We have studied graft-versus-host disease (GVHD) after transplantation of allogeneic peripheral blood stem cells (PBSC) mobilized by either recombinant canine granulocyte colony-stimulating factor (rG-CSF) alone or combined with stem cell factor (rSCF). These studies were prompted by the observation of extremely rapid and sustained engraftment of growth factor-mobilized PBSC in the autologous setting using genetically marked cells and changes in function of T lymphocytes from donors that had undergone mobilization. Specifically, lymphocytes from growth factor-treated donors were hyporesponsive in mixed leukocyte culture and in response to Con A, raising hopes that GVHD in dogs given growth factor mobilized allogeneic PBSC might be altered in a beneficial way. Eighteen dogs were given a median of 17.1 x 10⁶ PBSC/kg from littermate donors after 920 cGy of total body irradiation without postgrafting immunosuppression. Donors were either genotypically DLA-identical (n = 9) or DLA-haploidentical (n = 9). The median number of colony-forming unit-granulocyte macrophage (CFU-GM) infused was 27 x 10⁶/kg, and the number of CD3⁴ cells in the transplant was on the order of 4.6 x 10⁴/kg. The dogs received a median of 52.8 x 10⁴ CD4 cells/kg and 13.7 x 10⁴ CD8 cells/kg. All 18 dogs had prompt hematopoietic engraftment of donor cells as assessed by chimerism studies using variable number tandem repeat, as well as cytogenetic markers. Three of the nine dogs given grafts from DLA-identical littermates had fatal GVHD, five had transient GVHD, and one had no GVHD. All nine DLA-haploidentical recipients of PBSC developed fatal hyperacute GVHD. In conclusion, the expectation about rapid engraftment was fulfilled. However, incidence and severity of acute GVHD after transplantation of mobilized PBSC were not different than previously reported for nonmobilized PBSC or marrow. This model will allow for further studies, including T-cell depletion to minimize GVHD without increasing graft rejection.

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Materials and Methods

Litters of Beagles and Beagle/Harrier/Hound mix were either raised at the Fred Hutchinson Cancer Research Center (Seattle, WA) or purchased from commercial kennels in the State of Washington. The dogs weighed from 7.0 to 14.1 (median, 9.7) kg and were 6 to 11 (median, 8) months old. They were observed for disease for at least 2 months before study. All were immunized for leptospirosis, distemper, hepatitis, and parvovirus. Research was performed according to the principles outlined in the Guide for Laboratory Animal Facilities and Care prepared by the National Academy of Sciences, National Research Council. The protocol of the study was approved by the Internal Animal Care and Use Committee of the Fred Hutchin-
The following day, leukapheresis was performed in the first four donors, collecting 200 mL of blood each, collecting the buffy coat and returning it to the donor. At day 7 of growth factor treatment, a shunt connecting the carotid artery with the external jugular vein was placed in the PBSC donor. The following day, leukapheresis was performed in the first four animals (group 1, Table 1) by four cycles of centrifugation of 200 to 300 mL of blood each, collecting the buffy coat and returning the red blood cells and plasma to the animals. In the subsequent 14 donors, a 3-hour leukapheresis was performed by connecting the shunt to a Cobe 2997 continuous flow centrifuge (Cobe BCT, Inc, Blood Component Technology, Lakewood, CO) with a calculated processed volume of approximately 10 L of blood.

Recipient dogs were administered total body irradiation (TBI) at a dose of 920 cGy delivered at 7 cGy/minute from two opposing 60Co sources.20 PBSC were infused within 4 hours of TBI at a median dose of $1.1 \times 10^{10}$ cells/kg (Table 1). The day of PBSC transplant was designated as day 0.

Postgrafting care was given as previously described,22 which included twice daily oral nonabsorbable antibiotics, neomycin sulfate, and polymyxin sulfate, from day -5 until the day of recovery of white blood cell counts to more than 1,000/µL. Prophylactic systemic antibiotics twice daily (usually ceftazidime unless sensitivity tests indicated otherwise) were begun on the day of TBI and continued until the white blood cell count reached 1,000/µL. In addition, dogs received red blood cell and platelet transfusions if indicated based on the results of daily blood counts. All transfusions were irradiated in vitro with 2,000 cGy from a cesium source.

Four groups of recipients were studied (Table 1). Group 1 was comprised of four DLA-identical littermates receiving G-CSF/SCF-mobilized PBSC; group 2 included five DLA-identical littermates receiving G-CSF-mobilized PBSC; group 3 was composed of five DLA-haploidentical littermates given G-CSF/SCF-mobilized PBSC; and group 4 consisted of four DLA-haploidentical littermates receiving G-CSF-mobilized PBSC.

