Published in final edited form as:

Exp Hematol. 2006 March ; 34(3): 359–368.

Busulfan-conditioned bone marrow transplantation results in high-level allogeneic chimerism in mice made tolerant by in utero hematopoietic cell transplantation

Shuichi Ashizuka, William H. Peranteau, Satoshi Hayashi, and Alan W. Flake

The Children's Institute for Surgical Science, The Children's Hospital of Philadelphia, Philadelphia, Pa., USA

Abstract

Objective—In utero hematopoietic cell transplantation (IUHCT) is a nonablative approach that achieves mixed allogeneic chimerism and donor-specific tolerance. However, clinical application of IUHCT has been limited by minimal engraftment. We have previously demonstrated in the murine model that low-level allogeneic chimerism achieved by IUHCT can be enhanced to near-complete donor chimerism by postnatal minimally myeloablative total body irradiation (TBI) followed by same-donor bone marrow transplantation. Because of concerns of toxicity related to even low-dose TBI in early life, we wondered if a potentially less toxic strategy utilizing a single myelosuppressive agent, Busulfan (BU), would provide similar enhancement of engraftment.

Methods—In this study, mixed chimerism was created by IUHCT in a fully allogeneic strain combination. After birth, chimeric mice were conditioned with BU followed by transplantation of bone marrow cells congenic to the prenatal donor.

Results—We demonstrate that: 1) low-level chimerism after IUHCT can be converted to high-level chimerism by this protocol; 2) enhancement of chimerism is BU dose-dependent; and 3) BU reduces the proliferative potential of hematopoietic progenitor cells thus conferring a competitive advantage to the non-BU-treated postnatal donor cells.

Conclusion—This study confirms the potential of IUHCT for facilitation of minimally toxic postnatal regimens to achieve therapeutic levels of allogeneic engraftment.

In utero hematopoietic cell transplantation (IUHCT) is a nonmyeloablative approach to achieve mixed hematopoietic chimerism and donor-specific tolerance across full allogeneic barriers [1]. In experimental and clinical circumstances in which a competitive advantage exists for donor cells, high levels of allogeneic chimerism have been achieved. Unfortunately, when such an advantage does not exist, levels of engraftment following IUHCT are low and well below what would be considered therapeutic for most target diseases [2–77].

One strategy to achieve high levels of allogeneic engraftment following IUHCT is to take advantage of the donorspecific tolerance associated with mixed chimerism after IUHCT and enhance chimerism by minimally toxic postnatal bone marrow transplant (BMT) regimens. We have previously demonstrated that allogeneic engraftment can be enhanced in prenatally transplanted recipients by a strategy of nonmyeloablative total body irradiation (TBI) followed by postnatal BMT [8]. In this system, the TBI provides a competitive advantage to the nonirradiated postnatal donor cells, which are fully responsible for the enhancement in engraftment. Although highly effective and nontoxic in the murine model, the clinical

administration of even low-dose TBI to infants or children would raise significant concerns of long-term morbidity. Thus, safer and less toxic protocols are highly desirable.

Busulfan (BU; 1,4-butanediol dimethanesulfonate) is an alkylating agent with predominantly myelosuppressive and minimally immunosuppressive properties that has been relatively well tolerated in many human BMT protocols in adults and children [9–13]. In a postnatal congenic murine BMT model, transplant conditioning with BU alone resulted in high levels of engraftment and minimal transplant-related mortality [14–16]. We have previously shown that the allogeneic recipient made tolerant by IUHCT engrafts similarly to congenic recipients following postnatal minimally myeloablative BMT. Thus we hypothesized that BU, as a single-agent conditioning regimen, could effectively enhance allogeneic engraftment after IUHCT. The results of this study show that near complete allogeneic chimerism can be achieved without apparent toxicity by a strategy of IUHCT followed by postnatal BU-conditioned BMT.

Materials and methods

Mice

Balb/c (H-2K^d, CD45.2), C57BL/6 (H-2K^b, CD45.2; referred to as B6), and C57BL/6-Ly5.2 (H-2K^b, CD45.1; referred to as B6Ly5.2) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). CBA/J (H-2K^k, CD45.2) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). A minimum of five mice were assessed per group in the individual arms of the experiments to allow for statistical analysis. Fewer than five mice were used to compare chimerism levels following engraftment enhancement in those mice with initial chimerism levels following IUHCT of less than 1% because of the limitation in engraftment enhancement in this group. Animals were housed in the Animal Laboratory Facility of the Abramson Research Center at The Children's Hospital of Philadelphia. The experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia and followed guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Donor bone marrow harvest and T-Cell depletion

