Delayed Infusion of Immunocompetent Donor Cells After Bone Marrow Transplantation Breaks Graft-Host Tolerance and Allows for Persistent Antileukemic Reactivity Without Severe Graft-Versus-Host Disease

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The development of graft-host tolerance after bone marrow transplantation (BMT) is crucial to avoid the problems of graft-versus-host disease (GVHD) and graft rejection. GVHD can be eliminated by depleting mature donor T cells from the BM inoculum, thereby facilitating the development of graft-host tolerance. However, T-cell depletion often results in an increased incidence of graft rejection and an increased frequency of leukemia relapse. Thus, although graft-host tolerance is a desirable outcome, it can pose a significant threat to leukemia-bearing hosts. Using a major histocompatibility complex (MHC)-matched allogeneic model of BMT (B10. BR into AKR), we found that irradiated recipients given donor BM alone displayed mixed T-cell chimerism and did not develop GVHD. Graft-host tolerance developed by 8 weeks after BMT in these chimeras, and they were susceptible to low-dose leukemia challenge. When sufficient numbers of donor spleen cells, as a source of T cells, were added to the BM graft, AKR hosts developed severe and lethal GVHD. Antihost reactive donor T cells persisted in chimeras undergoing GVHD, indicating that graft-host tolerance did not develop. When administration of the spleen cells was delayed for 7 to 21 days after BMT, there was significantly less mortality because of GVHD. Day 21 was the optimal time for infusion of cells without development of GVHD. Graft-host tolerance was broken by the delayed infusion of donor cells, as indicated by the persistence of antihost-reactive donor T cells in these chimeras in T-cell receptor cross-linking and mixed lymphocyte reaction assays. Importantly, the persistence of antihost-reactive donor T cells correlated with a long-term antileukemic effect that was still present at 100 days after transplant. Multiple infusions of immunocompetent donor cells could be administered without increasing the risk for GVHD if delayed until 21 days post-BMT. Delayed infusions of donor spleen cells also resulted in a long-term antileukemic effect in the absence of GVHD in an MHC-haplotypemismatched model of BMT (SJL into [SJL x AKR]F1). Although delayed infusion of normal donor cells did not induce GVHD, spleen cells from donors previously sensitized to host alloantigens induced GVHD when infused 21 days after BMT. Thus, the ability of previously activated cells to induce GVHD was not inhibited in the same manner as naive cells. Results from limiting dilution analysis assays indicated that alloactivated interleukin-2–secreting CD4⁺ T cells were preferentially inhibited over cytolytic T cells. Because CD4⁺ T cells are required for the induction of GVHD in these murine BMT models, selective inhibition of CD4⁺ T-cell activation may explain why significant GVHD did not occur in chimeras infused with donor cells 21 days after BMT.

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SELF-TOLERANCE is a central tenet of immunology. Acquisation of immune tolerance is important for the success of allogeneic bone marrow transplantation (BMT), where the graft and host must develop a state of mutual tolerance to avoid graft-versus-host (GVH) or graft rejection outcomes, respectively. Although graft-host tolerance is desirable with regard to GVH disease (GVHD) and graft rejection, the acquisition of tolerance may be detrimental for patients with residual leukemia, i.e., those not cured by pretransplant chemoradiotherapy.