Hematopoietic engraftment was assessed by sustained increases in neutrophil counts, which was confirmed by conventional cytogenetics in eight cases where donor and recipient were sex mismatched.
in granulocyte and platelet counts after the postirradiation nadir, by histologic features of the marrow from biopsy or autopsy specimens, by documentation of cells with donor (CA), repeat polymorphisms or donor karyotype in specimens from peripheral blood and marrow, and by the development of GVHD. No posttransplantation immuno-suppression was administered.

A number of intra studies were performed on the harvested PBSC. These included a colony-forming unit granulocyte/macrophage (CFU-GM) assay. PBSC were separated over a Ficoll gradient (D = 1.074), and 10^5 to 10^6 cells were cultured per plate for 10 to 14 days at 37°C in a humidified 7% CO_2 incubator in 35 mm plastic petri dishes containing 1 mL of agar medium consisting of 0.33% Bacto agar (Difco, Detroit, MI), 20% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), Iscove’s medium (GIBCO), and 10% bovine serum albumin, Fraction 5 (Irvine, CA). G-CSF, SCF, and GM-CSF (gift from Richard Nash, Fred Hutchinson Cancer Research Center) were added at 100 ng/mL to stimulate colony formation.

In addition, MLCs were performed with cells from PBSC donors and recipients along with cells from unrelated dogs. MLCs were performed before growth factor treatment and on day 7 of growth factor treatment according to established methods. Briefly, 10^6 responder PBMC were cocultured with 10^5 irradiated (22 Gy) allogeneic stimulator PBMC at 10^7/mL density for 6 days. Additional PBMC from the same dogs used for MLC were separately cultured in the same medium containing 20 μg/mL of Con A for 72 hours. Cells were terminally labeled with 1 μCi of tritiated thymidine for 6 hours, with cells harvested onto glass fiber filters and counted for isotope incorporation.

PBMC were also studied by flow cytometry using monoclonal antibodies (MoAb) 1E4 (αCD4) and JD3 (αCD8) as previously described. In addition, their CD34^+ contents was determined with a rabbit polyclonal antibody to the canine CD34 (P. McSweeney, manuscript in preparation). For single- and double-label experiments, PBSC were incubated with titered amounts of fluorescein isothiocyanate (FITC)-, biotin-, or phycoerythrin (PE)-conjugated MoAb. Cells (1 × 10^7) were incubated with 10 μg/mL of various MoAb for 30 minutes at 4°C, washed three times with Hank’s balanced salt solution with 2% horse sera and 5 mM sodium azide, and incubated with the second MoAb in the cases where two-color analysis was performed. For unlabeled primary MoAb, FITC-conjugated goat-antimouse antibody (Dako Corp., Carpinteria, CA) was used in the second step. Cells were then washed three times, resuspended in 0.3 mL of 1% paraformaldehyde, and analyzed on a FACScan (Becton Dickinson, San Jose, CA) flow cytometer. Controls consisted of cells treated with FITC-conjugated goat-antimouse antibody alone and relevant control MoAb followed by secondary MoAb. For controls of the direct conjugates, irrelevant antibodies that were directly conjugated were used as appropriate controls.

A natural killer (NK) assay was performed with cells from all donor and recipient dogs. Canine thyroid adenocarcinoma cells (CTAC)^22-28 were used to quantitate NK activity. Two million target cells (CTAC) were incubated with 0.1 mL (about 300 μCi) of Na^26 CrO_4 (New England Nuclear, Boston, MA) at 37°C for 60 minutes, washed once, and incubated with RPMI + 10% FBS + 1% nonessential amino acids + 2% L-glutamine + 1% sodium pyruvate at 4°C for 30 minutes and washed twice. Target cells were adjusted to 5 × 10^5 cells/mL and added to 96 U-bottom wells (Costar, Cambridge, MA) at 0.1 mL per well. These were incubated at 37°C for 18 hours and centrifuged at 100 G for 2 minutes. One-tenth milliliter of the supernatant was then harvested and radioactivity was determined using a gamma scintillation counter (Packard, Meriden, CT). Maximum release was determined using 100 μL of 1% nonidet P-40 to lyse the labeled targets. Percent specific lysis was expressed as:

\[
\frac{\text{Experimental CPM} - \text{Spontaneous Release CPM}}{\text{Maximum Release CPM} - \text{Spontaneous Release CPM}} \times 100
\]

where CPM represents the mean of triplicate counts per minute.