Adult donor bone marrow cells were harvested from 6- to 8-week-old adult female mice after sacrifice by cervical dislocation by flushing the tibias and femurs with Ca⁺⁺/Mg⁺⁺ free phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) using a 26-gauge needle. The cells were processed into a single-cell suspension by gentle passage through the 26-gauge needle and subsequently filtered through a 70-μm nylon mesh filter and layered over Ficoll (Histopaque 1077, Sigma, St Louis, MO, USA). After centrifugation at 600g for 15 minutes at room temperature, the light-density mononuclear cell (LDMC) layer was removed and washed with sterile PBS. CD3⁺ T-cell depletion (TCD) was performed by labeling the bone marrow LDMC with fluorescein isothiocyanate (FITC)-conjugated, anti-CD3 monoclonal antibody (mAb; Pharmingen, San Diego, CA, USA) followed by incubation with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA, USA) and subsequent passage through a VarioMACs magnetic cell sorter (Miltenyi Biotec, Auburn, CA, USA). A CD3⁺ cell fraction of less than 0.5% of the donor bone marrow cells after depletion was confirmed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA). On average, CD3 depletion resulted in a loss of approximately 30% of the cells from our donor population. Cells were counted prior to transplantation and more than 95% viability was confirmed by trypan blue exclusion.

In utero BMT

Time-dated E14 Balb/c mice were injected as previously described [5,6,17]. Briefly, under methoxyflurane (Medical Developments Australia, Australia) anesthesia and sterile technique, a midline laparotomy was performed and the uterine horns exposed. All fetuses were injected intraperitoneally using a 100- μ m glass beveled pipette with 5×10^6 TCD B6 bone marrow cells in 5 μ L of PBS. The fetus and uterine horns were returned to the abdomen and the abdominal cavity was closed after administering a 1 mL intraperitoneal bolus of sterile PBS, which replaced fluid lost during the procedure. Control animals received 5 μ L of PBS instead of donor cells. A total of 25 fetuses were injected with PBS and 466 fetuses were injected with donor cells. Pups were carried to term and weaned at 3 weeks of age.

Postnatal BMT

At 4 or 5 weeks of life and either 1 or 7 days after treatment with BU, chimeric and control mice were injected with 30×10^6 TCD B6Ly5.2 bone marrow cells in 200 μ L of PBS via the lateral tail vein. A total of seven, five, and five mice were transplanted with donor cells 1 day after treatment with 5 mg/kg, 15 mg/kg, and 35 mg/kg BU, respectively. Similarly, a total of seven, six, and five mice were transplanted with donor cells 7 days after treatment with 5 mg/kg, 15 mg/kg, and 35 mg/kg BU, respectively. These numbers were chosen based on the number of chimeric mice born after IUHCT and to allow for adequate statistical comparisons between groups.

Busulfan treatment

BU (Sigma) was reconstituted as follows: 500 mg or 150 mg of BU was dissolved in 2.0 mL dimethylsulfoxide (DMSO; Sigma); 100 μ L of this solution was then mixed with 900 μ L DMSO and 4 mL warm (37°C) PBS (pH 7.4) to create a 5 mg/mL or 1.5 mg/mL solution. Mice were weighed and one of three doses of BU (35 mg/kg, 15 mg/kg, or 5 mg/kg) was injected intraperitoneally. A total of 19, 16, and 23 mice were treated with 5 mg/kg, 15 mg/kg, and 35 mg/kg BU, respectively. These doses were chosen based on studies demonstrating that 15 mg/kg of BU is approximately one-fifth the LD_{50/30} in adult mice as well as the finding that 35 mg/kg of BU was the minimal effective dose required to rescue the twitcher mouse in a congenic hematopoietic cell transplantation system [15,18,19].

Flow cytometric analysis

The FITC-conjugated mAbs included antibodies against H-2K^b, CD45, and Mouse IgG2a. Phycoerythrin (PE)-conjugated mAbs included antibodies against H-2K^d, CD45.1, and Mouse IgG2a. For lineage analysis biotinylated antibodies against CD3, B220, CD11b, Gr1, Ter119, rat IgG2a, rat IgG2b, and hamster IgG1 were developed with streptavidin-PE. Nonspecific Fc γ receptor binding was blocked by the mAb against mouse Fc γ receptor 2.4G2. Conjugated antibodies with irrelevant specificities listed above served as negative controls. Propidium iodide staining was used to exclude dead cells in dual-color flow cytometry. A minimum of 10,000 events were assessed for each individual flow cytometric analysis. All antibodies were purchased from Pharmingen and flow cytometry was performed on a FACScan (Becton Dickinson).