The antileukemic reaction associated with allogeneic BMT is referred to as the graft-versus-leukemia (GVL) effect. GVHD and the GVL effect are intimately associated, and separation of the two phenomena has been somewhat problematic. One strategy that is currently being used in an attempt to separate these two phenomena is the delayed administration of donor cells to BMT recipients for the treatment of relapsed leukemia. It was initially shown in 1962 that donor spleen cells could be given to mouse allogeneic BMT recipients during the posttransplant period without causing GVHD. Additional studies in dogs and mice during the 1970s confirmed that earlier observation, and more recent studies have also shown the ability to diminish GVHD by delayed administration of donor spleen cells. The potential for using infusions of donor cells post-BMT as a form of antitumor immunotherapy was not realized until recently. In 1990, Kolb et al first reported the successful use of post-BMT donor cell infusions for the treatment of relapsed chronic myeloid leukemia (CML). Since that report, a number of other clinical trials have also shown the antileukemic effect of delayed donor cell infusions. Clinically, treated patients have still developed GVHD; however, the GVHD has been relatively mild considering the large numbers of donor T cells infused. It is unknown why GVHD is less severe when the infusion of donor cells is delayed or how soon donor cells can be infused without causing severe GVHD. In addition, the impact of post-BMT donor cell infusions on graft-host tolerance has not been well studied.

Initial studies in our laboratory using major histocompatibility complex (MHC)-matched B10.BR donors and AKR hosts showed that a single injection of normal donor cells on day 21 after BMT provided for an in vivo antileukemic reaction in the absence of GVHD. The present report shows that delayed infusion of donor cells breaks the development of graft-host tolerance. The broken state of immune tolerance did not result in the development of GVHD, but allowed...
for the persistence of antileukemic reactivity in donor-cell infected chimeras. Furthermore, multiple infusions of matched or haplotype disparate donor spleen cells could be safely administered, resulting in even greater GVL reactivity.

MATERIALS AND METHODS

Animals. Female AKR/J (H-2^d), B10.BR (H-2^b), and SJL (H-2^b) mice (8 to 12 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). (SJL × AKR)F1 (H-2^k) were bred in the AAALAC accredited Animal Resource center of the Medical College of Wisconsin (Milwaukee, WI), where all mice were housed.

Marrow transplants. BM was flushed from donor femurs with Hank’s balanced salt solution (HBSS) using syringes fitted with 25 G needles. Spleens were pressed through wire mesh screens to obtain single cell suspensions. Erythrocytes were removed from the spleen cell suspensions by hypotonic lysis with sterile distilled water. BM and spleen cells were washed twice with HBSS and counted.

AKR/J or (SJL × AKR)F1 recipient mice were given a single dose of 9 Gy total body irradiation (TBI) using a Shepherd Mark I cesium irradiator (J.L. Shepherd and Associates, San Fernando, CA) 2 to 4 hours before transplant. Irradiated recipients received a single intravenous injection of 10^7 nucleated BM cells with or without added spleen cells. Additional groups of mice were intravenously injected with single or multiple injections of spleen cells at various time points after transplant with BM cells alone. In some experiments, donor spleen cells had been sensitized to host alloantigens by injecting the donor mice intraperitoneally with 1×10^9 host spleen cells 3 and 6 days before sacrifice.

Leukemia. To assess GVL reactivity, mice were challenged with an acute T-cell leukemia/lymphoma that spontaneously develops in AKR mice. The leukemia used in these experiments was obtained from a male AKR mouse with advanced spontaneous leukemia. After passage through healthy AKR mice, a frozen stock of leukemia was prepared to minimize experimental variability. The AKR leukemia cells are L3T4- (CD4^−), Thy 1.1^+ with variable expression of Lyt 2 (CD8).

T-cell receptor (TCR) cross-linking and mixed lymphocyte reaction (MLR) assays. For the TCR cross-linking assays, U-bottom microwells were coated with 30 μL of Vα2-specific monoclonal antibody (MoAb; 10 μg/mL) (PharMingen, San Diego, CA) or CD3-specific antibody (8 μg/mL) (clone 145-2C11, kindly provided by Jeff Bluestone, University of Chicago) for 3 hours at 37°C. The coated wells were washed twice with phosphate-buffered saline, and 1×10^5 responder cells were added to triplicate wells in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5×10^{-5} 2-mercaptoethanol, antibiotics, and additional amino acids (complete DMEM). The microwell plates were cultured at 37°C for 5 days with 1 μCi of ^3H-thymidine being added to each well for the final 18 hours of culture. Stimulation of the cultured cells was calculated from the amount of ^3H-thymidine uptake. Negative controls consisted of uncoated wells containing responder cells.