**RESULTS**

PBMC from the donor dogs were studied in one-way MLC before and after G-CSF or G-CSF/SCF administration. In seven of 13 donor-recipient pairs studied, there were significant decreases in the donor cells’ ability to respond to or stimulate cells from unrelated dogs after cytokine administration, consistent with either qualitative or quantitative changes in T cells and antigen-presenting cells. In addition, cells from nine of 13 of the cytokine-treated animals had a significant reduction in response to Con A as compared with baseline and controls. Results for one of the donor-recipient pairs in group 2 (D867→D869) are shown in Table 2. Cells from the donor D867 did not proliferate in response to allogeneic cells after G-CSF treatment, nor or did they stimulate allogeneic lymphocytes as well as before G-CSF. As all recipients had successful engraftment, the hyporesponsiveness in MLC did not impact on the in vivo function of the donor “graft facilitating” cells in either the DLA-identical or DLA-haploidentical setting. In addition, normal responsiveness or hyporesponsiveness did not predict for GVHD or its absence. Of 10 recipients with GVHD, four had donors that were hyporesponsive in MLC, and six had donors with normal responsiveness after cytokine mobilization.

NK function did not change in the 18 donors after cytokine mobilization, as shown by an example in Table 3.

The transplant results are shown in Table 1. A median of 17.1 × 10^6 PBSC were infused containing median numbers of 27 × 10^4 CFU-GM/kg, 52.8 × 10^6 CD34^+ cells/kg, and 13.7 × 10^7 CD8^+ cells/kg. Though the dogs in group 1 had a lower number of PBSC and CFU-GM infused due to the less efficient method of pheresis by discontinuous centrifugation of blood products, overall, there were no differences in the number of PBSC and CFU-GM infused between groups 2, 3, and 4. The addition of SCF to G-CSF did mobilize a higher number of CFU-GM per mL of blood (data not shown), which was demonstrated previously in the canine studies for autografts. All 18 recipients of PBSC engrafted successfully after 920 cGy TBI. In the more recent transplants, the number of CD34^+ cells, as determined by a polyclonal antibody against canine CD34, was found to be on the order of 4.6 × 10^6/kg.

Eight of the nine DLA-identical recipients developed acute GVHD, which was transient in five and fatal in three. One of the five dogs with transient GVHD died on day 68 with pancreatic failure, and the remaining four survived uneventfully as did the dog without GVHD. Three of the dogs were killed with intravenous (IV) Nembutal at the end of study on days 102, 110, and 118, while two were euthanized after 1 year. Marker studies (cytogenetics and/or (CA), repeats) showed complete donor cell engraftment at all time points and in all dogs studied.

All nine dogs receiving DLA-haploidentical littermate
PBSC engrafted very promptly but, along with engraftment, developed hyperacute GVHD. Dogs were euthanized with IV Nembutal injection because of deteriorating clinical condition. Autopsy findings confirmed the presence of a hematopoietic graft as well as of GVHD in skin, gut, and liver. Marker studies (cytogenetics and/or (CA), repeats) performed on peripheral blood and marrow showed the cells to be of donor type at all time points tested.

**DISCUSSION**

While the presence of hematopoietic stem cells in the peripheral blood of dogs has been known for more than 3 decades, recent developments have prompted us to reexamine the use of PBSC for allogeneic grafts. Developments have included the recognition that stem cells can be mobilized into the blood in large numbers by growth factors, the observation of faster engraftment with PBSC than with marrow, the increasing numbers of canine growth factors available for study, and, finally, the finding of diminished lymphocyte reactivity after growth factor treatment. The latter finding has raised both a concern and a hope. The concern was that the donors’ lymphocyte hyporesponsiveness would lead to problems with hematopoietic engraftment, particularly in the setting of DLA-haploidentical grafts, where donor lymphocytes have been shown to be crucial for engraftment of marrow. The hope was that diminished lymphocyte reactivity would result in lessened incidence and severity of GVHD with growth factor mobilized PBSC.

Despite the hyporesponsiveness in MLC of over half the donors tested after cytokine mobilization, all 18 animals engrafted. Furthermore, all of the DLA-haploidentical recipients developed three-system GVHD that required euthanasia. The incidence of GVHD in the DLA-identical littermates was similar to what had been previously observed in littermates given marrow alone after 920 cGy TBI in the absence of immunosuppression. While the animals in group 1 that received PBSC were mobilized from donors with G-CSF plus SCF received on the order of ½ to 1 log lower number of CD4 and CD8 cells than the dogs in group 2 that were mobilized with G-CSF alone, the incidence of GVHD was no different.