Blood sampling and donor chimerism and multilineage engraftment assessment

Chimerism was assessed at 4 or 5 weeks of life in recipients of IUHCT prior to treatment with BU and postnatal BMT. Chimerism was analyzed by dual-color flow cytometry for H-2K^b (donor cells) and H-2K^d (recipient cells) every week for the first 4 weeks following postnatal BMT in chimeric and control mice. Levels were then assessed every other week for the next 8 weeks and then every month. The contribution to engraftment of postnatal and prenatal donor cells was distinguished by dual-color flow cytometry for CD45.1 (present only on the postnatal

donor cells) at the same time points. All mice were analyzed at each time point (1 day post-BU: 5, 15, 35 mg/kg chimeric and 35 mg/kg naïve groups, $n = 7, 5, 5$, and 7 , respectively; 7 days post-BU: 5, 15, 35 mg/kg chimeric and 35 mg/kg naïve groups, $n = 7, 6, 5$, and 5 , respectively) and followed for at least 6 months after postnatal BMT. Donor cell lineage analysis was performed at 2 and 6 months after postnatal BMT by dual-color flow cytometry for H-2K^b (donor cells) and CD3, B220, and CD11b lineage markers. For each analysis, approximately 200 μ L of peripheral blood was collected in heparinized capillary tubes via retro-orbital vein puncture and diluted to 10 mL with heparinized PBS. The samples were layered over a Ficoll gradient. The LDMCs were collected after centrifugation at 600g for 15 minutes and subsequently washed in PBS. A minimum of 10,000 events was analyzed for each determination.

Mixed lymphocyte reaction

Donor-specific tolerance was assessed by mixed lymphocyte reaction (MLR) in chimeric mice after IUHCT and postnatal BU-conditioned BMT. Splenocyte responder cells were harvested by hemisplenectomy and subjected to mixed lymphocyte culture by standard methods. Briefly, 2×10^5 splenic responder cells were cultured in triplicate at 37°C in 5% CO₂ for 4 days with 4×10^5 mitomycin C-treated stimulator cells (host, donor, and third-party) in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Life Technologies), 50 mM 2-mercaptoethanol (Sigma), and antibiotics (penicillin, 100 U/mL; streptomycin, 100 mg/mL; Life Technologies). Eighteen hours prior to harvesting, cells were pulsed with [³H] thymidine. Radioactivity was counted using a liquid scintillation counter at the time of harvest. Stimulation indices (SIs) were calculated by dividing mean counts per minute (c.p.m.) by mean background c.p.m.

Assessment of hematologic parameters after BU treatment

Naïve (non-in utero transplanted) 4-week-old Balb/c mice were treated with 0 mg/kg, 5 mg/kg, 15 mg/kg, or 35 mg/kg of BU injected intraperitoneally ($n = 5, 5, 5$, and 6 , respectively, chosen to allow for statistical comparison between groups). Peripheral blood (50 μ L) was collected in ethylene-diamine-tetraacetic acid tubes (Kabe Labortechnik GMBH) by retro-orbital vein puncture at 3 days before BU treatment and 1, 4, 7, 11, 14, 21, and 28 days after treatment. Hematologic parameters (white blood cell count, hemoglobin, and platelets) were assessed with a Hema Vet CBC machine (Mascot, CDC Technologies, Oxford, CT, USA). The hematologic parameters of mice receiving 35 mg/kg were assessed every month for 6 months following BU injection due to prolonged thrombocytopenia.

Assessment of the effect of BU treatment on stem/progenitor cells

In a separate experiment, 4-week-old naïve Balb/c mice were treated with 0 mg/kg, 5 mg/kg, 15 mg/kg, or 35 mg/kg of BU injected intraperitoneally. Bone marrow cells were harvested and the LDMCs isolated as described above at 1 and 7 days after BU treatment ($n = 6$ for each harvest time point and dose of BU administered, $n = 13$ for naïve non-BU-treated control mice). The effect of BU treatment on stem/progenitor cells was assessed by colony-forming unit assays with LDMCs from treated and nontreated mice according to standard protocol. Briefly, 9×10^3 LDMCs were resuspended in 300 μ L Dulbecco's modified Eagle's medium supplemented with 30 U/mL human epogen and mixed with 2.7 mL methylcellulose. The cells were vortexed vigorously and subsequently plated in duplicate at 3×10^3 cells/well and cultured for 14 days at 37°C in 5% CO₂. On days 10 and 14 of culture, the frequency of colony-forming unit cells per 10^5 nucleated cells and the number of cells in the three biggest colonies were counted.

Assessment of graft-versus-host disease

Mice were weighed prior to receipt of postnatal BMT and weekly following transplantation. Mice were also monitored twice a week for clinical signs of graft-versus-host disease (GVHD) including runting, fur loss, and serositis and for side effects of BU treatment such as persistent alopecia.

Statistical analysis

Data are represented as the mean of the respective group $\pm$ 1 standard deviation. A two-tailed Student's *t* test for comparison of means with unequal variance was used for statistical analysis. A *p* value less than 0.05 was considered statistically significant.