For MLR assays, 2.5×10^5 responder cells were cocultured with 5×10^5 mitomycin C-treated, B-cell–enriched AKR stimulator cells in U-bottom microwell plates for 5 days at 37°C. AKR B-cell enrichment was performed by depleting CDS^+ T cells from normal spleen cell suspensions using the MACS Magnetic Cell Separation System (Miltenyi Biotec, Inc, Auburn, CA). One microcure of ^3H-thymidine was added to each well for the final 18 hours of culture, and proliferation was calculated from the amount of ^3H-thymidine uptake. Negative control wells consisted of responder cells alone. Stimulation indices (SI) for both the TCR cross-linking and MLR assays were calculated by using the following formula: SI = average cpm of triplicate experimental wells/average cpm of triplicate negative control wells.

Limiting dilution analysis (LDA) assays. Cytolytic T lymphocyte (CTL) LDA assays were performed as described by Traut et al^18 to quantitate the frequency of functionally competent alloreactive CTL in the spleens of chimeras. Briefly, responder cells were cocultured in 16 replicate U-bottom microwells at each of 6 to 8 cell concentrations with irradiated (30 Gy) AKR spleen cell stimulator cells in complete DMEM (15% FBS) supplemented with 40% (vol/vol) of concanavalin A (con A)-stimulated rat spleen cell culture supernatant as a source of exogenous interleukin-2 (IL-2) and other growth factors. The plates were cultured at 37°C in a humidified CO2 incubator for 7 to 8 days. After incubation, half the volume of culture supernatant (100 μL) was removed from each well and 5,000 51Cr-labeled AKR target cells in 100 μL (2-day con A–stimulated blast cells) were added. After 3.5 hours, the amount of 51Cr-release for each well was determined using a gamma scintillation counter. Spontaneous and maximum values were determined from 16 to 24 wells containing stimulators only (no responders) and detergent, respectively. The cells in individual wells were scored as positive (cytolytic) when 51Cr-release values exceed the mean spontaneous release by at least 2 SD. CTL frequency estimates were made by linear regression analysis of the Poisson distribution relationship between the log_{10} percentuale of noncytolytic wells and the number of responder cells per culture well.

Antigen-driven T-helper (Th) LDA assays were used to estimate the frequency of IL-2 secreting antigen-specific T-cells in the spleens of chimeras. A modification of the methods described by Miller and Stutman^26 was used. CD4^+ responder T cells were obtained by positive selection using the MACS Magnetic Cell Separation System. The responders were cocultured in 16 replicate U-bottom microwells at each of 6 to 8 cell concentrations with 2.5×10^5 irradiated AKR peritoneal exudate cells for 4 days. The culture supernatant was then collected from each microwell, transferred to a new microwell plate, and 2,500 cells of an IL-2–responsive murine T-cell clone (SAC 9.12) were added to each well along with 1 μCi of ^3H-thymidine. Proliferation of the indicator cells in response to IL-2 secreted by responding cells in the primary culture was measured by 24-hour uptake of ^3H-thymidine. IL-2 sensitivity of the indicator cells was confirmed in each experiment by including a titrated recombinant IL-2 standard. Spontaneous thymidine uptake values were determined from 48 wells containing irradiated peritoneal exudate stimulator cells only. Individual wells were scored as positive when ^3H-thymidine uptake values exceeded the mean spontaneous uptake by at least 2 standard deviations. Estimates of Th frequencies were made using the same methods described for calculating CTL frequencies.

Statistics. Survival data was analyzed using Kaplan-Meier product limit life table statistics and the resulting survival curves were compared using the log rank test. The Wilcoxon Rank Sum Test was used to compare stimulation indices for the TCR cross-linking assays and MLR assays.