Of significance is the observation of engraftment in nine of nine recipients of DLA-haploidentical PBSC. Historically, 92% of recipients of DLA-nonidentical littermate marrow rejected their grafts after conditioning with 920 cGy TBI in the absence of immunosuppression or additional buffy coat cells. The addition of viable PBMC reduced that rate of rejection to 5% in this model, which is similar to the current study. The enhanced ability of the cytokine-mobilized cells to engraft as compared with marrow alone suggests either an increase in number or a change in function of the cells responsible for engraftment. The cells obtained after mobilization with G-CSF and G-CSF plus SCF were examined in MLC and NK assays which test T-cell and NK function in vitro, respectively. The cells from seven of 13 donors that were mobilized with either regimen failed to proliferate in response to allo-antigen in MLC, and nine of 13 were hyporesponsive to Con A, though still maintained normal NK function. This observation of hyporesponsiveness of the lymphocytes in MLC may be due to either a decrease in the ability of the cells to proliferate directly in response to allo-antigen or due to the presence of a new cell population that is inhibitory in vitro. These findings are consistent with those made with human cells after in vitro treatment with G-CSF. Despite this unresponsiveness of the cells in vitro, they are able to function both to engraft and mount a GVH response. Four of 10 recipients that developed severe GVHD received the “hyporesponsive” T cells. These studies were performed in the absence of postgrafting immunosuppression so as to fully determine the function of the donor lymphocytes in vivo after the cytokine mobilization. Two of the animals were kept alive for 1 year after transplant from group 2 to evaluate chimerism in the absence of immunosuppression, and all cells remained donor and there was no evidence of GVHD. To understand the mechanisms of hyporesponsiveness of the mobilized cells, a more detailed characterization of the lymphocyte populations will be necessary, including investigations of other cytokine receptors or lymphocyte activation markers.

**Table 2. Effect of G-CSF Therapy on MLC Responses**

<table>
<thead>
<tr>
<th>Responder Cells</th>
<th>Before G-CSF</th>
<th>After G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog No.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D867x</td>
<td>2,436</td>
<td>38,104</td>
</tr>
<tr>
<td>D869x</td>
<td>2,766</td>
<td>30,532</td>
</tr>
<tr>
<td>D379x</td>
<td>55,804</td>
<td>39,205</td>
</tr>
<tr>
<td>D867 (Donor)</td>
<td>2,481</td>
<td>393</td>
</tr>
<tr>
<td>D869 (Recipient)</td>
<td>1,704</td>
<td>875</td>
</tr>
<tr>
<td>D379 (Unrelated)</td>
<td>45,638</td>
<td>14,026</td>
</tr>
</tbody>
</table>

Data are means of triplicate CPM of 3H-thymidine incorporation. D867 was treated with G-CSF, D867, and D869 are DLA-identical littermates, and D379 is an unrelated DLA-nonidentical dog. x = cells were irradiated with 2,200 cGy.

**Table 3. Effect of G-CSF Therapy on NK Activity**

<table>
<thead>
<tr>
<th>Effector Cells Dog No.</th>
<th>Before G-CSF</th>
<th>After G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>D867 (Donor)</td>
<td>39.2</td>
<td>30.0</td>
</tr>
<tr>
<td>D869 (Recipient)</td>
<td>49.7</td>
<td>38.1</td>
</tr>
</tbody>
</table>

Data are the mean percent specific lysis of triplicates at an effector to target ratio of 60 to 1. D867 was treated with G-CSF, whereas D869 was not treated and served as a control. D867 and D869 are DLA-identical littermates.
These results are comparable to what has been observed in the preliminary analysis of matched allogeneic PBSC transplants in humans where, overall, 17% developed grades 3 to 4 acute GVHD, while 56% developed some form of chronic GVHD. While the incidence and severity of acute GVHD is similar to that seen with marrow alone, the incidence of chronic GVHD is compatible with the previous experience of adding buffy coat to marrow in patients with aplastic anemia. The data in humans for PBSC transplants is derived in the presence of various immunosuppressive regimens that make direct comparisons of PBSC to marrow more difficult. The dog model allows data to be derived in the absence of GVHD prophylaxis/therapy. Further studies will be done in the dog model involving T-cell and antigen-presenting cell depletion by CD34 selection to minimize the incidence of GVHD without causing graft rejection.

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Allogenic transplant of canine peripheral blood stem cells mobilized by recombinant canine hematopoietic growth factors

BM Sandmaier, R Storb, EB Santos, L Krizanac-Bengez, T Lian, PA McSweeney, C Yu, FG Schuening, HJ Deeg and T Graham