Results

BU treatment results in transient suppression of peripheral white blood cell and platelet counts

To assess the hematologic effects of BU treatment, hematologic parameters were assessed in normal naïve mice not receiving in utero treatment. Transient dose-dependent decreases were seen in peripheral white blood cell (WBC) counts in mice receiving 15 mg/kg and 35 mg/kg of BU. Similarly, transient dose-dependent decreases in platelet counts were seen in mice receiving either of the three doses of BU studied (5 mg/kg, 15 mg/kg, or 35 mg/kg; Fig. 1). The effect of BU treatment on platelet counts was most severe, requiring over 100 days to recover when 35 mg/kg was administered. No decrease in hemoglobin levels was seen at all three BU doses studied.

IUHCT followed by postnatal BU treatment and congenic BMT results in high-level allogeneic donor cell chimerism

Donor cell engraftment levels were significantly enhanced in tolerant recipients by postnatal BU treatment followed by TCD BMT with cells congenic to the prenatal allogeneic donor (Fig. 2A). Enhancement of engraftment was BU dose-dependent. Levels plateaued by 20 to 24 weeks after postnatal transplantation and remained stable until sacrifice (6 to 12 months after postnatal transplantation). Tolerance by IUHCT is a necessity to achieve engraftment enhancement as demonstrated by the inability of 35 mg/kg BU followed by BMT to result in any significant fluorescein-activated cell sorting-detectable levels of chimerism in naïve mice. Similarly, BU treatment alone of mice receiving IUHCT does not result in any significant enhancement of donor cell chimerism without posttreatment BMT (Fig 2B).

Our initial studies indicated that BU had its most profound effect on hematologic parameters between 7 and 14 days after treatment. Thus, we sought to determine the optimal time interval between BU administration and postnatal BMT by comparing BMT at 1 or 7 days after BU treatment. As demonstrated in Figure 3, significant differences in engraftment were seen only when the highest dose of BU was used. These differences were transient and by 6 months post-BMT, no statistical differences were seen.

Allogeneic chimerism is stable and multilineage

Donor cell chimerism levels were followed and at 8 and 24 weeks after transplant the presence of multilineage donor cell engraftment as determined by staining for myeloid and lymphoid markers (CD11b, CD3, and B220) was assessed (Fig. 4A). Donor cell chimerism was multilineage at both time points and with all doses of BU studied. The chimerism is balanced with minimal change over time and is similar to the lineage composition of naïve control B6Ly5.2 mice. There was no significant difference in the multilineage engraftment of mice

receiving BMT 1 or 7 days after BU administration. The presence of stable multilineage engraftment at 6 months supports the engraftment hematopoietic stem cell (HSCs).

Postnatal donor cells are responsible for enhancement of engraftment following BU treatment and BMT

The postnatal donor was congenic to the prenatal donor, allowing us to distinguish the contribution of each population to total donor chimerism. At all doses of BU studied, regardless of the time interval between BU administration and BMT, the postnatal donor bone marrow that was not subjected to BU treatment was responsible for the entire enhancement of allogeneic engraftment (Fig. 4B and Fig. 4C).

Successful enhancement of engraftment is dependent on the initial level of chimerism following IUHCT

The ability of BU-conditioned BMT to enhance allogeneic engraftment after IUHCT was compared in two groups of mice, those with <1% and those with >1% donor cell engraftment. In mice with <1% chimerism, engraftment was enhanced in 60% (12 of 20) of the mice. Engraftment enhancement was seen at all doses of BU studied but with greater success when 35 mg/kg of BU was used (5 mg/kg BU: three of seven; 15 mg/kg BU: four of seven; 35 mg/kg BU: five of six). This stands in contrast to mice with >1% donor cell chimerism following IUHCT in which chimerism was successfully enhanced in 100% of the mice independent of the dose of BU administered. In mice in which chimerism was successfully enhanced, engraftment levels were similar at 4 weeks and 6 months post-BU-conditioned BMT independent of the initial level of chimerism (Fig. 5).

We hypothesized that the lack of successful engraftment enhancement in mice with <1% donor cell chimerism following IUHCT was due to inadequate donor-specific tolerance. Thus, splenocytes from mice with <1% chimerism that successfully enhanced were compared with splenocytes from mice with <1% chimerism that failed to enhance by MLR against B6 stimulator cells to evaluate the tolerance of these two groups to the B6 donor cells. As demonstrated in Figure 6, both groups of chimeric mice did not respond to host antigen stimulation but responded normally to third-party antigen stimulation. However, the SI against donor antigen (B6) was higher when responder splenocytes originated from mice that failed to enhance compared to mice in which engraftment was successfully enhanced ($SI = 2.51 \pm 1.1$ versus 1.49 ± 0.73 , $p = 0.059$). This supports the persistence of a specific alloresponse as a contributing factor to the inability to successfully enhance engraftment in this group of mice.

