The Feasibility of Using Blood Bank–Stored (4°C) Cord Blood, Unmatched for HLA for Marrow Transplantation

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Abstract

The main purpose for storing large numbers of umbilical cord blood (CB) units by cryopreservation is to obtain a close HLA match for use in bone marrow transplantation. The use of partially matched or unmatched CB has been suggested, and publications about the success of 3 antigen mismatches have given some credence to this suggestion. Graft vs *host disease still is considered a major barrier for successful CB transplantation. The cost per frozen CB unit of approximately $15,000 considerably limits its availability in developing countries. Eleven human umbilical cord specimens were stored in gas-permeable bags at 4°C for up to 3 weeks. Clonal growth, replating efficiency in methylcellulose cultures, differential count, and flow cytometric immunophenotyping results were examined at intervals up to 21 days. Mixed lymphocyte cultures were evaluated on 13 similarly stored specimens at intervals up to 14 days. When plated at 1, 10, and 21 days, the combined percentage of the more primitive colonies increased on days 10 and 21. Replating efficiency of blast cell colonies when stem cell factor was added was 81.2% and 67.8% on days 10 and 21, respectively. When mononuclear cells were immunophenotyped, the mean percentage of CD34$^+$ and CD117$^+$ cells, considered primitive stem cell markers, increased significantly from day 1 to day 21. The ability of stored CB cells to respond to phytohemagglutinin or alloantigens decreased progressively from day 1 to day 14. By day 14, the reactivity of CB responder cells, in mixed lymphocyte cultures, to fresh allogeneic CB stimulator cells declined significantly. These findings suggest that CB can be stored in existing blood bank facilities and retain its hematopoietic potential for transplantation. Furthermore, it may be feasible to combine individual CB samples to provide a sufficient number of viable stem cells for transplantation, substantially expanding the number of potential recipients.*

The benefits and advantages of using human umbilical cord blood (CB) for transplantation have been well-documented during the past several years[1,2] and may soon attain the status of being a treatment of choice for patients with selected diseases. To minimize the possibility of clinical complications of transplant rejection (failure to implant) or graft *vs* host disease (GVHD), the selection of CB donors that are closely matched with the recipient for HLA antigens is the most desirable choice. However, in earlier publications, Ende et al,[3] Lu and Ende,[4] and Ende et al[5] have suggested that even if HLA compatibility is disregarded, umbilical CB still might be used effectively in lieu of bone marrow for transplantation, and this is supported by the clinical results of patients with 1, 2, or 3 antigen mismatches who have successfully received allogeneic CB transplants.[2]

Previous results[4,5] have indicated that CB stored for 5 to 21 days under blood bank conditions at 4°C will rescue mice from the effects of lethal irradiation by acting as a temporary bridge and by stimulating endogenous hematopoiesis leading to self-reconstitution.[6] Furthermore, in methylcellulose cultures, blast colonies were generated from 21-day-old stored CB, and replating these colonies produced secondary colonies 62.5% of the time.[4]

If CB can be stored in existing blood bank facilities at 4°C and does not require a close HLA match for successful transplantation, it would transform what is now a valuable biologic commodity with limited availability into one that is widely available to many more patients, children and adults, at a much more affordable cost. Moreover, for developing countries, which currently do not have the necessary financial resources to establish frozen CB banks or have cultural barriers, this would be a major step forward in providing a source of hematopoietic cells for transplantation.

The studies described herein were undertaken to further evaluate the effects of storage of human umbilical CB

samples under blood-bank conditions at 4°C on the viability, phenotype, and functional capability of the surviving cells.

Materials and Methods

Sample Collection and Storage

Eleven human umbilical CB samples were obtained from the placentas of healthy full-term neonates. The study was approved by the institutional review board of the New Jersey Medical School (Newark) under federal regulations, Title 45, part 46, paragraph 46, 110 no. 2. Collection of CB was made into a 50-mL sterile polypropylene test tube containing 5 mL of citrate phosphate dextrose as an anticoagulant. The volume of CB collected varied from 20 mL to 40 mL. Samples were kept at room temperature until they were sent to the blood bank for storage and were evaluated within 24 hours after delivery (day 1). The remainder of the CB sample was then transferred into a special polyolefin blood collection bag (Cryocyte Freezing Container, Baxter Healthcare, Deerfield, IL) that allows gaseous transfer[7] and stored in a 4°C blood bank refrigerator for up to 3 weeks. The temperature and duration of storage were selected because blood banks routinely use blood within 21 days when citrate phosphate dextrose is the anticoagulant. Physical and functional assays were performed on the stored CB, including clonal growth, replating efficiency in methylcellulose cultures, immunophenotyping by flow cytometry, and mixed lymphocyte cultures.

