Lymphohematopoietic graft-vs.-host reactions can be induced without graft-vs.-host disease in murine mixed chimeras established with a cyclophosphamide-based nonmyeloablative conditioning regimen

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ABSTRACT

Mixed hematopoietic chimerism can be induced in mice receiving allogeneic bone marrow transplantation (BMT) after nonmyeloablative host conditioning with depletion T cells with of anti-T cell monoclonal antibodies (mAbs), low-dose (3 Gy) total-body irradiation (TBI), and local thymic irradiation (7 Gy). These mice are specifically tolerant to donor and host antigens. When nontolerant donor T cells are given to chimeras several months after BMT, full donor-type chimerism develops, but graft-vs.-host disease (GVHD) does not occur. The induction of such lymphohematopoietic GVH reactions without GVHD could provide an approach to separating graft-vs.-leukemia (GVL) from GVHD in patients with hematologic malignancies. To make the nonmyeloablative conditioning regimen described above more cytoreductive for such malignancies, we have now modified it by replacing TBI with cyclophosphamide (CP). Treatment with anti-CD4 and anti-CD8 mAbs on day –5, 200 mg/kg CP on day –1, and 7 Gy thymic irradiation on day 0 was only slightly myelosuppressive and allowed fully major histocompatibility complex (MHC)-mismatched (with or without multiple minor antigen disparities) allogeneic bone marrow to engraft and establish long-term mixed chimerism in 40 to 82% of recipients in three different strain combinations. The administration of nontolerant donor spleen cells at 5 weeks or at 5, 8, and 11 weeks posttransplant was capable of eliminating host hematopoietic cells, leading to full or nearly full donor chimerism in six of six and two of four chimeric animals in two different strain combinations. No clinical evidence of GVHD was observed in any recipients of these donor leukocyte infusions (DLI). These studies demonstrate that induction of mixed chimerism with nonmyeloablative conditioning followed at appropriate times by DLI might allow lymphohematopoietic GVH reactions, and hence GVL effects, to eliminate chronic hematologic malignancies without causing clinically significant GVHD.

KEY WORDS:
Mixed chimerism • Donor leukocyte infusion • Bone marrow transplantation • Graft-vs.-host disease

INTRODUCTION

BMT is the only curative therapy available for several hematologic malignancies. The most significant limitation to the transplantation of bone marrow across MHC barri-
phocytic leukemia, and chronic myelogenous leukemia [5]. Thus, host-reactive donor T cells can eliminate host cells that resist alloengraftment [6] and can also eliminate residual tumor cells. In patients with hematologic malignancies, such GVL effects are a major potential benefit of allogeneic BMT and might be further augmented if HLA-mismatched BMT could be performed routinely without excessive GVHD.

Mixed hematologic chimerism, the coexistence of two genetically different types of bone marrow, can be induced with lethal irradiation followed by BMT with a mixture of T cell–depleted host-type and allogeneic marrow cells [7]. Mixed chimerism also can be induced with allogeneic BMT after nonmyeloablative host conditioning, such as a regimen that includes anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) on day −5, followed by 3 Gy TBI and 7 Gy thymic irradiation on day 0. Mixed chimerism is associated with a state of donor- and host-specific transplantation tolerance [9], which is induced by an intrathymic deletional mechanism [9]. In these mice, GVHD is not induced by T cells present in the initial marrow inoculum because these are eliminated by anti-CD4 and anti-CD8 mAbs that persist in the recipient's serum for 1 to 3 weeks after BMT [10].

When nontolerant donor spleen cells were administered to stable mixed chimeras prepared with lethal irradiation and mixed syngeneic and allogeneic marrow transplants, complete repopulation with allogeneic cells ensued [11]. This lymphohematopoietic GVH reactivity exhibited by donor spleen cells was not accompanied by clinically evident GVHD. Similar to these results in mixed chimeras prepared with lethal irradiation [11], delayed DLI to mixed chimeras prepared with the previously described nonmyeloablative regimen could convert them to full donor-type chimeras without causing GVHD [M.G. Wang and M.S., unpublished data].

