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STEM CELL TRANSPLANTATION

Endogenous hematopoietic reconstitution induced by human umbilical cord blood cells in immunocompromised mice: Implications for adoptive therapy

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Human umbilical cord blood (HUCB) cells show promising advantages over bone marrow (BM) cells for a variety of diseases that require transplantation. We observed that lethally irradiated SJL/J mice given a single injection of HUCB cells survive, whereas vehicle-injected mice do not. Because survival is not due to long-term engraftment of HUCB cells, we used this HUCB/mouse model to investigate additional therapeutic benefits of HUCB cells. We investigated the mechanism by which HUCB cells accelerated endogenous hematopoiesis in mice that received either lethal (9.5 Gy) or lower-dose (8.0 Gy) radiation and then were given a single injection of HUCB mononuclear cells. Compared to irradiated control mice, the lethally irradiated, HUCB-injected group showed significant increases in peripheral white blood cell counts, red blood cell indices, and granulocyte-macrophage colony-forming units (CFU-GM) by 3 weeks. In contrast, no significant differences in these parameters were observed between control and HUCB-injected mice that received the lower dose of irradiation. Moreover, regardless of the radiation dose, only HUCB-injected mice exhibited immune responses comparable to those of age-matched normal mice. The clinical relevance of these observations was determined in long-term, culture-initiating cell assays with human BM stem cells and irradiated (γ-) HUCB cells. CFU-GM colonies were detectable in cultures containing γ-HUCB cells by day 15, but were undetectable in cultures without γ-HUCB cells until day 40, suggesting a hematopoietic stimulatory role for HUCB cells. Overall, the results indicate that in addition to their use for transplantation, HUCB cells also may be used as an adjuvant therapy to enhance hematopoietic reconstitution and immunocompetence of the host. This hematopoiesis-enhancing effect represents a heretofore unrecognized function of HUCB cells. **Published by Elsevier Science Inc.**

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Introduction

The relatively slow progress in the area of gene therapy for life-threatening diseases such as leukemia and other genetic disorders still makes bone marrow (BM) transplantation the treatment of choice. Unfortunately, BM transplantation is plagued with major clinical complications, especially the possibility of graft-versus-host disease (GVHD). Measures to prevent GVHD in BM transplant patients have been attempted by depletion of particular BM cell populations (e.g., T cells) and by transplantation of only BM stem cells. However, there are disadvantages with these strategies, such as the requirement for considerably large amounts of donor BM cells, graft failure, and recurrence of malignancy [1,2]. The clinical complications associated with BM transplantation imply a requirement for alternative strategies. Human umbilical cord blood (HUCB) is a rich source of hematopoietic stem cells and, compared to BM, it has more repopulation capabilities [3,4]. In fact, HUCB cells now are being widely used for transplantation in a variety of diseases, including patients undergoing therapy for hematologic disorders [5–7].

Successful engraftment of humn leukocyte antigen (HLA) mismatched HUCB cells has been reported, and in many cases is unaccompanied by symptoms attributable to GVHD [8]. Perhaps, this lack of requirement for an exact donor–recipient HLA match may be partly responsible for the successful use of HUCB for transplantation [9]. This may be partly explained by the immunologic immaturity and reduced functional properties of T-cells and other immune cells present in HUCB [10–14]. Another major advantage for use of HUCB cells is their low incidence of infection by cytomegalovirus and Epstein–Barr virus, both of which are associated with severe complications in BM

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transplantation [15,16]. However, despite the advantages of HUCB over BM cells for transplantation, there is some evidence which indicates that HUCB cells engraft slower than BM cells [17].

Ende et al. [18,19] previously reported that HUCB cells can increase the survival of lethally irradiated SJL/J mice compared to noninjected mice. Long-term engraftment of the HUCB cells did not appear to be responsible for survival, suggesting that other mechanisms were operative. Therefore, we investigated possible mechanisms by which HUCB cells might enhance endogenous hematopoietic reconstitution. In the present study, we took advantage of the fact that HUCB cells do not permanently engraft in the SJL/J mice, and used this model to explore other possible functions for HUCB cells. We specifically examined whether HUCB cells can enhance endogenous hematopoietic reconstitution by residual, radioresistant host BM cells in irradiated SJL/J mice. To do this, we measured peripheral blood indices and colony-forming activity during the first few weeks after irradiation. We also determined whether HUCB cells can function as an immune adjuvant by studying the responses of lymphoid cells obtained from irradiated, HUCB-injected mice to: 1) T-cell and B-cell polyclonal activators, 2) alloantigens, and 3) a syngeneic B-cell lymphoma that stimulates through a mouse mammary tumor viral-encoded superantigen, Mtv-29 (vSAg) [20]. Clinical relevance of the results obtained in the HUCB/mouse model also was addressed in this study, using a human stem cell assay.

Materials and methods

Cytokines and antibodies

Recombinant murine granulocyte-macrophage colony-stimulating factor (rMuGM-CSF) was kindly provided by the Immunology Department of Genetics Institute (Cambridge, MA). Murine monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (IgG1), murine monoclonal phycoerythrin (PE)-conjugated anti-human CD38 (IgG1), and PE- and FITC-conjugated isotype controls were purchased from Caltag Laboratories (Burlingame, CA). FITC-conjugated anti-CD45, CD3, and CD10 and PE-conjugated anti-CD19, CD14, and CD56 were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA).

HUCB and peripheral blood

Approximately 15–50 mL of HUCB was collected into citrate phosphate dextrose (Sigma, St. Louis, MO). Deliveries were routine, and subjects had no underlying disease or infection. Procedures for the collection and use of HUCB for this study were reviewed and approved by the Institutional Review Board of UMDNJ-New Jersey Medical School, Newark NJ. Mononuclear cells were separated by Ficoll Hypaque (Sigma) density gradient centrifugation within 24 hours of collection.

Peripheral blood was taken from healthy volunteers and placed into heparinized tubes. Informed consent was obtained from each donor according to the guidelines of the Institutional Review Board of UMDNJ-New Jersey Medical School. Peripheral blood mononuclear cells (HuPBL) were isolated by Ficoll Hypaque density gradient centrifugation.