RESULTS

GVHD in mice given donor cells at various times after transplant. In the present study, we used the B10.BR/AKR model of MHC-matched allogeneic BMT to see whether donor cells could be administered earlier than 21 days after transplant without inducing lethal GVHD (Fig 1). Previously, we showed that a single infusion of 3×10^7 donor B10.BR spleen cells could be administered on day 21 without inducing lethal GVHD. Whether cells could be infused earlier than day 21 without inducing GVHD was not established. The donor BM did not contain enough T cells to
induce GVHD in this transplant model, so spleen cells were added as a source of T cells. A single infusion of $3 \times 10^7$ donor spleen cells was administered on day 3, 7, 10, 14, 17, or 21 after transplant. GVH control mice were given the same spleen cell inoculum at the time of BMT (day 0) to confirm that the cells were capable of inducing GVHD. The data shown in Fig 1 are the combined results of three experiments. All treatment groups were studied in each experiment except for the day 3 data, which was obtained from separate experiments. GVH controls were included in all experiments. Mice that received spleen cells at the time of BMT exhibited clinical signs of GVHD, characterized by ruffled fur, sticky feces, a hunched lethargic appearance, and body weight loss by 2 weeks. These mice all died within 5 weeks after BMT. In contrast, only some of the mice infused with donor spleen cells on day 21 (15% to 25%) exhibited mild transient signs of GVHD characterized by body weight loss and erythema of the ears, and only 1 of 21 animals died in this group. Increased mortality from GVHD was observed when donor cells were infused earlier than day 21, and the day 21 infused group had a statistically higher survival rate (95.2%) than all other groups with the exception of those mice infused on day 17 (85.7%). But, infusion of cells anywhere between 7 and 21 days after transplant resulted in significantly less mortality than GVH controls.

Persistence of antihost reactive T cells in post-BMT-infused chimeras: Breaking of tolerance. AKR mice have an endogenous mammary tumor virus that determines their Mls-1" (minor lymphocyte stimulatory antigen) phenotype. T-cell reactivity to Mls-encoded superantigens correlates with the expression of certain TCR Vβ genes, and one gene associated with reactivity to Mls-1" is Vβ6. T cells are clonally deleted in the thymuses of AKR mice, but are present in the peripheral tissues of B10.BR mice that do not express Mls-1". TCR cross-linking assays, similar to those described by others, were used to determine the level of anti-Vβ6-mediated responsiveness in B10.BR/AKR chimeras over time (Fig 2). AKR hosts were transplanted by intravenous injection with $1 \times 10^7$ BM cells only (BM alone) or BM plus $2 \times 10^7$ added spleen cells (BMS-20), and killed at various times after BMT. Spleen cells from killed BMT chimeras were tested in the cross-linking assays. Cells from mice in the BM-alone group showed Vβ6 responses around 2 to 4 weeks after BMT that completely disappeared by 8 weeks after transplant. Cells from these mice also lost the capability to respond against AKR alloantigens in MLR assays by day 40 after BMT (stimulation index of 0.9). Thus, these chimeras appeared to attain graft-host tolerance between 4 and 8 weeks after transplant. In contrast, cells from chimeras undergoing GVHD (BMS-20) continued to exhibit responses to Vβ6 when tested longer than 8 weeks after BMT. The low responses to TCR cross-linking by Vβ6-specific MoAb during the early post-BMT period, particularly in the BMS-20 chimeras, did not appear to be the result of low T-cell content (Fig 2). This low responsiveness has been attributed in part to GVH-associated immunodeficiency because positively selected Thy 1.2" donor T cells were able to respond in the cross-linking assays (R. Truitt, manuscript in preparation).