We hypothesized that the establishment of mixed chimerism and specific transplantation tolerance through a nonmyeloablative regimen and the subsequent lymphohematopoietic GVH reactions induced by delayed leukocyte infusions might be exploited to treat indolent hematologic malignancies, such as chronic lymphocytic leukemia, in which immediate cytoablation by chemotherapy or radiotherapy (or both) would not be essential for survival. These diseases often afflict older persons who are subject to a high risk of complications after conventional BMT but who might be better able to tolerate a less toxic, nonmyeloablative conditioning approach. We hypothesized further that GVHD might not be induced by infusions given after a sufficient delay to recipients originally conditioned with a mild regimen and that this might be a useful approach to separating lymphohematopoietic GVH reactions, which can mediate potent GVL effects, from GVHD. We have now developed a cyclophosphamide (CP)-based, nonmyeloablative BMT treatment strategy that would be expected to have cytokinetic activity for a variety of hematologic malignancies and that allows the induction of mixed bone marrow chimerism. The ability of delayed donor leukocyte infusions to mediate lymphohematopoietic GVH reactions without causing GVHD, which could have potential as antileukemic immunotherapy, was evaluated.

MATERIALS AND METHODS

Animals

B10.BR (H-2b) male and female donor mice purchased from the Jackson Laboratory (Bar Harbor, ME) were used at 7 to 12 weeks of age. Female BALB/c (H-2k) recipient mice were purchased from Frederick Cancer Research Facility, National Cancer Institute, and used after 8 weeks of age. In other experiments, B10.A or A/J donors (both H-2b, KkIkDd), and C57BL/6 (B6) (H-2b) recipients, all purchased from the Frederick Cancer Research Facility, were used. All mice were housed in autoclaved microisolator environments, and all manipulations were performed in a laminar flow hood.

Preparation of chimeras

Control chimeras were prepared using our standard nonmyeloablative regimen, as previously described [8], and are referred to as “standard nonlethal regimen” (NLR) chimeras. Recipient BALB/c or B6 mice were treated on day −5 with depleting anti-CD8 mAb 2.43 [12] (1.4 mg) and depleting anti-CD4 mAb GK1.5 [13] (2.0 mg) intraperitoneally (i.p.) in sterile Hanks’ balanced salt solution as described. On day 0, recipient mice were treated with 7 Gy thymic irradiation from a cobalt-60 source and 3 Gy TBI from a cesium-137 source. CP chimeras were prepared identically except that CP was given instead of TBI.

CP (Mead Johnson and Pharmacia) was reconstituted using sterile water to a concentration of 20 mg/mL up to 24 hours before injection. Reconstituted CP was stored in polypropylene tubes as directed. On day −1, mice received 100 mg/kg or 200 mg/kg cyclophosphamide i.p.

Bone marrow was harvested and single-cell suspensions were prepared as described previously [14]. Approximately 4 hours after the completion of irradiation, each recipient was injected intravenously through a tail vein with 14–20×10⁶ donor bone marrow cells (BMC).

Flow cytometric analysis of peripheral white blood cells and hematopoietic tissues

Chimerism was assessed by two-color flow cytometry (FCM) using a Becton Dickinson (Mountain View, CA) FACScan cytometer. After preparation of white blood cells (WBC) by deionized water lysis, cells were incubated with monoclonal antibodies. To reduce nonspecific mAb binding, anti-Fc receptor mAb 2.4G2 [15] was added to all tubes. Cells were stained as described [16] with HOPC1-fluorescein isothiocyanate (FITC) (a nonreactive control antibody prepared in our laboratory); biotinylated (BIO) anti-H-2Dd mAb 34-2-12 [17], FITC-conjugated anti-H-2Kd mAb 36-7-5 [18], FITC-conjugated anti-H-2Kb antibody 3F1 [19], or FITC-conjugated anti-D2 mAb KH95 (Pharmingen, San Diego, CA); and phycoerythrin (PE)-conjugated anti-CD4 and anti-CD8 mAbs or PE-conjugated rat IgG2a (a nonreactive control antibody) purchased from Pharmingen. Staining by BIO mAbs was developed by incubation with FITC-conjugated avidin (FA) for 10 minutes followed by washing. Propidium iodide, which is taken up by dead cells, was added to all tubes immediately before FACScan analysis to allow gating of live cells. Ten thousand live events were collected and analyzed. Lymphocyte, monocyte, and granulocyte populations were distinguished using forward-by-side scatter dot plots in combination with staining of control cells with mAb.
Mac1. For quantitative analysis, percentages of positive cells were determined using the following formula:

\[
\frac{(\text{chimera % positive} - \text{negative control % positive})}{(\text{positive control % positive} - \text{negative control % positive})} \times 100\%
\]