Clonal Growth

These procedures were performed on each sample within 24 hours after delivery for day 1 and sequentially on day 10 and day 21 of storage. Low density (1.077) cells were isolated by sodium diatrizoate poly-sucrose gradient (Histopaque, Sigma, St Louis, MO) centrifugation at 1,500 rpm for 30 minutes. The low density cells were harvested, washed twice with 2% fetal bovine serum–Iscove modified Dulbecco medium, and plated at 1×10^5 cells per milliliter in semisolid culture medium in triplicate. The Iscove modified Dulbecco culture medium was supplemented with 30% fetal bovine serum and 1% methylcellulose. Dishes were incubated at 37°C in 5% carbon dioxide with full humidity for 14 days. Various cytokines were used individually or in combination in this culture system as follows: human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF), 100 U/mL (Sigma); human recombinant interleukin (IL)-3, 100 U/mL (Sigma); human recombinant erythropoietin (EPO), 1 U/mL (Procrit, Amgen, Thousand Oaks, CA); and human recombinant stem cell factor (SCF), 50 ng/mL (Life Technologies, Rockville, MD) ▮Table 1▮.

Colonies were scored under low-power phase inverted microscopy after 2 weeks of incubation. Colony-forming units, granulocyte macrophage (CFU-GM) colonies were defined as aggregates consisting of 50 or more cells. Cell aggregates that had the red color of hemoglobin and contained at least 50 cells, or were composed of at least 3 subcolonies containing a minimum of 10 cells each, were scored as burst-forming units, erythroid (BFU-E). Colony-forming units, granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) colonies seemed to have erythroid and nonerythroid cells but shared a single center. A colony consisting of more than 40 cells that appeared colorless and transparent and uniformly small and round with no signs of terminal differentiation was counted as a blast cell colony (CFU-BL).[8,9]

Replating Efficiency

The extensive proliferative capacity of hematopoietic progenitor cells in CB was measured by replating efficiency. A single, well-defined colony was selected as the primary colony, and a 200-µL adjustable micropipet with the volume adjusted to 30 µL was used to pick up the entire colony. The colony cells were gently suspended into the medium and mixed evenly for the secondary culture. All of the primary replated colonies

▮Table 1▮
Hematopoietic Progenitors in Human Cord Blood*

	Day 1			Day 10				Day 21			
Cytokines Added†	GM	BFU-E	GEMM	BL	GM	BFU-E	GEMM	BL	GM	BFU-E	GEMM
GM-CSF	50 ± 15	0	0	3 ± 1.8	31 ± 7.9	0	0	2 ± 1.2	3 ± 1.2	0	0
GM-CSF + 1L-3	138 ± 14.7	0	0	5 ± 4.9	43 ± 9	0	0	5.3 ± 2.5	4.6 ± 1.5	0	0
EPO	5 ± 1.6	13 ± 3.4	46 ± 4.6	0	3 ± 2.5	3 ± 0.8	22 ± 3.9	0	0	0.6 ± 0.4	7.2 ± 1.9
EPO + 1L-3	26 ± 5.4	8 ± 3.2	78 ± 5.1	1.9 ± 0.8	9 ± 1.7	4 ± 0.8	45 ± 5.3	1.2 ± 0.9	1.5 ± 0.6	1.7 ± 1.3	21 ± 4.9
GM-CSF + EPO + 1L-3	34 ± 4.1	7 ± 2.5	84 ± 4.3	5 ± 2	15 ± 3.3	6 ± 1	50 ± 5.7	2.9 ± 2.4	1.9 ± 0.5	3.5 ± 1	25 ± 5.4

GM = granulocyte-macrophage; BFU-E = burst-forming units, erythroid; GEMM = granulocyte, erythroid, macrophage, megakaryocyte colonies; BL = blasts cell colonies; GM-CSF = granulocyte-macrophage colony-stimulating factor; EPO = erythropoietin; IL = interleukin.
* Colony-forming assays were performed on mononuclear cells obtained from cord blood samples. Day 10 and day 21 samples were stored at 4°C until assayed as described in the "Materials and Methods" section. The results are given as the mean number of colonies ± SE per 1×10^5 mononuclear cells plated from 11 individual cord blood samples.
† Cytokines were added individually or in combination at the following concentrations: GM-CSF, 100 U/mL; EPO, 1 U/mL; IL-3, 100 U/mL.

were selected from the original dishes that were stimulated with the combined cytokines of GM-CSF plus EPO. Cultures for secondary colonies were incubated and scored using the same conditions described for primary cultures.