Absence of GVHD following BU treatment and postnatal BMT

Animals were assessed for weight gain as well as clinical signs of GVHD including runting, fur loss, and serositis following BU-conditioned BMT. No obvious signs of GVHD were noted in any of the mice receiving BU and a postnatal BMT. Similarly, there was no evidence of cataract formation in any of the treated mice. Weight gain was observed in all groups. However, a BU dose-dependent negative effect on weight gain was noted as demonstrated by the initial weight loss at 1 and 2 weeks after BU treatment in the high-dose treatment group (Fig. 7). Mice treated with 5 mg/kg, 15 mg/kg, and 35 mg/kg of BU demonstrated a body weight gain at 24 weeks posttreatment that was 97.98%, 85.67%, and 57.76% of control naïve Balb/c mice, which did not receive BU, respectively.

Effect of BU on bone marrow progenitor cells

To determine the effect of BU on bone marrow progenitor cells, we analyzed the frequency and proliferative potential of progenitor cells by colony assays. Four-week-old naïve non-in utero treated Balb/c mice were treated with one of four doses of BU (0 mg/kg, 5 mg/kg, 15

mg/kg, or 35 mg/kg) and their bone marrow cells were subsequently harvested at 1 or 7 days after BU treatment. The frequency of colony forming cells among bone marrow mononuclear cells from mice treated with BU was decreased compared with untreated control mice (Fig. 8A). This was most pronounced 7 days after treatment with 15 mg/kg BU as well as 1 and 7 days after treatment with 35 mg/kg BU (89 ± 62 , 172 ± 63 , and 17 ± 28 total colonies, respectively, versus 614 ± 235 total colonies for untreated controls, $p < 0.001$). In addition, the proliferative potential of hematopoietic progenitors, as assessed by colony size, was significantly reduced in mice treated with BU independent of the dose (Fig. 8B). This in vitro data supports a mechanism by which treatment with BU results in a competitive disadvantage at the level of the progenitor cell, resulting in an enhancement in engraftment following BU-conditioned postnatal BMT in tolerant mice created by IUHCT.

Discussion

Major limitations to postnatal HSC transplantation include the need for an HLA-matched donor and the myeloablative and immunosuppressive conditioning required for successful transplantation [20–22]. IUHCT is a potential strategy to achieve allogeneic engraftment and donor-specific tolerance without the need for myeloablation or immunosuppression [1,5,6]. Unfortunately, in the absence of a significant competitive advantage for the donor cell population, levels of engraftment following experimental and clinical attempts at IUHCT have been minimal [2–7,23,24]. A promising approach to increase levels of donor cell chimerism after IUHCT takes advantage of the donor-specific tolerance present with low-level mixed hematopoietic chimerism to facilitate postnatal minimally myeloablative strategies to enhance chimerism to more clinically relevant levels. Postnatal donor lymphocyte infusion (DLI) as well as postnatal nonmyeloablative TBI followed by BMT in chimeric mice created by IUHCT are two methods that have resulted in enhancement of allogeneic chimerism to levels of greater than 90% [15,16]. Although successful in the murine model, both DLI and TBI have potential clinical limitations. DLI was unable to enhance chimerism in mice with levels of donor cell engraftment less than 1% following IUHCT [17] and carries the potential risk of GVHD. TBI is known to induce a proinflammatory cytokine response and is associated with microvascular injury [25]. More importantly, the use of low-dose TBI in clinical settings, especially in pediatrics, has been associated with the development of iatrogenic cancers and cataracts [26–28]. Thus, safer methods for postnatal enhancement of chimerism are desirable.

BU is a well-known myelosuppressive agent with minimal immunosuppressive effects that has been relatively well tolerated in pediatric and adult BMT protocols [12,13]. It has been demonstrated in congenic postnatal mouse models that pretransplant conditioning with BU alone can result in high levels of engraftment and minimal mortality [14,15]. In the present study we demonstrate that similar results can be obtained in an allogeneic mouse model following IUHCT. We show that levels of allogeneic chimerism can be enhanced to greater than 90% following conditioning with 35 mg/kg BU and that lower doses of BU result in dose-dependent increases in allogeneic chimerism. Chimerism is stable and multilineage at 6 months posttransplant, supporting the engraftment of donor HSCs. Enhancement of engraftment results solely from the postnatal donor cells supporting a mechanism of a BU-induced competitive disadvantage of host and prenatally transplanted donor cells. This is further supported by our in vitro data demonstrating reduced frequency and proliferative capacity of host progenitors after BU conditioning.