after values were corrected by subtraction of cells staining non-specifically with nonreactive control mAbs. For each H-2 class I allele-specific mAb, the mouse strain (donor or host) not bearing the allele recognized by the mAb was the negative control and the strain expressing the allele was the positive control. In some studies, WBC analysis was performed by staining with FITC-labeled anti-IgM or B220 (Pharmingen) to detect B cells, anti-CD4 or anti-CD8 to detect T cell subsets, and Mac1 mAb to detect myeloid cells, vs. BIO H-2 allele-specific mAb, plus phycoerythrin-streptavidin (PEA). In these studies, the percentage of donor among B cells and T cell subsets was determined in the lymphoid gate, and that among Mac1-positive cells was determined in the granulocyte and monocyte gates. Control staining with irrelevant mAb HOPC-1 or rat IgG2a was subtracted from the percentage of donor and host-type cells detected in each quadrant of a two-color dot plot. Percentages of donor-derived cells in each marker-positive subpopulation were calculated as the fraction of all cells in that subpopulation that were donor-derived multiplied by 100%.

For analysis of bone marrow, splenocyte, and thymocyte populations, single-cell suspensions were prepared, and two-color FCM was performed using anti-donor class I-specific mAb vs. rat anti-mouse IgM or B220 mAb, anti-CD4 and CD8 mAbs, and Mac1 mAbs. Control staining with irrelevant mAb HOPC-1 or rat IgG2a was subtracted from the percentage of donor- and host-type cells detected in each quadrant of a two-color dot plot. Percentages of donor-derived cells in each marker-positive subpopulation were calculated as the fraction of all cells in that population that were donor derived multiplied by 100%. Percentages of donor-derived bone marrow cells and thymocytes are reported as the net percentage of cells staining with donor class I-specific mAb.

**Complete blood counts**

Peripheral blood samples obtained from tails were collected in heparin-coated Eppendorf tubes. Samples were diluted in diluent buffer and analyzed by a Serono Baker 9000 automated complete blood count (CBC) instrument (Allentown, PA). Results are presented as mean values with standard deviations.

**Donor spleen cell infusions to mixed chimeras**

Chimeras were prepared as described above. Peripheral blood chimerism was assessed by FCM on day 14. Using these results, similar groups of CP chimeras were chosen on the basis of the level of donor repopulation and the presence or absence of donor T cells. Intravenous injections of 30×10^6 spleen cells from mice syngeneic to the original marrow donor strain were given at day 35 and, where indicated, at 8 and 11 weeks. Spleens were harvested under sterile conditions, red cells were lysed in ACK lysing buffer, and single-cell suspensions were prepared and counted.

**Statistical analysis**

Group means were compared using Student’s t test. A p value <0.05 was considered significant.

## Results

**Effects on hematopoiesis of conditioning with a CP-based nonmyeloablative regimen**

To determine whether CP could replace TBI for the induction of mixed chimerism in a nonmyeloablative regimen that includes host T cell depletion and thymic irradiation, we transplanted fully MHC- and multiple minor histocompatibility antigen–mismatched marrow to mice receiving 100 mg/kg or 200 mg/kg CP in place of 3 Gy TBI. BALB/c mice were conditioned with anti–T cell mAbs on day −5, CP at 100 mg/kg or 200 mg/kg on day −1, and thymic irradiation (7 Gy) on day 0. They were then either left untreated or given B10.BR BMC on day 0. A control group received our standard NLR, in which 3 Gy TBI is given on day 0 instead of CP. These mice received B10.BR BMT similar to the standard NLR [8]. Both CP-based regimens were nonmyeloablative, as all animals survived, even without BMT. CBCs were performed at early time points, and mean values with standard deviations for each group are presented in Fig. 1. Although a transient decline in WBC counts was observed on day 4 in recipients of either CP dose, these counts recovered to just below the normal range by day 7. The group receiving the TBI-containing standard NLR showed persistence of the marked depression in WBC counts at day 7, whereas WBC counts in CP-treated mice had increased substantially above their nadir. In CP-treated mice, no differences were observed in the WBC nadir or in the rate of recovery in animals receiving BMT compared with those receiving conditioning alone, and a CP dose effect was not apparent. All of the groups recovered normal WBC counts by 27 to 42 days after BMT (Fig. 1). None of the CP-treated groups, regardless of whether BMT was given, showed significant declines in hemoglobin concentrations, with the exception of a slight and transient decrease on day 7 in recipients of 100 mg/kg CP plus BMT (Fig. 1, middle panel). The group that received the standard 3-Gy TBI-conditioning regimen without CP showed a significant decline in platelet count on day 7, but this recovered to normal levels by day 14. CP-treated groups did not show significant reductions in platelet counts in this period (Fig. 1, bottom panel).