Flow Cytometry

Fluorescein-isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antihuman antibodies (Becton Dickinson, San Jose, CA) were used for simultaneous 2-color determination of mononuclear cell subpopulations of CB. Negative control staining, using mouse monoclonal isotype controls (IgG1-FITC/IgG1-PE), was performed on each sample. Aliquots of 100 μL of CB were stained according to the manufacturer's recommendations using 10 μL of fluorochrome-conjugated monoclonal antibodies. Antibody combinations included CD19-FITC/CD2-PE, CD8-FITC/CD4-PE, CD45-FITC/CD14-PE, CD34-FITC/HLA-DR-PE, CD34-FITC/CD117 (c-kit)-PE, and CD18-FITC/glycophorin A-PE.

Phytohemagglutinins (PHA) and Mixed Lymphocyte Responses

Mononuclear cells were obtained from CB or from peripheral blood samples of adult donors by Ficoll-Hypaque density centrifugation. For all of these experiments, irradiated (γ) peripheral blood mononuclear cells (PBMCs) from the same 2 adult donors were used. The 2 adult donors (N.M.P., N.E.) were healthy men, 52 and 74 years old. On the day of collection, CB cells were typed for expression of HLA class I and class II determinants, and all of the CB samples were histoincompatible in varying degrees at class I and class II loci with the 2 stimulator cell donors in all instances (data not shown). For PHA responses, cells were cultured at a density of 1×10^5/well in 96 well plates in the presence or absence of PHA (5 μg/mL) for 72 hours. For mixed lymphocyte responses, responder cells (2×10^5 per well) were cultured with an equal number of irradiated (γ; 3,000R) stimulator cells for a total of 96 hours.[10] For both types of response, ^{3}H-thymidine (specific activity, 1.9 Ci:/mol; NEN Research Products, Boston, MA) was added (1 μCi per well) 16 to 18 hours before harvest. The levels of ^{3}H-thymidine incorporation for unstimulated responder cells and γ-irradiated cells were obtained from wells containing these cell types cultured alone for the time periods indicated. For PHA responses and mixed lymphocyte responses, cultures were performed in triplicate.

For PHA responses, the difference between stimulated and unstimulated cells (Δcpm) was determined by subtracting the counts per minute of resting cells from the counts per minute of PHA-stimulated cells. For mixed lymphocyte responses, the Δcpm was obtained by subtracting the counts per minute of responder and γ-stimulator cells cultured separately from the counts per minute of responder cells cocultured with γ-stimulator cells.

Manual Leukocyte Differential Count

To evaluate the change in absolute numbers of nucleated cells and lymphocytes after storage at 4°C, we examined a total of 63 aliquots of fresh and stored CB including the 11 individual CB samples in which sequential samples were taken. Blood smears were stained with Wright-Giemsa stain, and the identity of the samples was unknown to the examiner for total and differential counts.

Results

Clonal Growth

As shown in Table 1, large numbers of hematopoietic progenitor cells were found in the 11 samples of day-1 CB samples tested. The mean number of CFU-GM was 50 per 1×10^5 low density cells when stimulated by GM-CSF alone. These were usually large colonies containing more than 1,000 cells. Using EPO alone, the mean number of erythroid colonies that contained erythroid cells, including BFU-E (primarily in a primitive pattern) and CFU-GEMM, was 59 per 1×10^5 low density cells from day-1 CB (Table 1). Most of these BFU-E colonies in day-1 CB were actually CFU-GEMM[11] as determined by the lack of red color in some cells when the BFU-E colonies were aspirated. If, however, no red color was identified, the cells were considered nonerythroid.

The total number of colonies (GM + BFU-E + GEMM) in day-1 CB increased nearly 2-fold when stimulated by the combination of IL-3 with GM-CSF or EPO compared with stimulation by GM-CSF or EPO alone. In addition, day-1 CB had a high frequency of large CFU-GEMM, the characteristic growth pattern of CB, if EPO or EPO plus IL-3 was present in the culture system. There was no statistical difference with the number of colonies obtained between EPO plus IL-3 and EPO plus IL-3 plus GM-CSF. Similarly there was no statistical difference with CFU-GEMM when cultured with EPO plus IL-3 or EPO plus IL-3 plus GM-CSF (Table 1).