In a second series of experiments, chimeras were killed at 100 days post-BMT from groups of AKR hosts that had received B10.BR BM alone or BM and a single infusion of $3 \times 10^7$ donor spleen cells 21 days after transplant (BMS-30 [21d] chimeras). These experiments were designed to test for the persistence of host-reactive donor cells in the infused chimeras. The results, shown in Table 1, include data from cells of normal AKR and B10.BR mice. All long-term surviving chimeras exhibited proliferative responses to immobilized pan-T-cell–specific antibody (anti-CD3), and the average responses were not significantly different between groups. Chimeras given BM alone exhibited no response to immobilized anti-Vβ6 (Table 1). On the other hand, the spleens of BMS-30 (21d) chimeras all contained cells that responded to anti-Vβ6 cross-linking (Table 1). In the anti-Vβ6 cross-linking assays, the average stimulation index of spleen cells from BMS-30 (21d) chimeras was seven times the average of the BM-alone chimeras (Table 1; $P < .00001$).

The long-term persistence of antihost-reactive T cells in BMS-30 (21d) chimeras was confirmed through MLR assays (Fig 3). BMS-30 (21d) and BM-alone chimeras were killed at 114 and 132 days, respectively, and their spleen cells tested individually for proliferation in response to AKR allo-
antigen. The cells were tested simultaneously in TCR cross-linking assays for responses to immobilized anti-V/β6 and anti-CD3 MoAbs. Spleen cells from normal B10.BR mice were also tested in the assays for comparative analysis. Cells from the BM-alone chimeras did not respond to AKR alloantigen in MLR assays (average SI of 0.8) or immobilized anti-V/β6 in TCR cross-linking assays (average SI, 1.1), but exhibited good responses to immobilized anti-CD3 (average SI, 84.1). Cells from all seven long-term BMS-30 (21d) chimeras proliferated in response to AKR alloantigen (average SI, 5.8) (Fig 3). The responses were not significantly different from those of normal B10.BR mice (average SI, 7.7), but were significantly different from the BM-alone chimeras (P < .005). Together, the results of the TCR cross-linking and MLR assays, suggest that antihost-reactive donor T cells persist for relatively long periods of time in the infused chimeras.

Multiple infusions of donor cells given after day 21 did not induce severe GVHD. Experiments were performed to determine whether multiple injections of spleen cells could be administered beginning on day 21 after BMT without inducing severe and lethal GVHD. AKR hosts, previously transplanted with B10.BR BM alone, were given weekly injections of 3 x 10^7 donor spleen cells on days 21, 28, and 35 after BMT (Fig 4). The survival of these mice was not significantly different than those mice that had received a single injection of donor cells on day 21 post-BMT (95.8 % v 95.2%). A few of these mice exhibited some mild transient signs of GVHD, but the majority of animals showed no clinically evident GVHD.

Multiple post-BMT donor cell infusions were also given to hosts in an MHC–haplo-mismatched combination (SJL into (SJL × AKR)F1) to determine if GVHD could be avoided in a marrow transplant model with increased immunogenic disparity. Similar to the B10.BR/AKR BMT model, BM alone does not contain a sufficient number of T cells to induce acute GVHD in this model (data not shown). When 1 x 10^7 donor spleen cells were added to the marrow inoculum (day 0), transplanted recipients developed severe and lethal GVHD (Fig 5). There were no survivors by day 45. A second group of mice received weekly injections of 1 x 10^7 donor spleen cells on days 21, 28, and 35 after BMT. All mice survived the infusion protocol, and none of them exhibited any clinical signs of GVHD. Thus, GVHD could also be avoided in single haplotype mismatched chimeras by delaying the infusion of immunocompetent donor cells until 21 days post-BMT.

Long-term antileukemic effect of post-BMT donor cell–infused chimeras. Work in our laboratory showed that donor spleen cells infused into AKR hosts 21 days after transplant with MHC-matched B10.BR BM still exhibited antileukemic reactivity. In the present study, we tested whether normal SJL donor cells infused into haplo-mismatched (SJL × AKR)F1 hosts could also provide an antileukemic effect (Fig 6). (SJL × AKR)F1 mice were transplanted with SJL BM alone, then randomized to receive no additional treatment or 10^7 SJL spleen cells 21 days after transplant. The two groups of mice were then challenged 7 days later (day 28 post-BMT) by intravenous injection with 5 x 10^7 AKR leukemia/lymphoma cells. Mice that received BM alone and leukemia all died within 65 days of leukemia challenge.