In contrast to our results with donor lymphocyte infusion in which it was not possible to enhance engraftment in mice with less than 1% chimerism following IUHCT, we did observe enhancement of engraftment in 60% of mice in this population using BU treatment followed by BMT. These results are similar to what we have seen using a conditioning regimen consisting of postnatal nonmyeloablative TBI followed by BMT in which engraftment

enhancement was possible in a portion of mice with chimerism levels less than 1% following IUHCT (unpublished data). We have previously shown that only a portion of mice with chimerism levels less than 1% following IUHCT are tolerant to the allogeneic donor and that tolerance in microchimeric animals correlates with the presence of clonal deletion [6]. This is supported in the current study where we demonstrate differential donor reactivity by MLR of splenocytes from recipient mice in which allogeneic chimerism was enhanced compared with splenocytes from recipient mice in which enhancement of engraftment did not occur. The ability to enhance engraftment using a conditioning regimen consisting of either BU or TBI and postnatal BMT in mice with less than 1% chimerism following IUHCT, and the inability to do so using DLI, may be understandable when one considers the population of cells responsible for the increase in donor chimerism. In mice treated with BU or TBI, engraftment enhancement results solely from the postnatal donor cell population [8]. In these systems, IUHCT serves only to tolerize the recipient to the postnatal donor. Conditioning with BU or TBI then provides a competitive advantage to the unexposed HSCs present in the postnatal donor cell inoculum. In mice receiving DLI after IUHCT, the increase in donor chimerism derives from the prenatal donor-derived HSC [17]. Thus, in this system, in addition to tolerizing the host to the allogeneic donor, the prenatal donor cells must provide a sufficient number of engrafted HSCs to contribute to an increase in donor cell chimerism following postnatal DLI. In mice with less than 1% donor cell engraftment following IUHCT, the microchimerism may not represent the engraftment of HSC, or the number of HSCs engrafted may be too small to allow for an enhancement in engraftment following postnatal DLI.

After an initial period of weight loss in mice receiving the highest dose of BU, all mice subsequently demonstrated appropriate weight gain. No additional toxic effects were noted in any of the mice including those receiving the highest dose of BU and at no time did any mouse demonstrate signs of GVHD. GVHD is a primary concern following allogeneic BMT. Risk of GVHD is increased after BMT in tolerant or immunosuppressed recipients. The strategy of tolerance induction by IUHCT combined with the minimal myelosuppression provided by BU conditioning might favor the onset of GVHD following BMT. The absence of GVHD in the present study can, in part, be attributed to two factors. TCD bone marrow (<0.5% CD3⁺ cells) was used as the postnatal donor cell source, thus reducing the exposure of the host to mature donor T cells. Additionally, previous studies using the same strain combination have demonstrated that HSC-derived T cells with antihost allo-reactivity undergo near complete deletion following postnatal BMT in mice made tolerant by IUHCT [8].

The current study demonstrates that high levels of allogeneic chimerism can be achieved following IUHCT combined with postnatal BU conditioned BMT. Although not directly applicable to humans without further studies in large-animal models, this study, in combination with previous studies demonstrating high levels of allogeneic chimerism following IUHCT and postnatal DLI or postnatal nonmyeloablative TBI/BMT, support the strategy of prenatal tolerance induction to facilitate postnatal allogeneic cellular or organ transplantation. The ability to enhance engraftment postnatally using nontoxic conditioning regimens in individuals made tolerant by IUHCT remains a promising strategy that, if translatable to the human, could greatly expand the clinical application of in utero cellular therapy. The development of safer protocols for postnatal enhancement of engraftment is therefore an important goal for eventual clinical application of this approach.

Acknowledgments

This work was supported by grants R01 HL/DK63434, R01 HL64715, and U54 HL070596-01 from the National Institutes of Health as well as funds from the Ruth and Tristram C. Colket, Jr. Chair of Pediatric Surgery (A.W.F.).