Although colony-forming capacity decreased when CB was stored at 4°C for 10 days (Table 1), there was no significant difference between the total number of colonies measured on day 10 and day 1 when EPO, IL-3, and GM-CSF were added to the cultures ($P > .4$). However, the total colony formation using these 3 cytokines decreased significantly ($P < .01$) with the prolongation of storage for 21 days at 4°C (Table 1). Despite the decline in the absolute number of colonies, the percentage of primitive colonies, including

CFU-BL and CFU-GEMM, actually increased after storage at 4°C **Figure 1**. The combined percentages of CFU-BL and CFU-GEMM of the total number of colonies formed was 67% on day 1, 72% on day 10, and 84% on day 21 for CB samples assayed in the presence of EPO, IL-3, and GM-CSF (day 1 *vs* day 21, $P < .01$). CFU-BL colonies usually were obscured in day-1 samples by a majority of large CFU-GM colonies, which consisted of more than 1,000 cells. In contrast, CFU-BL were identified readily by their unique pattern on day 10 and day 21; however, the CB cultures on these days contained fewer colonies and were smaller. Nevertheless, the percentage of CFU-BL among the nonerythroid colonies (CFU-BL + CFU-GM) also increased significantly with longer storage times, representing 25% on day 10 and 60% on day 21 (Figure 1).

Replating Efficiency

Whereas clonal growth in vitro reflects the existence of hematopoietic progenitor cells by the patterns of their progeny, the replating capacity of primary colonies gives direct evidence of renewal ability by producing progeny of multiple lineages. The replating efficiency is shown as the percentage of single primary colonies that develop into at least 1 secondary colony.[12] A total of 241 single primary colonies from 11 CB samples stored at 4°C were replated. Among these were 115 primary colonies derived from day-10 CB and 126 primary colonies derived from day-21 CB samples **Table 2**.

The replating efficiency of primary colonies obtained from day-10 CB was similar for CFU-BL and CFU-GEMM, regardless of whether SCF was added along with GM-CSF, EPO, and IL-3. With day-21 cord blood, however, the replating potential was largely dependent on the addition of SCF, and this was seen for CFU-BL and CFU-GEMM primary colonies. The addition of SCF with the combined cytokines GM-CSF, EPO, and IL-3 achieved the greatest replating efficiency for day 21-CB: 67.7% among CFU-BL primary colonies and 48.8% among CFU-GEMM primary colonies. However, the total number of secondary colonies formed per primary CFU-BL colony replated decreased significantly from 28.6 to 4.8 if SCF was not added to the cultures (Table 2). In the presence of SCF, individual primary CFU-BL in day-21 CB developed diverse secondary colonies consisting of CFU-BL, CFU-GEMM, BFU-E, and CFU-GM. These results indicate that SCF has an essential role in the stimulation and differentiation of hematopoietic progenitor cells in CB that has been stored for 21 days at 4°C.

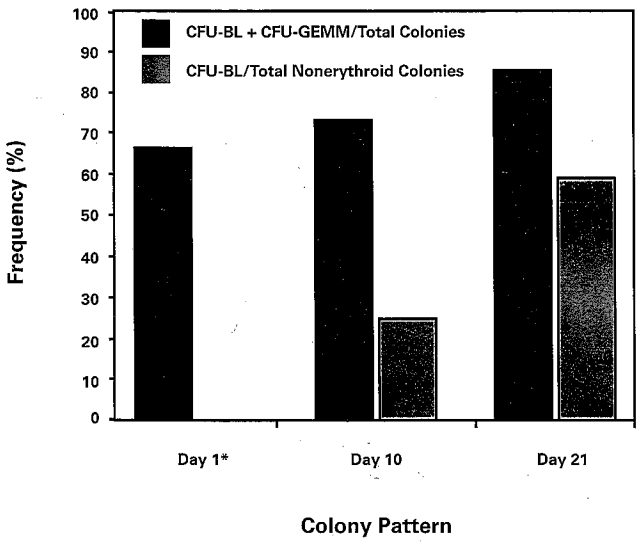

Figure 1 Distribution of colony-forming units, blasts (CFU-BL) and colony-forming units, granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) colonies in stored cord blood. *Blast colonies may be obscured.