Adoptive transfer

Female SJL/J mice, 6–8 weeks, were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed in the AAALAC-accredited Research Animal Facility at UMDNJ-New Jersey Medical School. Mice were depleted of natural killer (NK) cells by retro-orbital intravenous (IV) injection of 100-μL rabbit anti-asialo GM1 (Wako Pure Chemicals, Osaka, Japan). Two weeks after this injection, NK cells remain undetectable, based on a cytotoxicity assay that utilizes splenic effector cells and the NK-susceptible target cells, YAC-1 [18,21].

Twenty four hours later, mice were irradiated either lethally (9.5 Gy) or sublethally (8.0 Gy) by a cesium source (Mark I model 68-A-3 gamma irradiator, J.L. Shepherd, San Fernando, CA). After 1–2 hours, mice were injected IV with either 10^7 HUCB mononuclear cells or 10^7 HuPBL, resuspended in phosphate-buffered saline. Control mice were injected with a comparable volume of phosphate-buffered saline (vehicle control). Mice were housed in a laminar flow environment in sterile cages with sterile bedding, food, and water. At various times thereafter, mice were analyzed for routine peripheral blood indices (RBC [red blood cell] and white blood cell [WBC] counts, hematocrit, hemoglobulin), lymphocyte functional assays, and granulocyte-macrophage colony-forming units (CFU-GM) in BM and spleen.

Immunofluorescence assays

The phenotypic profile for the expression of CD34, CD38, CD45, CD3, CD19, CD14, CD10, and CD56 in HUCB mononuclear cells was determined in random samples. Cells were labeled for 30 minutes at 4°C with specific FITC- or PE-conjugated antibodies. After labeling, cells were washed to remove unbound antibodies, fixed by resuspending in 1% paraformaldehyde, and then analyzed by flow cytometry (Becton Dickinson).

Clonogenic assays

Single cell suspensions from mice were prepared from either the femurs or spleens and then used in clonogenic assays for CFU-GM as previously described [22]. Briefly, cells were resuspended in culture medium and then plated in duplicate in methylcellulose at 10^5/plate in a total volume of 1 mL. Due to the low numbers of cells recovered in non-HUCB–injected, lethally irradiated mice, for these cultures, the total numbers of recovered cells from two femurs (up to 3×10^5) were plated in a single dish. Cultures were supplemented with 4 U of rMuGM-CSF. Colonies ≥20 cells were enumerated at day 10 of culture.

Lymphocyte responses

A single cell suspension of responder (R) cells was prepared from the lymph nodes or spleen of the HUCB-injected or non-injected SJL/J mice. Responder cells were cultured with stimulator (S) cells obtained from either syngeneic γ-irradiated (7.5 Gy) B-lymphoma cells expressing vSAg (R/S = 4), γ-irradiated (2.5 Gy) allogeneic ($H\text{-}2^b$) spleen cells (R/S = 1), or the following mitogens: concanavalin A (Con A) at 1 μg/mL; lipopolysaccharide (LPS) at 5 μg/mL. Both mitogens were purchased from Sigma. Cultures stimulated with γ-lymphoma cells or γ-allogeneic cells were incubated for a total of 96 hours, and those stimulated with mitogens were incubated for a total of 72 hours. Cell proliferation was based on the incorporation of 1 μCi tritiated thymidine (^{3}H-TdR; 1 μCi/mL; specific activ-

ity 1.9 Ci/mM; Amersham Life Sciences Inc., Arlington Height, IL). ^{3}H-TdR incorporation was determined by harvesting cells onto glass fiber filters with an automated harvester (Cambridge Technology Inc., Cambridge, MA). For each experiment, stimulation was performed in triplicate. The background counts per minute (cpm) (responder cells alone or responder + stimulator cells alone) was subtracted from the cpm of stimulated cultures (Δcpm).

BM stroma

BM aspirates were obtained from the posterior iliac crest of normal healthy volunteers. Samples were immediately placed into Iscove's medium (Life Technologies, Grand Island, NY) containing 50 U/mL preservative-free heparin. Informed consent was obtained from each donor according to the guidelines of the Institutional Review Board of UMDNJ-New Jersey Medical School.

BM aspirate cells (4×10^6) were cultured in 12-well plates (Corning Costar, Cambridge, MA) in 2 mL of "stromal" medium consisting alpha minimal essential media (α-MEM) (Life Technologies) containing 12.5% FCS (Hyclone Laboratories, Logan, UT), 12.5% horse serum (Hyclone Laboratories), 10^{-7}M hydrocortisone (Sigma), 10^{-4}M 2-ME (Sigma), and 1.6 mM glutamine (Cellgro, Mediatech). Cultures were incubated for 3 days at 33°C, after which the mononuclear cells (BMNC) were separated from the nonadherent population by Ficoll Hypaque density gradient centrifugation. BMNC were replated into culture plates, which were reincubated with weekly 50% change of medium until confluency occurred.

Long-term culture-initiating cell assay

Confluent BM stroma cultures prepared in 12-well plates were irradiated with 150 Gy delivered by a cesium source (Mark 1 Model 68-A-3). After 24 hours, nonadherent cells were replaced with fresh media containing quiescent BM mononuclear cells (1–10/well). Parallel cultures consisted of wells with 10^5 γ-irradiated (100 Gy) HUCB cells. This radiation dose was established in long-term culture-initiating cell (LTC-IC) assays with HUCB cells that were subjected to various doses of radiation (30–150 Gy). HUCB cells that were given <100 Gy proliferated in culture. During the culture period, 50% stromal medium was replaced weekly. At various time periods, cells from each well were trypsinized and cultured in duplicate in short-term clonogenic assays with rHuGM-CSF.

Quiescent BM mononuclear cells were prepared by incubating cells with 5-fluorouracil (5-FU; Hoffman La Roche Inc., Nutley, NJ). This drug preferentially kill cells in cycling phase, whereas the quiescent population remains viable [23]. Cells (10^7) were resuspended in 5 mL α-MEM containing 20% FCS and 200 μg/mL 5-FU for 7–10 days. The cycling state of the cells was determined by pulsing 10^5 cells with 1 μCi [^{3}H]TdR (35 Ci/mM; ICN Biomedicals Inc., Irvine, CA) for 24 hours. [^{3}H]TdR incorporation was determined as described for lymphocyte responses. By day 7 of 5-FU treatment, the cpm showed a plateau at 215 ± 24.