**Table 1. TCR Cross-Linking Induced Proliferation of Spleen Cells From Long-Term Surviving B10.BR/AKR Chimeras**

<table>
<thead>
<tr>
<th>Spleen Cell Source*</th>
<th>No.</th>
<th>Anti-CD3</th>
<th>Anti-V/β6</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM alone</td>
<td>12</td>
<td>41.6 (22.7)</td>
<td>1.3 (0.4)</td>
</tr>
<tr>
<td>BMS-30 (21d)</td>
<td>17</td>
<td>36.0 (30.3)</td>
<td>9.1 (4.2)</td>
</tr>
<tr>
<td>Normal B10.BR</td>
<td>3</td>
<td>46.3 (9.7)</td>
<td>22.9 (8.1)</td>
</tr>
<tr>
<td>Normal AKR</td>
<td>3</td>
<td>28.4 (13.7)</td>
<td>1.9 (0.7)</td>
</tr>
</tbody>
</table>

* AKR mice, preconditioned with 9 Gy TBI, were injected intravenously with 1 X 10^7 B10.BR BM cells alone (BM alone), or BM plus 2 x 10^7 BM spleen cells (BMS-30). Normal mice were untreated.

† Spleen cells from individual chimeras were tested in TCR cross-linking assays (see Materials and Methods) for responsiveness to immobilized CD3 and V/β6-specific antibody at 88 to 92 days after transplant for mice in the BM-alone group, and 104 to 114 days after transplant for the BMS-30 (21d) group. The data for normal B10.BR and AKR mice was taken from Fig 2. The average stimulation indices and SDs for each group of mice are shown.

† The average V/β6 response of the BMS-30 (21d) group was significantly greater than that of the BM alone group (P < .00001).
Fig 3. Proliferation of spleen cells from B10.BR/AKR chimeras and normal B10.BR mice in response to AKR alloantigen in MLR assays. AKR hosts, preconditioned with 9 Gy TBI, were injected with 1 x 10^7 B10 BR BM cells alone (BM alone; n = 6), or B10 BR BM alone followed 21 days later by 3 x 10^7 B10 BR spleen cells (BMS-30 [21d]; n = 7). BMS-30 (21d) and BM-alone chimeras were killed 114 days and 132 days after BMT, respectively. Spleen cells from individual chimeras were tested in MLR assays versus AKR alloantigen and TCR crosslinking assays for responsiveness to immobilized Vβ6-specific and CD3-specific MoAbs (see Materials and Methods). Spleen cells from normal B10 BR mice (n = 5) were also tested in the assays for comparative analysis. The mean responses for each experimental group of chimeras is indicated by the solid horizontal bars. Statistical analyses: for the MLR and anti-Vβ6 responses, BMS-30 (21d) and normal B10 BR responses were significantly different from the BM alone (P < .005), but not significantly different from each other; anti-CD3 responses: the only significant difference was between the BMS-30 (21d) and normal B10 BR responses (P = .018).

Many of these mice lost considerable body weight and exhibited a sickly appearance before death. Animals that were necropsied from this group had enlarged spleens, which are associated with the presence of leukemia. In the chimeras infused with donor cells on day 21, only 1 of 20 animals died after leukemia challenge (95% survival) (Fig 6). The lone mouse that died had massive splenomegaly, suggesting the presence of leukemia. All other mice in this group exhibited a healthy clinical appearance and maintained their body weight throughout the observation period.