**Mixed chimerism in recipients of CP-based conditioning**

Two-color FCM analysis was performed on peripheral WBC to determine the level of donor reconstitution in various lymphoid and myeloid lineages over time in the BMT groups receiving CP-based conditioning. Variable levels of donor reconstitution were detected in mice receiving the two different CP doses (Fig. 2). Although persistent chimerism developed in only one of five mice receiving BMT after anti–T cell mAbs, thymic irradiation, and 100 mg/kg CP, higher rates were achieved in the group that received 200 mg/kg CP. Four of five mice in this group showed chimerism for the first 6 weeks after BMT, and three of these animals showed persistent chimerism at the last day of follow-up, between 10 and 31 weeks posttransplant. All chimeras conditioned with the CP-based regimen showed higher levels of donor reconstitution in the lymphoid than in the myeloid lineages, with only minimal granulocyte and monocyte chimerism detectable (Fig. 2).
In two repeat experiments, persistent multilineage WBC chimerism (follow-up 6 to 17 weeks) was achieved in six of nine mice receiving the regimen containing 200 mg/kg CP. Combining all three experiments, the overall persistent WBC chimerism rate was 64% in mice receiving this regimen. Mice were killed at 9 and 12 weeks post-BMT to allow evaluation of chimerism in additional lymphohematopoietic tissues. Data from representative animals are presented in Fig. 3. Five of five animals showed measurable chimerism to varying degrees in some or all of the thymus, spleen, lymph node, and marrow. One animal killed at 9 weeks had already shown a loss of WBC chimerism by 7 weeks post-BMT and had low levels of donor T and B cells in the spleen and lymph node but did not show marrow or thymic chimerism. A second animal killed at 12 weeks (animal 1 in Fig. 3), which had declining but measurable WBC chimerism at 11 weeks, showed a similar pattern of chimerism. The other three CP-chimeras, which had shown higher levels of WBC chimerism before death, all showed significant chimerism among splenic and lymph node T and B cells and low but measurable donor representation among mature thymocytes and bone marrow cells (e.g., animals 2 and 3 in Fig. 3).

To determine whether chimerism could be achieved with a CP-based regimen in additional strain combinations, we performed transplants in the B10.A to B6 (full MHC disparity) and A/J to B6 (full MHC plus multiple minor antigen disparities) strain combinations using the higher-dose CP-based conditioning regimen. In the B10.A to B6 combination, lasting WBC chimerism developed in a total of nine of 11 recipients in two separate experiments followed up to 25 weeks (data not shown). The chimerism rate was somewhat lower in the A/J to B6 combination, with a total of four of 10 animals showing lasting (up to 25 weeks) chimerism in two separate experiments (data not shown). In both strain combinations, similar to results described above for the B10. BR to BALB/c combination, chimerism was higher in the lymphoid lineages than in the myeloid lineages.

Animals in both the B10.A→B6 and A/J→B6 strain combinations were killed 21 to 28 weeks post-BMT. As shown in Table 1, six of nine A/J→B6 and seven of seven B10.A→B6 chimeras showed the presence of donor T
and/or B cells in the spleen at the time of sacrifice; five of nine and four of nine, respectively, showed thymic chimerism, and six of nine and seven of seven, respectively, showed low levels of marrow chimerism at the time of death.

**Donor leukocyte infusions beginning at 5 weeks can increase chimerism without inducing clinical GVHD**

We next evaluated the ability of delayed donor leukocyte infusions (DLI) to eliminate host-type hematopoietic cells without causing GVHD. BALB/c mice received anti-T cell mAbs on day –5, 200 mg/kg CP on day –1, and 7 Gy thymic irradiation on day 0, followed by $20 \times 10^6$ B10.BR donor BMC on day 0. Three groups of animals ($n=3$ each) with similar levels of chimerism were selected, and B10.BR donor-type spleen cells ($3 \times 10^7$) were administered at various times thereafter to two of the three groups. As shown in Fig. 4, DLI at 5 weeks resulted in a dramatic increase in repopulation by donor cells in three of three animals, which was evident in all lineages by 10 weeks post-BMT and persisted at 16 weeks post-BMT. Multiple DLI at weeks 5, 8, and 11 post-BMT also led to conversion to complete donor repopulation in the monocyte and granulocyte lineages by 10 weeks post-BMT in three additional chimeras. While host monocytes, granulocytes, and most B cells (not shown) had been eliminated by 16 weeks in mice receiving DLI at 5 weeks and those receiving DLI at 5, 8, and 11 weeks, elimination of host T cells after DLI occurred more slowly. In these mice, up to 40% of all T cells were still of host origin at 16 weeks. Overall, compared with non-DLI controls in the same experiment, a dramatic increase in donor reconstitution was seen in six of six mice receiving DLI (Fig. 4). The fact that conversion occurred in all lineages, including myeloid cells that turn over rapidly, indicates that the increase in donor WBC reflected a change in the balance of donor vs. host hematopoiesis rather than mere expansion of the T cells administered in the DLI.

