</sup> HSC assume that a cytokine based (predominantly G-CSF) stem cell mobilisation regimen, with or without chemotherapy, was used to enrich the peripheral blood with HSC immediately prior to harvest. Given that most of the collections had taken place before the routine clinical availability of haematopoietic cytokines, none of the patients in our study group had received any form of cytokine-based mobilisation prior to harvest. All collections were carried out after chemotherapy-alone mobilisations which are known to contain considerably fewer CD34<sup>+</sup> HSC compared to G-CSF mobilised harvests [8,20]. It is likely that many of these harvests, whilst containing fewer total CD34<sup>+</sup> cells, would possess equivalent numbers of the primitive CD34<sup>+</sup>CD38<sup>-</sup> subset responsible for long-term engraftment [10,26] and hence may in actuality be suitable for transplantation. The fact that 7 of these PBSC harvests had been successfully transplanted provides evidence to support this. Unfortunately, 4 colour analysis of CD45<sup>+</sup>34<sup>+</sup>38<sup>-</sup>/7AAD<sup>-</sup> cells exceeds the technical capacity of the FACScan flow cytometer. It is our contention that the low levels of CD34<sup>+</sup> cells observed in most samples were largely due to the initial collection of low numbers of CD34<sup>+</sup> cells, rather than cell losses during subsequent processing, freezing, and storage.

Due to changing CFU-GM assay conditions, this study could not quantitatively assess whether deterioration of clonogenic activity has occurred, although findings of deterioration over extended cryostorage of cord blood collections has been previously reported [7,24]. Despite precautions to maintain a constant ultra-low temperature storage environment, temperature fluctuations that may compromise HSC quality and viability are inevitable in any HSC banking facility. Although samples had been stored in the liquid phase of liquid nitrogen, an environment considered relatively

stable and less susceptible to temperature fluctuations than the vapour phase [5,15], the design of the storage tank and inventory system meant that adding and removing collections from cryostorage was associated with transiently exposing other samples stored in the same area of the tank to ambient temperatures. The inventory systems of newer storage tanks are designed in a manner that reduces this problem. It has also previously been suggested that selective loss of more mature progenitors, including CFU-GM, may occur during cryostorage [9,25], particularly if the patient has received extensive pretreatment, which appears to increase the susceptibility of CFU-GM to damage during storage [9,15,28]. Given these findings it is possible that deterioration occurred to samples in the present study, but not to the extent that rendered them inadequate for transplantation. This is consistent with other studies which have demonstrated clonogenic activity in samples stored for similar periods of time [19,32].

The clinical significance of this study lies in the determination of whether HSC harvests retain engraftment potential following long-term cryostorage. When results of both post-storage assays were considered together, it was apparent that 16 of the 39 samples would definitely be suitable for transplantation by our current CD34<sup>+</sup> and CFU-GM criteria, rating as adequate for safe transplantation by both assays. In contrast, six samples that were currently assessed to be inadequate by both assays would definitely not be transplanted as this almost certainly would result in failure to engraft. However there were 17 samples which had an adequate CFU-GM content but an inadequate number of CD34<sup>+</sup> HSC. We would consider these harvests to be marginally adequate for safe transplantation, as it is our clinical experience that such collections may be successfully transplanted if required, albeit with delayed megakaryocytic engraftment (RM Lowenthal, unpublished observations). The CD34<sup>+</sup> HSC count may be underestimating the true engraftment potential of these harvests as none of these patients received G-CSF mobilisation prior to collection.

This study has demonstrated that HSCs remain functional after long-term cryostorage and hence the performance of prophylactic insurance/"rainy day" stem cell harvests is a clinically viable procedure. However, there is a possibility that collections may deteriorate with storage, and it is therefore recommended that higher than required numbers of HSCs are harvested where the intention is long-term cryostorage. This would

allow for some deterioration yet ensure that transplantable numbers of HSCs were present after storage, and repeat evaluations of engraftment potential after long-term storage should be performed to verify that adequate numbers of functional HSCs remain for transplantation. Overall this study has demonstrated that HSCs can retain their engraftment potential after up to 14 years of cryostorage.

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