References

1. Flake AW, Zanjani ED. In utero hematologic stem cell transplantation: ontogenic opportunities and biological barriers. *Blood* 1999;94:2179–2191. [PubMed: 10498587]
2. Blazer BR, Taylor PA, Vallera DA. Adult bone marrow-derived pluripotent hematopoietic stem cells are engraftable when transferred in utero into moderately anemic fetal recipients. *Blood* 1995;85:833–841. [PubMed: 7833485]
3. Carrier E, Lee TH, Busch MP, Cowen MJ. Induction of tolerance in nondefective mice after in utero transplantation of major histocompatibility complex-mismatched fetal hematopoietic stem cells. *Blood* 1995;85:4681–4690. [PubMed: 8541562]
4. Harrison MR, Slotnick RN, Clombleholme TM, Golbus MS, Tarantal AF, Zanjani ED. In-utero transplantation of fetal liver haemopoietic stem cell in monkeys. *Lancet* 1989;2:1425–1427. [PubMed: 2574363]
5. Kim HB, Shaaban AF, Milner R, Fitcher C, Flake AW. In utero bone marrow transplantation induces tolerance by a combination of clonal deletion and anergy. *J Pediatr Surg* 1999;34:726–730. [PubMed: 10359172]
6. Kim HB, Shaaban AF, Yang EY, Liechty KW, Flake AW. Microchimerism and tolerance after in utero bone marrow transplantation in mice. *J Surg Res* 1998;77:1–5. [PubMed: 9698523]
7. Pearce RD, Kiehm D, Armstrong DT, et al. Induction of hematopoietic chimerism in the caprine fetus by intraperitoneal injection of fetal liver cells. *Experientia* 1989;45:307–308. [PubMed: 2564345]
8. Peranteau WH, Hayashi S, Hsieh M, Shaaban AF, Flake AW. High-level allogeneic chimerism achieved by prenatal tolerance induction and postnatal nonmyeloablative bone marrow transplantation. *Blood* 2002;100:2225–2234. [PubMed: 12200389]
9. Dunn CDR. The chemical and biological properties of Busulfan (Myleran). *Exp Hematol* 1974;2:101–107. [PubMed: 4616840]
10. Santos GW, Tutschka PJ. Effect of busulfan on antibody production and skin allograft survival in the rat. *J Natl Cancer Inst* 1974;53:1775–1780. [PubMed: 4612167]
11. Tutschka PJ, Santos GW. Bone marrow transplantation in the busulfan-treated rat. III. Relationship between myelosuppression and immunosuppression for conditioning bone marrow recipients. *Transplantation* 1977;24:52–62. [PubMed: 18820]
12. Hassan M. The role of busulfan in bone marrow transplantation. *Med Oncol* 1999;16:166–176. [PubMed: 10523796]
13. Matsuyama T, Kojima S, Kato K. Allogeneic bone marrow transplantation for childhood leukemia following a busulfan and melphalan preparative regimen. *Bone Marrow Transplant* 1998;22:21–26. [PubMed: 9678791]
14. Yeager AM, Shinn C, Pardoll DM. Lymphoid reconstruction after transplantation of congenic hematopoietic cells in busulfan-treated mice. *Transplantation* 1991;78:3312–3316.
15. Yeager A, Shinn C, Shinohara M, Pardoll H. Hematopoietic cell transplantation in the twitcher mouse. *Transplantation* 1993;56:185–190. [PubMed: 8101401]
16. Yoder MC, Cumming FG, Hiatt K, Mukberjee P, Williams DA. A novel method of myeloablation to enhance of adult bone marrow cells in newborn mice. *Biol Blood Marrow Transplant* 1996;2:59–67. [PubMed: 9118300]
17. Hayashi S, Peranteau WH, Shaaban AF, Flake AW. Complete allogeneic hematopoietic chimerism achieved by a combined strategy of in utero hematopoietic stem cell transplantation and postnatal donor lymphocyte infusion. *Blood* 2002;100:804–812. [PubMed: 12130490]
18. Mauch P, Down JD, Warhol M, Hellman S. Recipient preparation for bone marrow transplantation. I. Efficacy of total-body irradiation and busulfan. *Transplantation* 1988;46:205–210. [PubMed: 3043777]
19. Morley A, Blake J. An animal model of chronic aplastic marrow failure. I. Late marrow failure after busulfan. *Blood* 1974;44:49–56. [PubMed: 4834516]
20. Kreiter S, Winkelmann N, Schneider PM, et al. Failure of sustained engraftment after non-myeloablative conditioning with low-dose TBI and T cell-reduced allogeneic peripheral stem cell transplantation. *Bone Marrow Transplant* 2001;28:157–161. [PubMed: 11509933]