Statistical analysis

Data were analyzed using the Student's *t*-test to determine the significance (*p* value) between experimental and control values.

Results

It was shown previously that a majority of lethally irradiated, HUCB-injected mice survive for a significant period beyond the time (2–3 wks) when non-HUCB–injected mice died [18,24]. Indeed, in these studies, 40% of HUCB-injected mice survived until 180 days compared to 0% survival in irradiated non-HUCB–injected mice. Because evidence of permanent engraftment of HUCB cells was not obtained in these mice, it appeared that endogenous hematopoietic repopulation was responsible for the long-term survival. We therefore addressed the mechanism by which such endogenous reconstitution might occur in HUCB-injected mice.

Phenotypic and localization properties of injected HUCB cells

The phenotypic profile within the samples of HUCB mononuclear cells used in the study was determined in seven randomly selected samples. We measured the distribution of progenitor/stem cells (CD34), more matured progenitors (CD34/CD38), T-cell (CD3), B-cell (CD19), Thy1 (CD45), and NK cells (CD56). The phenotypic distribution within the mononuclear fractions used in our studies (Table 1) was consistent with published reports of other investigators [25].

NK cells can affect hematopoietic activity of HUCB cells [26]. However, in this study, because we did not attempt to engraft HUCB cells in the mice, the significance of the 4% NK cells within the HUCB cells was not a concern for this particular HUCB/mouse model. Furthermore, the human NK cells would be irrelevant to the long-term endogenous hematopoietic reconstitution in the mice, because the repopulating cells are not of human origin [18,24]. However, we previously observed that recipient murine NK cells can affect endogenous hematopoietic reconstitution in HUCB-injected mice [24]. Therefore, recipient mice were depleted of NK cells by a single injection of polyclonal asialo GM1 antiserum [18,21] prior to transfer of HUCB cells.

We next determined the initial anatomic localization of the HUCB cells following IV injection. ^{51}Cr-Labeled HUCB cells were injected into mice that had received 9.5 Gy. On days 1 and 2, groups of animals were sacrificed, and the radioactivity in various tissues was determined as a percentage of the injected cpm (data not shown). Control irradiated

Table 1. Phenotypic distribution in cord blood mononuclear cells

	% of HUCB mononuclear cells (n = 7, ±SE)
CD34	2 ± 0.6
CD38	19 ± 5.6
CD34/CD38	3 ± 1.8
CD45	16 ± 5.6
CD3	9 ± 2.8
CD19	6 ± 1.9
CD10	2 ± 0.7
CD14	1.2 ± 0.4
CD56	3.6 ± 0.4

Human umbilical cord blood (HUCB) mononuclear cells were labeled with either fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies. The precentages of labeled cells were determined by FACScan.

mice received syngeneic ^{51}Cr-labeled BM cells. Following IV injection of HUCB cells, the highest percentage of injected cpm (50%) was found in the liver on day 1, and this percentage was essentially unchanged on day 2. When syngeneic BM cells were injected, high counts (15%) also were found in the liver, but by day 2, this dropped to about 9% of injected cpm. Differences between HUCB-injected and syngeneic BM-injected recipients also were noted on day 1 for spleen (2.5% vs. 7.2%), BM (0.14% vs. 0.5%), and lung (0.07% vs. 0.7%). However, by day 2, the differences between these groups in these organs decreased.

Effects on hematopoietic activity by HUCB in lethally irradiated mice

Due to the radioresistance of NK cells and their influence on hematopoiesis [27,28], mice were injected with anti-NK antibody 24 hours prior to administration of lethal radiation (9.5 Gy). This was followed by injection with either HUCB mononuclear cells or vehicle. At various time periods up to 3 weeks, peripheral blood indices were determined at selected intervals. In both HUCB-injected and vehicle-injected mice, there was a precipitous drop in the WBC count to a nadir on day 10 (Fig. 1A). After day 10, however, HUCB-injected mice showed accelerated return of WBC in comparison to noninjected mice. Indeed, by days 15–18, the peripheral blood WBC counts were 2,200/μL in HUCB-injected mice compared to only 500/μL in noninjected mice (Fig. 1A). The WBC counts in normal age-matched controls were 8,800 ± 600/μL (n = 26; ± SE). In contrast, erythropoietic-related peripheral blood cell parameters exhibited little, if any, decreases at day 5 in comparison to the levels observed in age-matched controls (n = 26, ± SE; hematocrit: 32 ± 1%; hemoglobulin: 11.6 ± 0.4g/dL; RBC: 7 ± 0.2 × 10^6/μL) (Fig. 2). There were decreases in these parameters in both groups of mice up to day 14. Beyond day 14, however, although the RBC indices continued to fall in the non-HUCB–injected mice, these values increased to normal levels in mice that received HUCB cells.

We next studied myelopoiesis in the spleen and BM from both groups of mice (HUCB-injected and noninjected) using clonogenic cultures that contained 10^5 cells/plate. Based on the localization patterns, we cultured cells that were obtained from the liver, spleen, and BM. In BM and spleen, there was an increase in CFU-GM at day 10 that continued through days 15–18 (Fig. 1B). By this time, the numbers of CFU/10^5 cells (60 and 40 in BM and spleen, respectively), were approaching the levels measured in tissues from normal age-matched control SJL/J mice (68 ± 4 for BM and 30 ± 6 for spleen, ± SE; n = 9). At none of these time intervals, however, was CFU-GM detected in cells taken from the livers of these mice. CFU-GM also were undetectable at these times in BM or spleens of vehicle-injected mice, despite the plating of up to 3 × 10^5 cells per culture.