Immunophenotype

Eleven samples of CB were immunophenotyped concurrent with the clonogenic assays performed on day 1, day 10, and day 21 **Table 3**. The mean percentage of $CD34^+$ cells in samples taken from whole CB was 1 ± 0.1 on day 1, 1 ± 0.4 on day 10, and 4 ± 2.8 on day 21, which represents a significant increase ($P < .01$) by day 21. The percentage of $CD117^+$ cells, which are the receptors for SCF,[13] also showed a significant increase ($P < .05$) after storage for 21 days at 4°C. In contrast, the percentage of cells that coexpressed CD34 and HLA-DR showed no significant difference within this period. The increase in percentage of $CD34^+$ cells after storage was inversely proportional to the total colony formation but was consistent with the increase in the percentage of CFU-BL and CFU-GEMM, which is representative of more primitive hematopoietic progenitor cells (Table 3). Glycophorin A, which is a marker for the erythroid series, also showed a statistically significant percentage increase from day 1 to day 21 ($P < .01$, Table 3).

Ability of CB Cells To Respond to PHA and Alloantigens

Mononuclear cells from CB samples obtained on the day of collection or after storage at 4°C also were tested for the ability to respond to PHA or after stimulation with allogeneic adult PBMCs in mixed lymphocyte cultures (MLCs). As shown in **Table 4**, fresh CB cells responded very well to PHA (mean Δcpm, $43.6 \pm 6.71 \times 10^3$) in comparison to the response of PBMCs obtained from the same 2 adult donors tested in parallel on 18 occasions (donor A: mean Δcpm, $20.5 \pm 2.42 \times 10^3$; donor B: mean Δcpm, $20.4 \pm 2.41 \times 10^3$). After 7 days of storage at 4°C, CB cells maintained an ability to respond to PHA (mean Δcpm, $37.3 \pm 7.65 \times 10^3$) that was not significantly different ($P > .05$) from their response on the day of collection. After storage at 4°C for 14 days, however, the response of CB cells to PHA (Δcpm = 15.7 ±

Table 2
Replating Efficiency of Stored Cord Blood*

1° Colony Replated	Cytokines Added†	Day 10 n‡	Day 10 Replating (%)	Day 10 No. of 2° Colonies per 1° Colony	Day 21 n‡	Day 21 Replating (%)	Day 21 No. of 2° Colonies per 1° Colony
CFU-BL	With SCF	27	81.2	23.4	34	67.7	28.6
	Without SCF	11	81.8	19	14	35.7	4.8
CFU-GEMM	With SCF	43	86	42.5	43	48.8	12.9
	Without SCF	34	76.5	19.7	35	20	1.1

CFU = colony-forming units; BL = blasts; SCF = stem cell factor; GEMM = granulocyte, erythroid, macrophage, megakaryocyte colonies.
* Individual CFU-BL or CFU-GEMM colonies from primary (1°) CFU assays established from day-10 or day-21 4°C-stored cord blood samples were replated in secondary (2°) methylcellulose cultures. Results are given as the percentage and number of 2° colonies scored as described in the "Materials and Methods" section.
† All 2° cultures contained granulocyte-macrophage colony-stimulating factor, 100 U/mL; erythropoietin, 1 U/mL, and interleukin-3, 100 U/mL, with or without added SCF (50 ng/mL).
‡ The number of 1° colonies replated after storage at each time point.

Table 3
Immunophenotype of Fresh or 4°C-Stored Cord Blood*

Marker Expression	Time of Storage Day 1	Time of Storage Day 10	Time of Storage Day 21	*P* Day 1–10	*P* Day 1–21	*P* Day 10–21
CD34	1.02 ± 0.11	0.95 ± 0.42	3.95 ± 2.82	NS	<.01	<.01
CD117	0.38 ± 0.12	0.66 ± 0.15	0.84 ± 0.17	NS	<.05	NS
CD34/HLA-DR	0.91 ± 0.34	0.92 ± 0.19	1.1 ± 0.22	NS	NS	NS
CD2	19.82 ± 3.84	21.73 ± 2.12	18.27 ± 2.86	NS	NS	NS
CD19	4.45 ± 0.99	5.91 ± 0.81	7 ± 1.22	NS	NS	NS
CD4	13.36 ± 2.45	12.27 ± 1.88	12.18 ± 2.89	NS	NS	NS
CD8	8.55 ± 1.8	6.64 ± 1.55	5 ± 0.86	NS	NS	NS
GPA	19.4 ± 5.35	47.5 ± 7.61	47.2 ± 6.21	<.01	<.01	NS
CD45	88.11 ± 5.33	51.44 ± 6.52	41.66 ± 5.63	<.001	<.001	NS
CD18	77.52 ± 7.33	46.33 ± 6.63	35.5 ± 5.32	<.01	<.001	NS
CD14	7.23 ± 1.85	23.57 ± 4.56	19.32 ± 1.22	<.01	<.001	NS

NS = not significant; GPA = glycophorin A.
* Mononuclear cells obtained from 11 individual cord blood samples that were stored for the indicated times were analyzed for expression of cell surface markers as described in the "Materials and Methods" section. The results are given as the mean percentage ± SE of cells that expressed a particular marker.