Two recipients each of single DLI (at 5 weeks) and of multiple DLI (at 5, 8, and 11 weeks), were killed more than a year after BMT (~14 months). All of these animals showed complete donor reconstitution among splenic T and B cells. Both recipients of multiple DLI and one recipient of a single DLI showed full donor marrow repopulation, but one recipient of a single DLI still contained about one-third host-derived cells in the marrow at the time of sacrifice. No evidence of lymphoid atrophy, which could be a subtle manifestation of GVHD, was seen, as lymphoid tissues were similar in size to those of mice of the same age killed at the same time (data not shown). Unfortunately, all of the non-DLI CP chimeras had died by this time and were not available for chimerism analysis.

One non-DLI chimera in this experiment died on day 61 of unknown cause. All DLI recipients survived and showed no clinical evidence of GVHD. As shown in Fig. 4, there was no difference in the weights of the mice receiving DLI compared with the non-DLI controls. Thus, donor leukocyte infusions given at 5 weeks or at 5, 8, and 11 weeks led to a significant lymphohematopoietic GVH reaction that was associated with destruction of host-type hematopoietic cells without causing clinically significant GVHD.

We also evaluated the effect of DLI in the A/J→B6 strain combination. Six A/J→B6 BMT recipients were given DLI on day 35 post-BMT. Two of these mice had already lost chimerism in most lineages before DLI, and these did not recover any measurable chimerism after DLI. Two additional mice still had low levels of chimerism at 4 weeks and did not show an increase in chimerism after DLI. Both of these mice eventually lost WBC chimerism (Fig. 5). Finally, the two mice showing the highest levels of chimerism at 4 weeks, before DLI, converted to become full donor chimeras by about 7 to 10 weeks post-BMT. One of these animals remained fully donor-type in all lineages, whereas the other later developed “split” chimerism, with the

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**Figure 3.**

Chimerism in bone marrow, spleen, and thymus 12 weeks after bone marrow transplantation (BMT) of BALB/c mice that received T cell–depleting monoclonal antibodies on day –5, 7 Gy thymic irradiation on day 0, and 200 mg/kg cyclophosphamide on day –1, followed by B10.BR BMT on day 0. **Top panel:** thymic chimerism. **Middle panel:** splenic chimerism. ■, percent donor CD4 cells; □, percent donor CD8 cells; □, percent donor B cells. Data are normalized to 100% for each lineage, so that donor- plus host-derived cells total 100%. **Bottom panel:** Bone marrow chimerism. ■, percent donor-derived cells among low forward scatter/low side scatter cell population; □, percent donor-derived cells in high forward scatter/high side scatter cell population. Results from individual animals shown here are similar to those obtained for animals sacrificed at 9 weeks post-BMT (not shown).
appearance of ~50% host-derived myeloid cells and 12% host-derived B cells at 14 weeks, while CD4 (Fig. 5) and CD8 (not shown) T cells remained fully donor in origin. Non-DLI controls in the same experiment did not show an increase in donor repopulation over time (e.g., an average of 12.1, 15.8, 1.1, 4.5, and 3.9% of WBC CD4 cells, CD8 cells, B cells, granulocytes, and monocytes, respectively, were donor derived at 18 weeks; n=4), indicating that the DLI was responsible for this increase.