We ruled out the possibility that the injected HUCB cells might contribute to the number of CFU-GM measured in the clonogenic assays. HUCB cells were used for CFU-GM assays in which rMuGM-CSF was added. No colony growth occurred when HUCB cells were included with rMuGM-CSF, but they responded well to rhuGM-CSF in these assays (data not shown). Therefore, it is unlikely that the injected HUCB are a direct source of any of the CFU-GM seen in cells obtained from the mice in these experiments. Overall, these results indicate that HUCB cells are clearly involved in the process of hematopoietic recovery observed in the γ-irradiated mice. Thus, it appears that this hematopoiesis-enhancing effect (HEE) of HUCB cells influences the ability of surviving murine stem cells to begin endogenous repopulation.

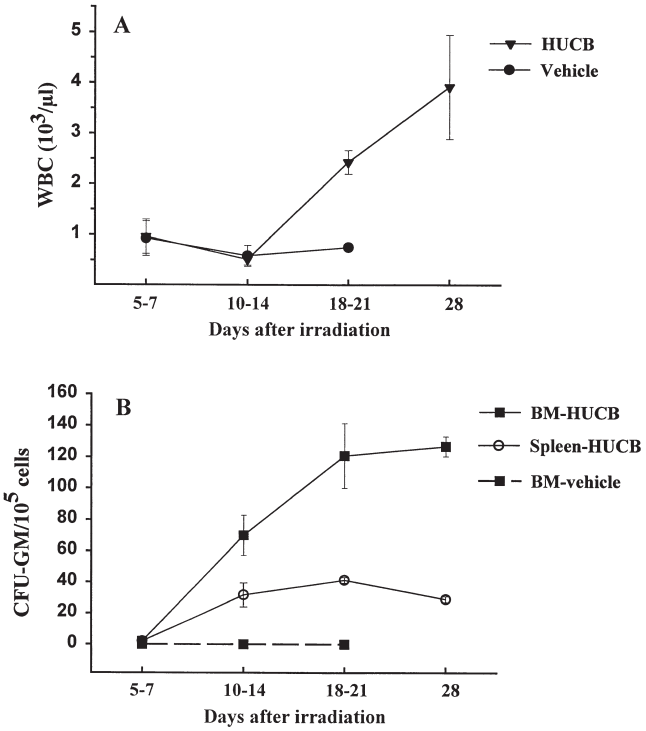

Figure 1. White blood cell (WBC) counts in peripheral blood and CFU-GM in the bone marrow (BM) and spleen of HUCB-injected mice. NK cell-depleted SJL/J mice were lethally irradiated and then injected with either HUCB cells or vehicle. At various times, either peripheral blood WBC counts were determined **(A)** or mice were sacrificed and the number of CFU-GM in the BM and spleen was determined in clonogenic assays **(B)** as described in Materials and Methods. The number of CFU-GM colonies in the BM and spleen of normal, age-matched mice was 68 ± 4 and 30 ± 6, respectively (n = 9; ± SE).

Hematopoietic activity in lower-dose, γ-irradiated mice

We next determined if the period during which HUCB potentiates hematopoietic recovery could be shortened in mice that received a lower dose of radiation. Our rationale here was that, at a lower dose, the surviving murine hematopoietic cells would include totipotent and multipotent cells compared to only totipotent cells remaining in lethally irradiated mice. Thus, following HUCB cell injection, the recovery period should be shorter. A secondary reason for using a lower

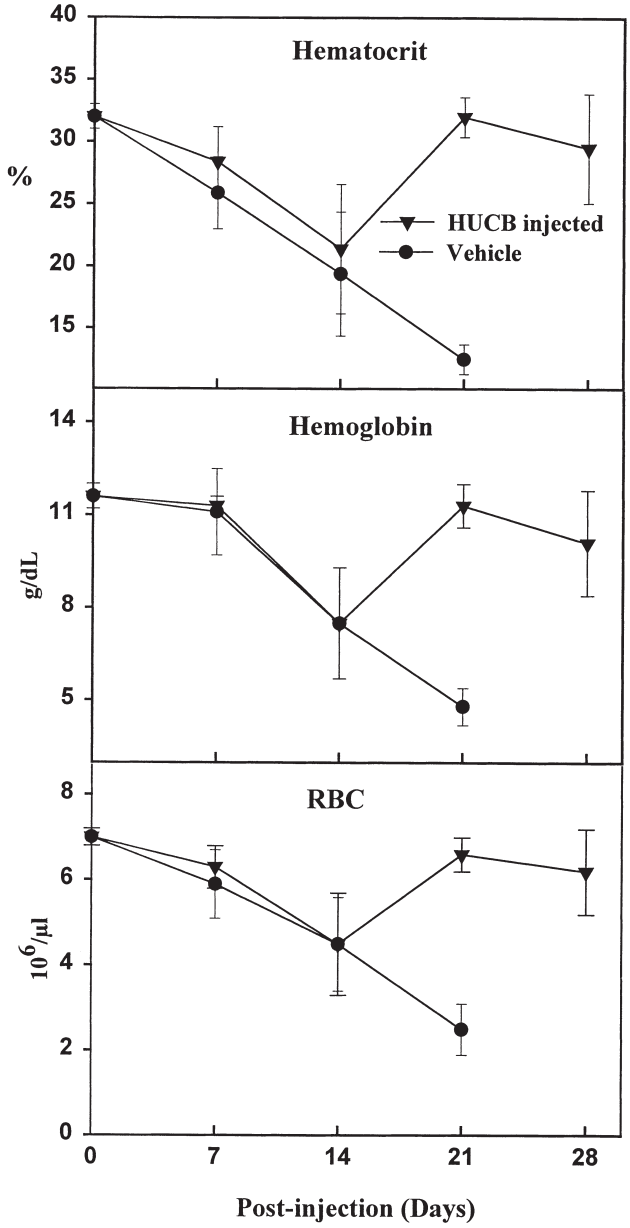

Figure 2. Erythropoietic indices in peripheral blood of HUCB-injected mice. NK cell-depleted SJL/J mice were lethally irradiated and then injected with either HUCB cells or vehicle. At various times, a sample of peripheral blood was obtained for determination of red blood cell (RBC) counts and hemoglobulin and hematocrit levels.

dose was to be able to analyze the noninjected controls for a longer period of time, because at 9.5 Gy, practically all of the vehicle-injected mice died between 2 and 3 weeks after irradiation. For these experiments, NK-depleted mice were irradiated with 8.0 Gy and then injected IV with 10^7 HUCB cells. At various time periods, WBC levels and CFU-GM in BM and spleen were determined. Although WBC levels and CFU-GM in HUCB-injected mice were greater than in vehicle-injected animals, the differences were not statistically significant ($p > 0.5$) (Figs. 3A and 3B). These results indicate that with a lower dose of radiation, the degree of endogenous reconstitution determined by the levels of hematopoietic progenitors and differentiated cells are comparable for mice that were injected with either HUCB cells or vehicle.