Fig 4. GVH-associated mortality in MHC-matched B10.BR/AKR chimeras given multiple infusions of donor cells after BMT. Survival curves are shown for irradiated (9 Gy) AKR hosts given 1 x 10^7 B10 BR BM cells admixed with 3 x 10^7 B10 BR spleen cells (II; n = 12), or 1 x 10^7 BM alone followed by infusions of 3 x 10^7 donor spleen cells on days 21, 28, and 35 after transplant (III; n = 24). The data represent the combined results of three replicate experiments.

Fig 5. GVH-associated mortality in haplo-mismatched SJL/ISJL x AKR/F1 chimeras given multiple infusions of donor cells after BMT. Survival curves are shown for SJL x AKR/F1 hosts conditioned with 9 Gy TBI before intravenous injection with 1 x 10^7 SJL BM cells admixed with 1 x 10^7 SJL spleen cells (e; n = 12) or 1 x 10^7 BM cells alone followed by infusions of 1 x 10^7 donor spleen cells on days 21, 28, and 35 after transplant (C; n = 20). The data represent the combined results of two experiments.
Experiments to this point involved challenging mice with leukemia after infusion of donor cells. The next series of experiments were designed to determine if infused donor cells could manifest an antileukemic effect in animals with leukemia in situ. AKR hosts were transplanted with B10.BR BM alone, then injected 18 days later with a relatively low dose (100 cells) of AKR leukemia. The leukemic mice were randomized into two groups: one group received no additional treatment, whereas the second received infusions of $3 \times 10^7$ B10.BR donor spleen cells on days 21, 28, and 35 after BMT. A group of normal, nontransplanted AKR mice was also injected with 100 AKR leukemia cells. The survival results are shown in Fig 7A. Mice infused with donor cells had a better survival rate than those given BM alone at day 100 post-BMT (58.3% vs 33.3%), but the increased survival rate did not reach statistical significance with the group sizes used.

Survivors of the experiment in Fig 7A were rechallenged with 100 AKR leukemia cells between 99 and 111 days after transplant to determine whether the donor cell infusions post-BMT provided long-term antileukemic reactivity (Fig 7B). Mice that received BM alone all died by day 45 after secondary challenge with leukemia. Nine of 13 donor-infused mice survived the secondary challenge, and their overall survival rate was significantly better than that of the BM-alone group (69.2% vs 0%; $P = 0.001$). The survival curve is shown until day 70 postrechallenge, but 5 of 9 donor-infused survivors were followed until 100 days after rechallenge, and all 5 chimeras survived.

The long-term antileukemic effect of post-BMT-infused donor cells was also investigated in the haplo-mismatched transplant combination. For this experiment, two groups of (SJL × AKR)F1 mice were challenged with a supralethal dose of $5 \times 10^7$ AKR leukemia cells 102 days after transplant. The groups consisted of (1) chimeras that received infusions of $10^7$ donor SJL spleen cells on days 21, 28, and 35 after BMT and (2) mice previously given a single injection of $10^7$ donor spleen cells on day 21 after BMT and leukemia on day 28. Normal (SJL × AKR)F1 mice were also injected with leukemia. Chimeras that received multiple donor-cell infusions had a better overall survival rate than the mice given a single infusion of donor cells (40.0% vs 12.5%) (Fig 8), but the increased survival rate was not statistically significant. Normal (SJL × AKR)F1 mice all died.
rapidly because of leukemia progression. Thus, both groups of long-term survivors exhibited some level of antileukemic reactivity.