The animals receiving DLI at 5 weeks, which increased

Table 1. Chimerism in tissues of A/J→B6 and B10.A→B6 bone marrow transplant recipients

|                 | Spleen |  |  |  |  |
|-----------------|--------|--------|--------|--------|--------|--------|
|                 | CD4    | CD8    | B cells | Thymus  | Marrow |
| A/J controls (n=5)*  | 99.9 ± 0.2 | 99.9 ± 0.2 | 99.9 ± 0.2 | 64.5 ± 12.8 | 93.4 ± 4.9 |
| B10.A controls (n=6)* | 99.9 ± 0.2 | 99.9 ± 0.2 | 99.9 ± 0.2 | 63.6 ± 13.0 | 95.4 ± 4.7 |
| B6 controls (n=12–13)* | 0.6 ± 1.1 | 0.1 ± 0.3 | 1.2 ± 1.4 | 0.2 ± 0.4 | 0.7 ± 1.9 |
| A/J→B6 BMT‡ | 31.5 ± 35.4 | 24.4 ± 40.5 | 29.2 ± 30.0 | 11.7 ± 6.9 | 12.7 ± 13.3 |
| B10.A→B6 BMT‡ | 9.7 ± 6.5 | 3.4 ± 2.3 | 9.4 ± 3.6 | 4.9 ± 5.1 | 5.5 ± 5.4 |
| Chimeras/total‡ | 6/7 | 5/7 | 7/7 | 4/7 | 7/7 |

Data are mean ± SD.

*Controls are untreated mice of the indicated strain that were analyzed at the time the mixed chimeras included in Table 1 were killed.

†Number of animals showing chimerism/total number of animals analyzed. Chimerism is defined as having % Dd+ cells more than 2SD above the mean for B6 controls.

‡Mean % Dd+ cells for animals defined as showing chimerism.

Figure 4.

Effect of day 35 donor lymphocyte infusions (DLI) in mixed chimeras prepared with the CP-based regimen in the B10.BR→BALB/c strain combination. In a single experiment, animals received no DLI (n=3), DLI at 5 weeks (n=3), or repeated DLI at 5, 8, and 11 weeks (n=3). Chimerism in the T cell (CD4 plus CD8 positive) and granulocyte populations of white blood cells is shown. Mean weights are shown for each group in the upper right panel. For the group that received a single DLI at 5 weeks, p < 0.05 for granulocytes at 10 weeks and 17 weeks, and for T cells at 17 weeks, compared with the group not given DLI. For the group that received repeat DLI, p < 0.05 at 10 and 17 weeks compared with the no-DLI group for both T cells and granulocytes.
their level of donor chimerism, showed perfect health throughout the experiment, with no weight loss or clinical evidence of GVHD (Fig. 5). Thus, DLI at 5 weeks could convert a subset of mixed chimeras to full donor chimeras in the A/J→B6 strain combination without causing clinically significant GVHD. However, conversion to full donor chimerism occurred only in the animals showing the highest levels of chimerism shortly before the time of DLI (Fig. 5). At the time of death 26 weeks posttransplant, spleen cell yields were similar to those of controls not receiving DLI, but thymocyte yields were lower than those of controls in one of two DLI mice that converted to full chimerism, raising the possibility that the GVH reaction may have induced thymic atrophy in this animal.

DISCUSSION

In the studies reported here, we have modified a previously established nonmyeloablative method of inducing mixed chimerism by using CP in place of low-dose TBI in the conditioning regimen. The original regimen on which the present studies were based was developed for the purpose of inducing mixed chimerism and transplantation tolerance while avoiding the use of toxic, myeloablative chemoradiotherapy [8]. The goal of the present studies was to modify the regimen to make it applicable to the treatment of patients with chronic hematologic malignancies. Successful engraftment of donor marrow after conditioning with a milder, nonmyeloablative conditioning regimen might be used to set the stage for donor leukocyte infusions as immunotherapy. Infusions of donor leukocytes that contained large numbers of T cells converted mixed chimeras prepared with another regimen to full donor-type chimeras, without causing GVHD [11]. Because the number of T cells administered in these DLI would have been sufficient to cause rapidly lethal GVHD in freshly conditioned mice, we hypothesized that resistance to GVHD increased with time after conditioning and BMT [11]. A similar demonstration in mice conditioned with a nonmyeloablative regimen for the induction of mixed chimerism [M.G. Wang and M. S., unpublished data] confirmed that GVH alloreactions directed at host alloantigens could eliminate host hematopoietic cells without causing GVHD. “Lymphohematopoietic GVH reactions” suggested a novel approach to separating GVHD and GVL. The present studies were aimed at modi-