Recovery of lymphocyte function in lethally irradiated, HUCB-injected mice

Prompted by the accelerated recovery of WBC and CFU-GM seen in lethally irradiated mice injected with HUCB cells, we also examined the recovery of lymphocyte function as measured by the ability of spleen cells to mount in vitro proliferative responses to murine alloantigens.

Three weeks after lethal irradiation and injection with either syngeneic BM or HUCB cells, SJL (H-2^s) splenic responder cells were stimulated in mixed lymphocyte reaction with irradiated Balb.B (H-2^b) or Balb/c (H-2^d) stimulator cells. As shown in Figure 4, spleen cells from HUCB-injected mice proliferated in response to alloantigens to a similar degree as spleen cells taken from syngeneic BM-injected mice. None of the mice that received 9.5-Gy irradiation alone survived for 3 weeks in this series of experiments. These results demonstrate that injection of HUCB cells into lethally irradiated mice also influences lymphopoiesis, causing a recovery of mature, alloantigen-responsive lymphocytes within a time period similar to that observed for erythroid and myeloid compartments (Figs. 1A, 1B, and 2).

Recovery of lymphocyte function in mice given a lower dose of radiation

In mice irradiated with 8.0 Gy, because no significant differences were observed in the myelopoietic compartment between the HUCB-injected mice irradiated with the lower dose and the noninjected controls (Figs. 3A and 3B), we determined if HUCB cells can influence their immunocompetence levels. The data presented in Figure 5 indicate that, 3 weeks after administration of 8.0 Gy, responses to the polyclonal lymphocyte activators Con A (T-cell) and LPS (B-cell) are more prominent in irradiated mice that received HUCB cells than in mice that were irradiated but not injected with HUCB cells. At 9 weeks after irradiation, T-cell responses to Con A were comparable in both groups of mice, but B-cell responses to LPS in the HUCB-injected mice were closer to the response of age-matched, unirradiated controls than in mice that only received irradiation (Fig. 5).

The proliferative response of cells from these same mice to a syngeneic B-cell lymphoma was measured (Fig. 6). Tsiagbe et al. [29] showed that the response of SJL lymphoid cells to these lymphoma cells is stimulated by the expression of a mammary tumor provirus (Mtv)-encoded superantigen (vSAg) on the tumor cells (Mtv-29). Furthermore, the Mtv-29 vSAg stimulates T-helper (TH) cells that use a specific β-chain (Vβ16) in their T-cell receptor (TCR) [30]. The data in Figure 6 show that the presence of tumor-responsive Vβ16$^+$ TH cells is comparably low in both

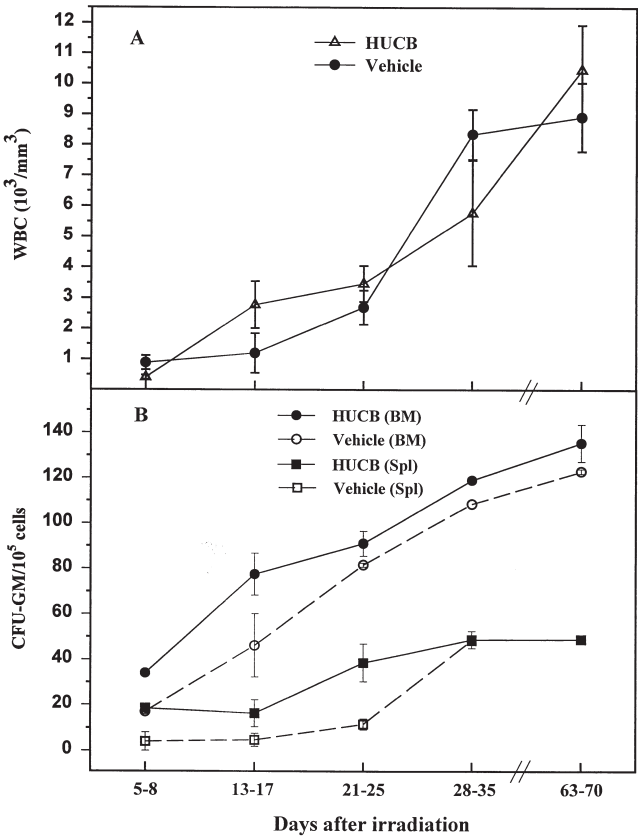

Figure 3. CFU-GM and white blood cell (WBC) counts in peripheral blood in HUCB-injected mice that received 8.0 Gy irradiation. NK cell-depleted SJL/J mice were irradiated with 8.0 Gy and then injected with either HUCB cells or vehicle. At various time periods, mice were either **(A)** bled for WBC counts or **(B)** sacrificed for CFU-GM determination in the spleen and BM.