Table 4
Ability of 4°C-Stored Cord Blood Cells To Proliferate in Response to Different Stimuli*

Period of 4°C Storage (d)	Stimulus†	n‡	Δcpm × 10^3 ± SE
1	PHA	13	43.6 ± 6.71
	γ-Allogeneic PBL (donor A)	11	16.6 ± 2.04
	γ-Allogeneic PBL (donor B)	10	15.8 ± 2.06
	γ-Allogeneic CB	26	11.2 ± 1.57
7	PHA	10	37.3 ± 7.65
	γ-Allogeneic PBL (donors A and B combined)	17	8.5 ± 1.62
	γ-Allogeneic CB	11	1.7 ± 2.41
14	PHA	13	15.7 ± 6.71
	γ-Allogeneic PBL (donors A and B combined)	14	3.0 ± 1.79
	γ-Allogeneic CB	18	−0.4 ± 1.89

Δcpm = the difference between stimulated and unstimulated cells; PHA = phytohemagglutinins; PBL = peripheral blood leukocytes; CB = cord blood.
* Mononuclear cells from CB samples that were stored for the indicated time periods were used as responder or stimulator cells in mixed lymphocyte cultures as described in the "Materials and Methods" section.
† Stimulator cells were given 3,000R (γ) before culture. The response (Δcpm) to PHA for peripheral blood mononuclear cells from 2 adult donors tested on 18 different occasions during this series of experiments was 20.5 ± 2.42 × 10^3 (donor A) and 20.4 ± 2.41 × 10^3 (donor B).
‡ The number of individual CB samples that were tested.

6.71×10^3) was significantly lower ($P < .01$) than their responses on day 1 and day 7.

Fresh and stored CB cells also were tested for their ability to respond to allostimulation in MLCs. As given in Table 4, the ability of freshly obtained CB cells to respond in MLCs to each of the donor stimulator cells was comparable (mean Δcpm to donor A, $16.6 \pm 2.04 \times 10^3$; donor B, $15.8 \pm 2.06 \times 10^3$). For further analysis, therefore, the data from MLCs in which donor A and donor B were used as stimulator cells have been combined.

After storage at 4°C for 7 and 14 days, CB cells demonstrated responses to allogeneic PBMCs that were significantly lower than the response measured on day 1. The response on day 7 showed a mean Δcpm response of $8.5 \pm 1.62 \times 10^3$ ($P < .05$), and the mean Δcpm response on day 14 of storage was $3.0 \pm 1.79 \times 10^3$ ($P < .01$).

The effect of 4°C storage on the ability of CB cells to respond to freshly obtained, irradiated (γ) allogeneic CB cells also was tested in this series of experiments. As shown in Table 4, at the time of collection (day 1), CB responder cells showed a level of response to γ-allogeneic CB stimulator cells that was similar to their response to γ-allogeneic adult PBMCs. After 7 and 14 days of storage at 4°C, however, the response of those same CB responder cells to freshly obtained γ-allogeneic CB stimulator cells declined significantly. Overall, the results of these in vitro functional assays indicate that CB cells have the capacity to respond to mitogens and alloantigens, but these responses decline progressively and significantly over the 14-day period of storage at 4°C.

Leukocyte and Differential Counts

The number of stem cells in fresh CB is higher than in adult peripheral blood. However, after storage of CB samples at 4°C for 21 days, the absolute count of nucleated cells decreased significantly to only about one half of the day-1 value [Table 5]. The majority of cells that disappeared were polymorphonuclear leukocytes (58.6% on day 1 *vs* 3.5% on day 21, data not shown), while the mean percentage of lymphocytes increased significantly from 35% on day 1 to 82% on day 21 ($P < .001$). Therefore, the absolute number of lymphocytes in day-21 CB was not significantly different ($P > .05$) from that seen on day 1 (Table 5). Relative to the number at day 1, the number of $CD34^+$, $CD117^+$, and GPA-positive cells also increased proportionally in day 21-CB ($P < .01$, $P < .05$, and $P < .01$, respectively; Table 3).

Discussion

The enormous potential benefits of using umbilical cord blood cells for transplantation in lieu of bone marrow have been well-documented during the past decade.[2] However, some of the problems that remain to be completely addressed before CB transplantation becomes more widely used include the potential for HLA-mismatched CB cells to cause GVHD, the number of stem cells that can be obtained from a single CB sample for transplantation, and the efficacy of hematopoietic progenitor cells in CB cells that are cryopreserved for prolonged periods.