Figure 5.
Effect of day 35 donor leukocyte infusions on white blood cell chimerism among CD4 T cells (top left), B cells (top right), and granulocytes (bottom left), and on weights (bottom right) in CP chimeras prepared in the A/J-B6 strain combination. B6 mice received T cell-depleting mAbs on day –5, 7 Gy thymic irradiation on day 0, and 200 mg/kg CP on day –1, followed by A/J bone marrow transplantation on day 0. Each line represents an individual animal in all four panels (n=6). The weight curves are shown with the same symbols used in the chimerism curves. CD8 cells had chimerism patterns (not shown) similar to those of CD4 cells. A single experiment was performed.
fying our existing nonmyeloablative conditioning regimen to include an agent that would be cytoreductive for a variety of lymphohematopoietic malignancies and at determining whether delayed DLI could then be used to mediate lymphohematopoietic GVH reactions (and hence GVL) without inducing GVHD.

The results presented here demonstrate that it is possible to establish and maintain mixed chimerism using a modification of our nonmyeloablative regimen that replaces TBI with CP. The higher dose of 200 mg/kg CP was more successful in permitting long-lasting multilineage chimerism than was the lower dose of 100 mg/kg. Although transient and mild myelosuppression occurred in mice conditioned with this regimen, the leukopenia was less marked than that observed in mice receiving our standard TBI-containing nonmyeloablative regimen, and no significant thrombocytopenia, anemia, or other toxicity was observed. Lasting chimerism was achieved in three different strain combinations involving BMT across complete MHC barriers, although the success rate varied from ~40% in the A/J→B6 strain combination to 64% in the B10.BR→BALB/c and 82% in the B10.A→B6 strain combinations. Because the A/J→B6 and B10.A→B6 strain combinations both involved H-2^a donors and H-2^b recipients, the non-MHC background of the donor strain or the presence of multiple minor histoincompatibilities in addition to complete MHC disparity (or both), influenced the ability to achieve lasting chimerism. Although this variability in results in different strain combinations raises concern about the applicability of this approach to outbred human populations, it should be borne in mind that it is unlikely that transplantation from fully MHC-mismatched donors, as we have done in these animal studies, would be necessitated in humans with hematologic malignancies. Most individuals have a relative who shares at least one HLA haplotype, and often one or more loci on the second haplotype also share identical alleles. Thus, it will be important to repeat our studies in mouse strain combinations differing at one MHC haplotype or less. We predict that chimerism and conversion to full donor chimerism with DLI will be more universally achievable with lesser degrees of MHC disparity.

In all three strain combinations, higher percentages of donor cells were observed among the lymphoid lineages than among monocytes and granulocytes. These results contrast significantly with those obtained in chimeras prepared with our standard TBI-containing nonmyeloablative regimen, in which high levels of myeloid chimerism are achieved and in which B cell and myeloid chimerism are usually observed at similar levels at steady state [20]. These results may reflect the higher degree of immunosuppression achieved with CP compared with 3-Gy TBI, which appears to be more myelosuppressive (Fig. 1) [21,22]. Selective depletion of recipient lymphocytes may allow higher levels of initial reconstitution by donor-derived common lymphoid progenitors [23] than by donor myeloid progenitors. The higher degree of myelosuppression achieved with TBI probably allows higher levels of donor pluripotent hematopoietic stem cell engraftment to be achieved, thus explaining the higher long-term plateau levels of donor chimerism achieved with the standard TBI-containing regimen [20,8] compared with the CP-based regimen described here.

Delayed administration of donor leukocytes at day 35 and later was able to convert some of the mixed chimeras to full or nearly-full donor-type chimeras without inducing clinically significant GVHD in any mice. This effect was more consistent in the B10.BR→BALB/c strain combination than in the A/J→B6 combination, in which the incidence of lasting chimerism tended to be lower in non-DLI controls. The number of donor T cells administered in the DLI here is sufficient to cause severe, lethal, acute GVHD in fresh, lethally irradiated recipients [24–26]. Studies in fully allogeneic chimeras, which are also resistant to the induction of GVHD by delayed allogeneic T cell administration [11,27–30], indicate that the time available for host recovery from conditioning treatment is an important factor in determining resistance to GVHD. Conditioning therapy is likely to cause some epithelial tissue injury, with production of proinflammatory cytokines [31,32] and chemokines, adhesion molecule upregulation on endothelial cells, and hence T cell migration into the GVHD target tissues. By waiting sufficiently long for the recipient to recover from conditioning treatment, it may be possible to induce GVH reactions that are confined within the lymphohematopoietic system only. This type of allosresponse is highly desirable, as it may be associated with GVL reactions [28]. Consistent with these results, DLI have been used to induce remissions in patients with chronic myelogenous leukemia who relapse after allogeneic BMT. Although many of these patients develop GVHD, it has been suggested that the incidence and severity may be lower than expected for the large number of donor T cells given [33–35].