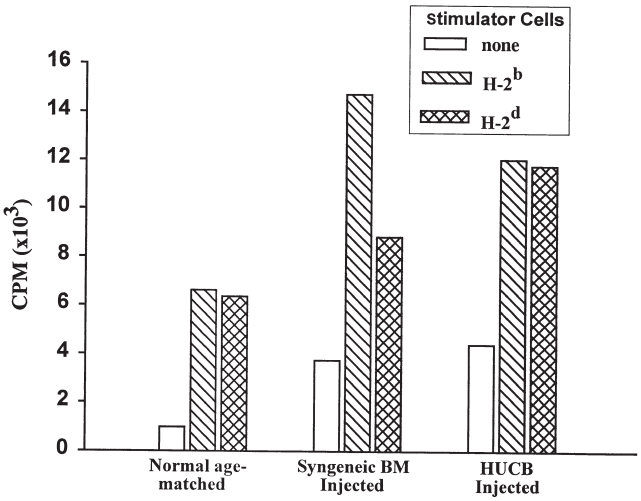

Figure 4. Allogeneic responses by lymphoid cells from HUCB-injected, lethally irradiated SJL/J mice. NK-depleted SJL/J mice (H-2^s) were lethally irradiated and then injected with either HUCB mononuclear cells or syngeneic BM. At day 17 after injection, lymph node cells from these mice were stimulated with γ-irradiated allogeneic spleen cells obtained from either Balb/c (H-2^d) or Balb.B (H-2^b) mice. The responses of lymph node cells obtained from age-matched, unirradiated control mice are shown for comparison. Cell proliferation, shown on the Y-axis as CPM × 10^3, was based on the amount of [^{3}H]TdR incorporated during the last 16–18 hours of a 96-hour incubation. Details of the procedure are described in the Materials and methods.

groups of mice at 3 weeks after radiation. At 9 weeks, however, the Vβ16$^+$ TH cell response to syngeneic lymphoma cells is significantly reconstituted only in the irradiated mice that received HUCB cells, although the response was not the same as that of age-matched normal control mice.

Overall, these results suggest that the ability to mount polyclonal T- and B-cell responses is reconstituted earlier in 8.0-Gy irradiated mice that receive HUCB cells. This is in keeping with the results seen at this dose with the recovery of cells of other hematopoietic lineage (i.e., erythroid and myeloid). However, the results using more specific stimuli, such as the ability to mount a TCR Vβ-restricted TH cell response to Mtv-vSAg, suggest that the return of selected, antigen-specific lymphocyte subsets proceeds more rapidly in irradiated mice that also receive HUCB cells. To what degree this is true for other populations of antigen-specific lymphocytes remains to be fully determined.

Hematopoietic recovery of lethally irradiated mice injected with HUCB or HuPBL

Although the rescue of lethally irradiated mice by injection of HUCB cells demonstrated the biologic relevance of the model, we could not exclude the possibility that the HEE activity exerted by HUCB cells was due to a "xenogeneic" effect. Therefore, we designed the next set of experiments to determine if another source of human cells also could exhibit HEE. For these experiments, we compared hematopoietic recovery in lethally irradiated mice injected with either HUCB or HuPBL. In three different experiments, we observed that the cellularity of BM from mice injected with HUCB was significantly higher than in mice injected with HuPBL (Table 2). Also, compared to HuPBL injection, HUCB injection resulted in 3- and 7- fold more CFU-GM in the spleen and BM, respectively (Table 2). Comparison of peripheral blood indices in the two groups of mice indicated twice the number of WBC in mice injected with HUCB over those injected with HuPBL. However, we observed no differences in erythroid indices in mice injected with either HUCB or HuPBL (data not shown). These results suggest that although HUCB cells exhibit a greater enhancing effect than HuPBL in this mouse model, HEE may not be a unique property of HUCB cells.

Effects of irradiated HUCB cells in LTC-IC cultures

The next series of experiments was designed to determine if the ability of HUCB cells to stimulate hematopoiesis in the xenogeneic murine model also could be demonstrated with allogeneic human hematopoietic stem cells, using the LTC-IC

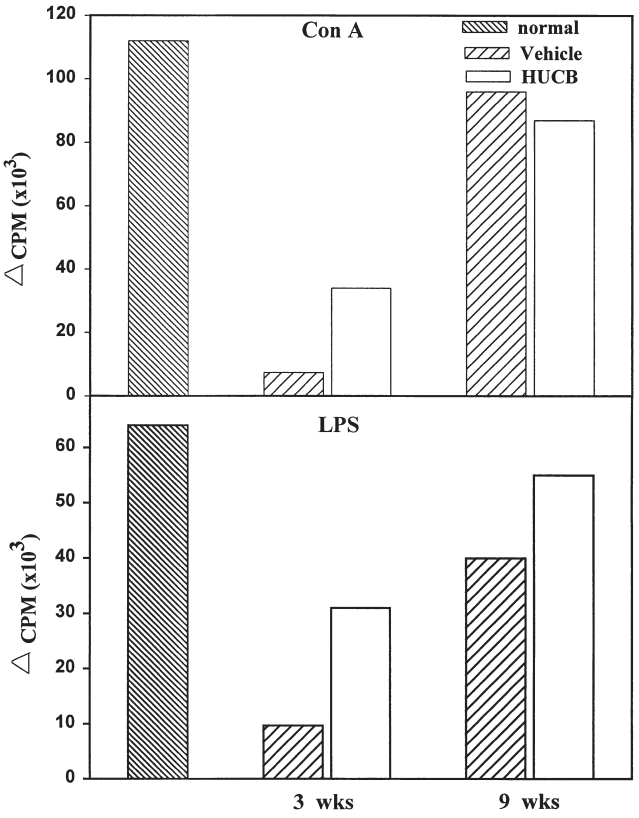

Figure 5. Proliferative responses to T- and B-cell mitogens by splenocytes from HUCB-injected mice given a lower dose of radiation. NK cell-depleted SJL/J mice were irradiated with 8.0 Gy and then injected with either HUCB cells or vehicle. At 3 and 9 weeks after injection, splenocytes were cultured with either concanavalin A (Con A) or lipopolysaccharide (LPS) for 72 hours. During the final 16–18 hours, cell proliferation was determined by [^{3}H]TdR incorporation. The ΔCPM are given on the Y-axis. Details of the procedure are described in the Materials and methods.

assay. Modified LTC-IC assays were performed with quiescent (5-FU–resistant) human BM cells in the presence or absence of γ-HUCB mononuclear cells. Beginning on day 10, cells from each well were trypsinized and the number of CFU-GM was determined in short-term clonogenic assays. As shown in Figure 7, the presence of γ-HUCB cells considerably shortened the period by which the quiescent human stem cells developed hematopoietic colonies. CFU-GM were detected by day 15 in γ-HUCB-containing cultures and maximal CFU-GM were observed at day 30. By contrast, in control cultures without HUCB, CFU-GM were not detected until day 40. Furthermore, it took twice as long (60 days) for the control cultures to reach maximal levels of colony formation compared to the time observed for HUCB-containing cultures. No CFU-GM were observed in parallel cultures with γ-HUCB cells alone. These results show that HUCB cells also can exhibit HEE on human hematopoietic stem cells by a mechanism that does not require their own proliferation and suggest the potential clinical benefits of using HUCB cells therapeutically.