The results we present indicate that cells obtained from CB that is stored at 4°C for up to 3 weeks not only retain a significant level of hematopoietic efficacy (judged by CFU activity), but also, if judged by an absence of MLC reactivity, may show little or no capacity to cause GVHD. Our results strongly suggest the possibility that CB can be routinely stored under conditions that exist in any hospital blood bank, thus significantly expanding the number of patients who can benefit from the use of CB cells for hematopoietic transplantation.

Previous studies have demonstrated that human cord blood stored for 7 to 21 days at 4°C in gas-permeable bags produced significant survival of lethally irradiated mice.[4,5] In this xenogeneic mouse model, the human CB cells seem to serve as a transient bridge, giving the animal's own stem cells an opportunity (as well as stimulation) to repopulate the hematopoietic compartments.[3,6,14] This ability of CB to stimulate the host hematopoietic and immunologic systems could be useful for humans as an adjunct to radiation or chemotherapy.[6] Earlier results also indicated that blood bank–stored CB cells had a considerable ability to produce blast colonies.[4] However, it was unclear in these preliminary experiments whether there was a proportional increase in $CD34^+$ cells, $CD117^+$ cells, or both. The present flow cytometry results indicate that the percentages of the most immature hematopoietic progenitor cells in cord blood ($CD34^+$ and $CD117^+$) actually increase significantly, after storage at 4°C,while the more mature or lineage-committed cells (primarily granulocytes) tend to decrease. It is of interest to note that $CD34^+$/HLA-DR-positive cells, which is a more lineage-committed phenotype than $CD34^+$/HLA-DR-negative, remains proportionately the same. As shown in Table 5, the end result of cold storage for 3 weeks is that despite the loss of half of the WBCs present in CB samples, the absolute number of $CD34^+$ cells (based on percentage) almost doubles. It also is noteworthy that cells that express CD117, the receptor for SCF,[13] also increase after cold storage and that SCF has a critical role in the repopulation of hematopoietic progenitor cells in day-21 stored CB (Table 2). The number of nucleated RBCs, when compared with the number of leukocytes and the percentage of GPA-positive cells, also increased significantly. Nucleated RBCs on fresh CB averaged 8 and, on day 21, averaged 54 per 100 leukocytes. This further indicates that storage of CB in polyolefin gas-permeable bags at 4°C for 14 to 21 days is not deleterious to immature

Table 5
Quantitative Analysis of Subpopulations Present in Fresh and 21-Day Stored Cord Blood*

Variable Measured†	Day 1 (n = 63)	Day 21 (n = 63)	*P*
WBC, /μL (×10⁹/L)	12,890 ± 1,290 (12.9 ± 1.3)	6,210 ± 570 (6.2 ± 0.6)	<.001
Lymphocytes, %	35 ± 1.57 (0.35 ± 0.02)	83 ± 1.55 (0.83 ± 0.02)	<.001
Lymphocytes, /μL (×10⁹/L)	4,302 ± 240 (4.3 ± 0.2)	5,260 ± 360 (5.3 ± 0.4)	>.05
CD cells			
34⁺, %	1.02 ± 0.11	3.95 ± 2.82	<.01
34⁺, /μL (×10⁹/L)	131.48 (0.13)	245.30 (0.24)	—
117⁺, %	0.38 ± 0.12	0.84 ± 0.17	<.05
117⁺, /μL (×10⁹/L)	48.98 (0.05)	52.16 (0.05)	—

* Cells were obtained from cord blood samples that were stored for the indicated periods and were analyzed for total number of nucleated cells (WBC/μL) and the percentage of lymphocytes by morphologic criteria to calculate the absolute number of lymphocytes (n = 63). Data are given as the mean ± SE.
† The WBC count as reported by the automated hematology analyzer (Coulter STKS, Coulter, Hialeah, FL) also includes nucleated RBCs. By using the immunophenotype percentages obtained from Table 3 (n = 11), the absolute number of cell subpopulations was calculated.

cells. The replating efficiency of 10- and 21-day-old stored CB as shown in Table 2 is similar to previous results and appears to indicate a greater replating potential than the published data on frozen cord blood.[4] Therefore, there is a reasonable possibility that cord blood stored at 4°C may be an equally effective, if not a more effective, source of hematopoietic stem cells than fresh frozen stored CB for achieving successful marrow transplantation.