A second factor that may confer resistance to GVHD may be the mixed chimeric state itself. Consistent with a role for host hematopoietic cells in suppressing GVHD, the initial presence of host hematopoietic elements has been shown to mitigate the severity of GVHD in rodents [29,36]. “Natural suppressor” cells, which recover first from host marrow in mixed marrow recipients [37] and possibly other host-derived cell populations, may play a role in this effect. The spontaneous development of mixed chimerism has been associated with a lower incidence of GVHD (and a higher incidence of graft failure) in some human studies of conventional allogeneic BMT [38,39], but not in others [40]. Because animals receiving DLI demonstrate lymphohematopoietic GVH reactions that convert them to full chimeras, any regulatory cell populations that play a role in preventing GVHD must have highly restricted activities, perhaps preventing adhesion molecule upregulation or the production of critical cytokines by donor T cells, without globally preventing GVH alloreactivity.

Preliminary data from a pilot clinical trial that we have initiated based on the murine studies reported here are strongly supportive of the possibility that mixed chimerism is associated with increased resistance to GVHD. Less severe GVHD than expected has been observed in patients receiving allogeneic BMT after conditioning with anti-thymocyte globulin, high-dose CP, and thymic irradiation, followed by DLI on day 35 or later [41–43]. Similar to the murine studies described here, these patients develop mixed chimerism initially, and the delayed administration of DLI can convert them to complete donor chimeras without causing GVHD. Preliminary results have shown striking anti-lymphoma responses in these patients, even in the absence of GVHD.
demonstrating that this approach can indeed be used to separate GVHD from GVL. Our clinical study also demonstrates that lasting, high-level, multilineage mixed chimerism can be induced intentionally across MHC barriers in adult humans receiving cells treated with nonmyeloablative conditioning protocol based on the strategy described here. Other BMT regimens involving HLA-identical sibling or matched-unrelated BMT after nonmyeloablative conditioning with purine analog–containing combinations of chemotherapeutic agents led to high levels of donor reconstitution (rather than lasting mixed chimerism) in recipients of HLA-matched sibling marrow [44,45], but matched unrelated donor marrow failed to engraft in two of two patients [46]. The dominant donor reconstitution and failure of engraftment of matched-unrelated marrow transplants in one of these studies [46] suggests that the host conditioning used by these groups may be less immunosuppressive, and perhaps more myelosuppressive, than our strategy, which involves depleting T cells with antibody and thymocytes with irradiation, along with CP for additional immunosuppression and myelosuppression. Using a similar strategy, HLA-mismatched marrow engraftment is clearly achievable [42].

One potential toxic effect of lymphohematopoietic GVH reactions might be atrophy of recipient lymphoid tissues and associated GVH-induced immunodeficiency [47]. Indeed, we have previously shown that DLI administered to mixed chimeras prepared with lethal conditioning induces transient immunodeficiency without clinical GVHD, with eventual recovery of immune function in most animals [11]. Although we did not see clear evidence of lymphoid atrophy in most long-term DLI recipients at the time of death in this study, further studies are needed to evaluate the degree of immune responsiveness remaining in these mice.

In summary, the studies presented here demonstrate that lasting, low-level multilineage mixed chimerism can be induced across complete histocompatibility barriers in mice using a nonmyeloablative conditioning regimen that consists of T-cell-depleting mAbs, CP, and thymic irradiation and that administration of DLI at 5 weeks and later can convert the mixed chimeras to full donor-type chimeras without causing clinically evident GVHD. The incidence of chimerism and the ability of DLI to cause this conversion in fully mismatched strain combinations is somewhat variable, and further studies are needed to determine the outcome of this approach in more clinically relevant, less disparate strain combinations. In view of results (to be presented elsewhere) showing that earlier DLI can have adverse consequences, including loss of chimerism or the development of GVHD, additional work is needed to elucidate the kinetics and mechanisms of the development of host-vs.-graft tolerance and of resistance to GVHD by day 35 in this model. However, the relative safety with which DLI can be administered at late times post-BMT while mediating potent lymphohematopoietic graft-vs.-host responses in mixed chimeras prepared with nonmyeloablative conditioning suggests a rational approach to separating GVHD from GVL.

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