21. Mohty M, Fegueux N, Exbrayat C, et al. Enhanced graft-versus-tumor effect following dose-reduced conditioning and allogeneic transplantation for refractory lymphoid malignancies after high-dose therapy. *Bone Marrow Transplant* 2001;28:335–339. [PubMed: 11571504]
22. Lee KH, Lee JH, Lee JH, Kim WK, Chi HS, Lee JS. Nonmyeloablative conditioning regimen of fludarabine, busulfan, anti-thymocyte globulin, and methylprednisolone for allogeneic peripheral blood hematopoietic cell transplantation. *Haematologica* 2001;86:999–1001. [PubMed: 11532635]
23. Flake AW, Roncarolo MG, Puck JM, et al. Treatment of X-linked severe combined immunodeficiency by in utero transplantation of paternal bone marrow. *N Engl J Med* 1996;335:1806–1810. [PubMed: 8943162]
24. Wengler GS, Lanfranchi A, Frusca T, et al. In utero transplantation of prenatal CD34 hematopoietic progenitor cells in a patient with X-linked severe combined immunodeficiency (SCIDXI). *Lancet* 1996;348:1484–1487. [PubMed: 8942778]
25. Samlowski WE, Araneo BA, Butler MO, Fung MC, Johnson HM. Peripheral lymph node helper T-cell recovery after syngeneic bone marrow transplantation in mice prepared with either γ -Irradiation or busulfan. *Blood* 1989;74:1436–1445. [PubMed: 2527572]
26. Schlumberger M, Cailleux AF, Suarez HG, de Vathaire F. Irradiation and second cancers. The thyroid as a case in point. *C R Acad Sci III* 1999;322:205–213. [PubMed: 10196674]
27. Modan B, Chetrit A, Alfandary E, Katz L. Increased risk of breast cancer after low-dose irradiation [Erratum appears in *Lancet* 1989;1:916]. *Lancet* 1989;1:629–631. [PubMed: 2564456]
28. Wilde G, Sjostrand J. A clinical study of radiation cataract formation in adult life following gamma irradiation of the lens in early childhood. *Br J Ophthalmol* 1997;81:261–266. [PubMed: 9215051]

Figure 1. Effects of BU treatment on hematologic parameters. Analysis of WBC (A) and platelet (B) counts in mice injected with one of three doses of BU. * $p < 0.05$, *** $p < 0.001$ when comparing the hematologic parameters after BU treatment to those before treatment.

Figure 2.

BU-conditioned BMT results in high levels of alloengraftment in chimeric mice formed by IUHCT. Four- to 5-week old chimeric mice received one of three doses of BU 24 hours prior to TCD BMT with 30×10^6 B6Ly5.2 cells. Control mice were 4-week-old Balb/c females that received 35 mg/kg BU followed by 30×10^6 TCD B6Ly5.2 bone marrow cells (A). Levels of chimerism were statistically different between each BU dose with the exception of the points marked with an asterisk (*) in which $p > 0.05$. The effect of 35 mg/kg BU on prenatally engrafted cells was assessed in chimeric mice that received BU treatment at 4 weeks of age without subsequent BMT ($n = 5$) (B).

Figure 3.

Engraftment profiles following BU-conditioned BMT at different time intervals between BU treatment and postnatal BMT in chimeric mice formed by IUHCT. Chimeric mice were transplanted with 30×10^6 TCD B6Ly5.2 bone marrow cells via tail vein infusion 1 or 7 days after BU treatment. Engraftment profiles were compared at the two time intervals in mice receiving one of three doses of BU: (A) 5 mg/kg; (B) 15 mg/kg; (C) 35 mg/kg. * $p < 0.05$ when comparing BU treatment 1 day before BMT to BU treatment 7 days before BMT.

Figure 4.

Stable multilineage chimerism predominantly resulting from the postnatal donor. Peripheral blood multilineage chimerism was assessed at 8 and 24 weeks after BU-conditioned postnatal BMT. Results were similar at both 8 and 24 weeks as well as at 1- and 7-day time intervals between BU conditioning and BMT. Thus only the 1-day time interval analyzed at 24 weeks posttransplant is shown (A). The postnatal donor (CD45.1) was congenic to the prenatal donor (CD45.2) and was distinguished by flow cytometry based on the CD45 isoform expression (B). The contribution to the total chimerism made by each donor assessed at all time points at which total donor peripheral blood chimerism was analyzed (C).

Figure 5.

Engraftment enhancement of <1% versus 1 to 3% chimeric mice. Chimerism levels were assessed in mice with initial levels of chimerism following IUHCT of <1% versus 1 to 3%, which were successfully enhanced by BU-conditioned BMT. Similar levels of engraftment enhancement were seen at all doses of BU studied [35 mg/kg (A), 15 mg/kg (B), and 5 mg/kg (C)] independent of the initial levels of chimerism.

Figure 6.

MLR in 4-week-old chimeric mice. SIs were assessed with responder cells from mice with initial levels of chimerism <1% following IUHCT in which engraftment was and was not successfully enhanced by BU-conditioned BMT. Stimulator splenocytes are on the x-axis.

Figure 7.

Body weight assessment following BU-conditioned BMT. Mice were weighed serially after BU-conditioned BMT at 4 weeks of age. Body weight assessment is expressed as a percentage of body weight change. The control group consists of naïve 4-week-old Balb/c mice.

Figure 8.

Colony formation after BU treatment. The frequency (A) of colonies/ 10^5 bone marrow cells as well as the size of the colonies (B) grown from bone marrow cells from mice treated with one of three doses of BU at 1 or 7 days prior to bone marrow harvest was assessed. *** $p < 0.001$ when comparing the frequency of total colonies originating from bone marrow cells of BU-treated mice to bone marrow cells from nontreated mice. n in B represents the number of colonies assessed.