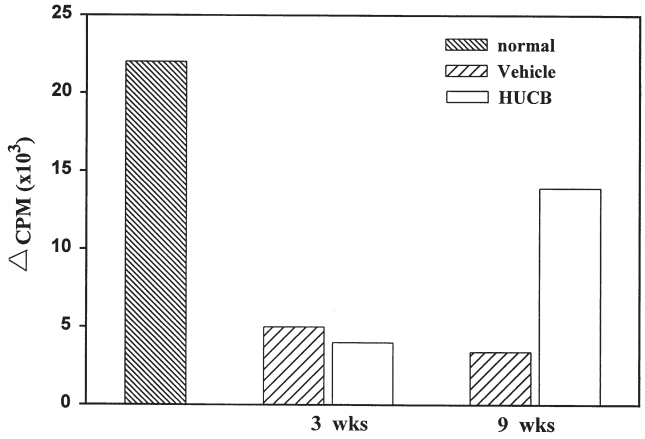

Figure 6. Proliferative responses to mouse mammary tumor virus superantigen by lymphoid cells from HUCB-injected SJL/J mice given a lower dose of radiation. Lymph node cells were obtained from mice that were irradiated with 8.0 Gy and then injected with either HUCB cells or vehicle. Cells were cultured with vSAg-expressing, γ-irradiated RCS tumor cells for 96 hours. During the final 16–18 hours, cell proliferation was determined by [^{3}H]TdR incorporation. The ΔCPM values are given on the Y-axis. Details of the procedure are described in the Materials and methods.

Discussion

HUCB cell therapy rescues mice from irradiation death [18]. It appears that most of the injected HUCB cells initially localize to the liver, although the significance for this localization has yet to be determined. The data indicate that HUCB cells mediate the recovery of the endogenous hematopoietic and immunologic systems in NK-depleted, lethally irradiated SJL/J mice. Therefore, this mouse model is a potentially useful experimental system to study a heretofore unrecognized property (i.e., HEE) of HUCB cells in clinical application. Although the HUCB cells injected into

Table 2. Hematopoietic recovery in lethally irradiated mice injected with either HUCB or HuPBL

	Number of cells × 10^6		CFU-GM/10^5 cells	
Group	BM*	Spleen	BM*	Spleen*
HUCB (n = 7)	3.4 ± 0.6	67 ± 17	61 ± 6.6	39 ± 4.1
HuPBL (n = 3)	0.9 ± 0.2	42 ± 16	9 ± 2.2	11 ± 1.9

Anti-asialo GM1-treated SJL/J mice were lethally irradiated (9.5 Gy) and then injected with either vehicle, 10^7 HuPBL or 10^7 HUCB. At day 17–22, the cellularity and CFU-GM were determined in the BM and spleen of mice. CFU-GM/10^5 cells in nonirradiated mice were 132 and 42 in BM and spleen, respectively. None of the lethally irradiated, vehicle-injected mice survived to day 17. Cellularity is expressed as the total number of viable nucleated cells in either the spleen or one femur. Techniques are described in the Materials and methods section.

*For these values, there was a significant difference ($p < 0.01$) between mice injected with HUCB and mice injected with HuPBL.

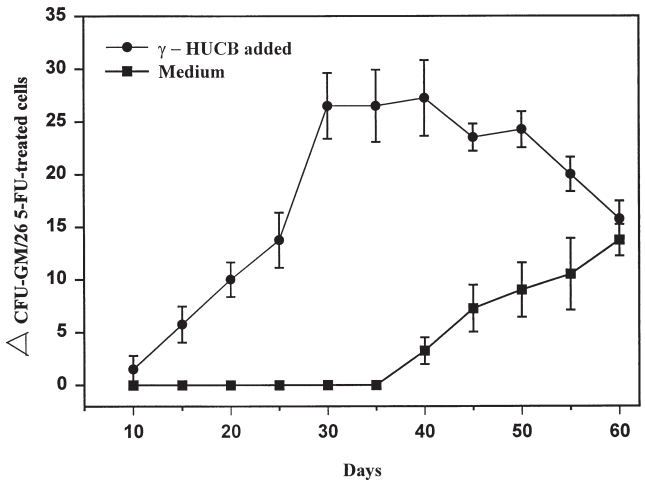

Figure 7. Effect of HUCB cells on 5-fluorouracil (5-FU)–treated BM mononuclear cells in LTC-IC assays. Human BM stromal cells were irradiated (150 Gy) 1 day prior to co-culture with 5-FU–treated BM mononuclear cells (10, 8, 5, 2, 1/well) with or without γ-irradiated (100 Gy) HUCB mononuclear cells. At various time periods, cells from each well were trypsinized and analyzed for CFU-GM in short-term clonogenic assays. The change (Δ) in CFU-GM is given at each time point as the mean (±SD) of four different experiments. In each of the four experiments, cultures were performed in duplicate. At each time period, the number of colonies obtained for cultures at each of the five cell densities was totaled and the numbers of CFU-GM/26 5FU-cells are given on the Y-axis. CFU-GM were not detected in cultures containing only γ-irradiated HUCB mononuclear cells. Details of the procedure are described in the Materials and methods.

lethally irradiated SJL/J mice may initially survive and provide transient protection from acute radiation damage, long-term engraftment of HUCB cells in these mice is unlikely [18,19,24]. Nonetheless, our results using the SJL/J model show that HUCB cells can provide significant benefits for enhanced hematopoietic reconstitution by endogenous stem cells in lethally irradiated mice (Figs. 1A, 1B, 3A, and 3B).