Although controversial, reactivity of donor cells to the recipient's alloantigens in MLCs has been used to predict the probability of GVHD in bone marrow transplant recipients.[15,16] Despite HLA mismatch between donor and recipient, the probability of complications due to GVHD is lower when CB is compared with adult bone marrow for transplantation. However, in summarizing the clinical experience with CB transplantation across HLA barriers, Gluckman et al[2] concluded that acute GVHD remains a substantial problem to be resolved. Our results demonstrate that the alloreactivity seen in fresh CB cells can be avoided by using a procedure that does not require depletion or enrichment of specific cell subpopulations. To what degree our observation can be further exploited to decrease the potential of CB cells to cause GVHD remains to be explored.

The cumulative results we present in Table 4 indicate that fresh CB cells have the capacity to mount a significant response to PHA and to histoincompatible stimulator cells. Our results are in contrast with those reported by Harris et al,[17] in which little or no reactivity to PHA or alloantigens by freshly isolated CB cells was seen. We cannot explain why our results differ in this regard; however, these discrepancies may be due to differences in selection of CB donors and/or methods of mononuclear cell isolation used in each study.

Nevertheless, despite the reactivity that was consistently seen in fresh CB cells in our experiments, the capacity of cells from the same CB donors to mount these responses declined significantly after storage for 7 days at 4°C, and after 14 days of storage, these responses were less than 20% of the day-1 responses. The reduction in MLC reactivity does not seem to be due to an overall loss of lymphocytes, since the percentage of cells scored as lymphocytes by morphologic criteria actually increased in CB that was stored at 4°C (35% on day 1 to 82% on day 21). It is possible that mature (ie, alloreactive) lymphocytes exhibit a differential sensitivity to prolonged storage at 4°C in comparison with their immature counterparts; however, this explanation remains to be studied. When CB cells were cocultured with allogeneic CB cells, there was essentially no reactivity (Δcpm = –0.4) after 14 days of cold storage.

Furthermore, CB cells that were stored for 14 days at 4°C also showed a significantly reduced ability to provide allostimulation to histoincompatible responder cells (data not shown). Considered together, these results suggest that it may be possible to combine CB cells from different donors after storing them at 4°C with less concern for their ability to cause GVHD after transplantation than now exists for HLA-mismatched fresh or fresh cryopreserved CB cells.

It has been well recognized that the number of colonies per milliliter that an individual cord blood sample can produce is quite variable,[18] and the quantity of CB obtained per donor also varies considerably. In addition to our own finding with MLCs, it also was shown that when 2 CB samples were mixed and incubated together, there was no evidence of inhibition of colony formation of either sample.[19] If CB samples can be combined without increasing the likelihood of untoward adverse effects, the possibility of greatly increasing the number of pluripotential progenitor cells given to an individual recipient exists. Although validation of the safety and efficacy of such a strategy is required, our results support the feasibility of conducting additional studies on combining CB samples for transplantation. This represents an important advantage of using CB cells, since the number of stem cells that are injected may be the most critical factor for successful engraftment when CB is used for transplantation in adults. Among many possibilities, it

also could provide adequate donor cells for certain ethnic groups within which suitable HLA-matched donors are difficult to find (such as groups affected by sickle cell anemia).[20]

Two of the most vexing ethical problems related to the use of CB for transplantation (particularly for allotransplantation) are to whom the CB sample belongs and what amount of CB can safely be obtained at the time of birth in relation to clamping of the umbilical cord.[21,22] Both of these ethical issues could be resolved readily if CB does not need be closely matched and if multiple donor samples can be combined. In addition, as was the practice for peripheral blood donation, the CB donor's family can be promised a similar specimen in the future, if the need arises.

Although the feasibility of using unmatched CB and fetal liver cells for transplantation has not received substantial support in the United States, there have been attempts in other countries to use these procedures with varying degrees of success.[23,24] In contrast, the use of HLA-matched fresh or cryopreserved umbilical CB is accepted widely in many countries but demands considerable financial support. The feasibility of developing countries being able to provide a similar degree of economic support is unlikely for many years. However, the possibility of using HLA-unmatched or partially matched umbilical CB that is blood bank–stored at 4°C has considerable appeal for these developing nations. In all likelihood, blood banks that already exist could readily incorporate these procedures into their existing structure.

Potentially, from our findings, CB could be stored for 10 or 14 days, pooled (after appropriate viral screening for pathogenic viruses), and then used immediately or frozen depending on the clinical and economic feasibility. This would allow the benefits of CB to be available to all patients who may need it, including children and adults, and also as an adjunct therapy for patients receiving high-dose chemotherapy, irradiation, or both.

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