However, it appears that HEE may not be a property unique to HUCB cells, because similar activity (albeit at a significantly reduced level) also was seen when HuPBL were injected into lethally irradiated SJL/J mice. Therefore, a current focus of our ongoing effort is to determine if hematopoietic cells from other sources (e.g., human BM) exhibit HEE and to identify the phenotype of the cell that mediates this function. A likely candidate may be the $CD34^+$ stem cell itself, or one of its early descendants. To what degree the HEE observed in our experiments with HUCB is similar to the graft "facilitating cell" described in BM [31,32] remains to be explored.

Although HEE was seen after injection of HUCB cells into lethally irradiated mice, there was not a significant effect on hematopoiesis following injection of HUCB cells into mice that received a lower dose (8.0 Gy) of irradiation. There are two possible explanations for these results. First, there may be survival of a sufficient number of stem/progenitor cells in the mice that received the lower irradiation dose to initiate endogenous hematopoiesis without a need for exogenous stimulation. Second, although not tested, it is possible that the 8.0-Gy–irradiated, HUCB-injected mice can mount an immune response that destroys the HUCB cells before they can fully perform their HEE function. Moreover, these two mechanisms are not mutually exclusive.

Our results also show that HUCB cells exhibit an adjuvant-like activity for reconstitution of selected immune responses (Figs. 4–6). Especially significant is the enhancement of antigen-specific responses by mice that received HUCB cells (Fig. 6). These latter results are important because, in addition to the advantages of HUCB cells over BM cells for transplantation, our results suggest additional clinical benefits of using HUCB cells. The adjuvant-like functions of HUCB cells suggest that they can potentially be used in situations where immune stimulation may be necessary, such as in patients with cancer or infectious disease. Furthermore, with regard to application in humans, the immunologic adjuvant property of HUCB gives these cells a dual role, because they can simultaneously engraft and diminish the immunosuppression that can lead to secondary opportunistic infections.

Cell surface markers on HUCB cells may partially explain the combined hematopoietic and immune adjuvant effects observed in this study. Compared to BM, MHC class II molecules are more densely expressed on HUCB stem cells [33]. Recent studies indicate that MHC class II is involved in autologous hematopoietic reconstitution in sublethally irradiated dogs [34]. This suggests that part of the hematopoietic effects observed by HUCB cells could be attributed to the high expression of MHC class II molecules on their stem cells. In addition, CD10 expression on HUCB cells also might be important, because this cell surface marker has an endogenous endopeptidase activity that can utilize, as its substrate, several peptides that are relevant to hematopoiesis [35–37].

In vitro, irradiated (γ-) HUCB cells enhance the proliferation of human stem cells (Fig. 7). Despite their inability to proliferate, this effect could be mediated by the release of early acting cytokines by the γ-HUCB cells. However, Sautois et al. [38] showed that HUCB cells do not exhibit a dramatic difference in their ability to produce relevant hematopoietic cytokines when compared to peripheral blood mononuclear cells. Therefore, it is more likely that the γ-HUCB cells stimulate the BM stroma in these LTC-IC assays to produce cytokines that, in turn, are capable of upregulating stem cell activity. The mechanisms by which this stem cell activation occurs is a current focus of our ongoing investigation, because the interaction between HUCB and stromal cells appears important to both the immune adjuvant as well as the HEEs observed in HUCB-injected mice.

In addition to inducing endogenous stem cell proliferation (Fig. 7), injection of HUCB cells also leads to reconstitution of differentiated hematopoietic cells as judged by the reappearance of WBC in the peripheral blood of lethally ir-

radiated mice (Fig. 1A). This suggests that the presence of HUCB cells not only influences the induction of stem cell proliferation, but also their differentiation. In fact, this is supported by the results of the LTC-IC cultures, where we observed both an accelerated appearance and increased quantity of CFU-GM (Fig. 7) in cultures containing γ-HUCB cells. The reason for the decrease at later time periods in the number of CFU-GM in cultures with γ-HUCB is not readily apparent, but could be due to cell death. Trypan blue dye exclusion indicated good viability of cells in these cultures; however, a time-dependent induction of apoptosis cannot be excluded at this time. This area of investigation currently is being addressed and will provide further insight into the mechanisms of HUCB-mediated endogenous, hematopoietic reconstitution.

The results of our study can be significantly useful for application to the current problems associated with the continued use of allogeneic BM transplantation for treatment of many hematopoietic disorders [39]. Complications associated with GVHD continue to be a medical challenge. Although depletion of T-cells from the donor BM has reduced the incidence of GVHD, loss of this cell population can have negative effects on engraftment, such as an increased rate of recurrence of disease [40,41]. Our results suggest that HUCB cells could very well be replacing the engraftment benefits of having T-cells present, but without their deleterious effects of potentiating GVHD. Associated with a lower risk of developing GVHD appears to be a less stringent need for exact donor–recipient HLA matching when HUCB is used for transplantation [42], benefits that have been attributed to the immaturity of HUCB cells [42].

Our results suggest yet another unrecognized benefit of using HUCB cells in lieu of BM for such patients, namely, the ability of HUCB cells to stimulate endogenous hematopoietic repopulation. Because the HUCB cells do not show long-term engraftment in the mouse model, we were able to focus our analysis on this HEE function of HUCB, exclusively. However, in situations where HUCB transplants have been performed in human patients, engraftment of the transplanted cells would actually mask the hematopoiesis-enhancing function of the transplanted HUCB cells. Indeed, because care usually is taken to match the donor–recipient for HLA loci, the hematopoiesis-enhancing function of HUCB would be difficult to measure and would go largely unnoticed.

In summary, the data presented in this study indicate that HUCB cells facilitate the ability of radioresistant endogenous stem cells to reconstitute the hematopoietic and immunologic systems of lethally irradiated SJL/J mice. Although the mechanisms by which this occurs have yet to be determined fully, the data suggest that HUCB can be used in novel treatment regimens to stimulate endogenous hematopoietic repopulation in patients who currently require BM transplantation. These results also suggest that HUCB may be of potential therapeutic value for immune stimulation. If, as our data suggest, these properties of HUCB cells also occur following transplantation in humans, our observations would be highly significant in view of the shortage of human donors and the widespread controversy regarding xenotransplantation.

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