**Delayed infusion of donor cells previously sensitized to host alloantigens.** Donor cells were sensitized to host alloantigens to determine if preactivated cells could induce GVHD when administered 21 days after BMT. B10.BR donors were sensitized to host alloantigens by injection with normal nonirradiated AKR spleen cells intraperitoneally on 13 and 6 days before tissue collection. Both normal and sensitized spleen cells (3 X 10^7) induced lethal GVHD when given at the time of BMT (Fig 8). As previously observed, a single injection of normal donor spleen cells on day 21 post-BMT did not induce GVHD (95.2% survival). Mice that received 3 X 10^7 host-sensitized cells on day 21 developed clinically evident GVHD with ruffled fur and body weight loss (data not shown). Their survival rate was significantly lower at 100 days post-BMT than mice that received normal donor cells on day 21 (66.7% v 95.2%) (Fig 9). Because several of these mice exhibited a poor clinical appearance on day 100, this group was followed longer. Several additional mice died from ongoing chronic GVHD between days 100 and 140, and the overall survival rate was 22.2% at termination.

**Quantitative analysis of CTL and TH frequencies.** LDA assays were used to assess the frequencies of alloreactive CTL and IL-2-secreting TH cells in chimeras at different time points after donor cell infusion post-BMT. Three groups of B10.BR/AKR chimeras were tested: (1) mice that received B10.BR BM only (BM alone); (2) mice given a single infusion of 3 X 10^7 B10.BR donor spleen cells 21 days after transplant with BM alone (BMS-30 [21d]); and (3) mice...
DISCUSSION

The infusion of donor leukocytes after BMT is an exciting new form of immunotherapy that is being used clinically to induce remissions in patients with relapsed leukemia. It offers hope for patients that have a poor prognosis of survival. Unfortunately, little experimental data exists to help optimize this form of immunotherapy. Previous studies in animal models showed that delayed infusion of allogeneic donor cells could be given to recipients without causing GVHD, or with less severe GVHD, but those studies did not investigate the antitumor potential of the infused cells. Recently, we reported that a single infusion of donor spleen cells, administered 21 days after MHC-matched allogeneic murine BMT, could provide antileukemic reactivity shortly after infusion without inducing GVHD. Important observations from the present report regarding delayed infusion of donor cells in mice are that (1) donor spleen cells could be infused earlier than 21 days after BMT, but day 21 was the optimal time for delaying infusion of donor cells; (2) graft-host tolerance is broken by the presence of antihost donor T cells correlates with the presence of a long-term antileukemic effect in chimeras given donor cell infusions; (4) donor cells could also be infused into MHC-haplo-mismatched murine BMT chimeras after transplant to provide antileukemic reactivity without inducing severe GVHD; (5) multiple infusion of donor cells starting on day 21 after BMT did not increase the risk for developing GVHD; and (6) antihost alloactivated donor cells were able to induce lethal GVHD after delayed infusion; and (7) activation of alloreactive T cells was inhibited, which may account for diminished GVHD in post-BMT-infused chimeras.

GVHD could be avoided in MHC-matched and haplo-mismatched BMT if infusions of donor cells were delayed until 21 days after BMT (Figs 1 and 5). Work by others showed that GVHD could also be minimized in MHC-mismatched murine BMT when infusion of donor cells was delayed a sufficient amount of time after BMT. The same phenomenon held true for canine recipients. Clinically, patients given donor leukocyte infusions after BMT may still...
develop some GVHD. Kolb et al\textsuperscript{12} suggested that it may be because of species differences, differences in age, or differences in previous antigen exposure. They also suggested that peculiarities may exist for human GVHD and that prior chemotherapy may play a role. It is still surprising that relatively large numbers of donor cells, capable of causing severe lethal GVHD if given at the time of BMT, can be administered to patients after BMT without inducing the same level of GVHD.

The mechanism of suppressed GVH reactivity in patients or animals given delayed infusions of donor cells is unknown. In B10.BR/AKR chimeras, the mechanism evolves during the first 21 days after BMT (Fig 1). Suppression of GVHD was noticeable as early as 3 days post-BMT, where significant prolongation of survival was observed as compared with GVH controls, and was most effective by day 21. We demonstrated that donor cells previously sensitized to host alloantigens bypassed the mechanism of suppression and caused GVHD (Fig 9). These results are in agreement with other investigators who found that large numbers of normal donor lymphocytes could be infused into murine or canine BMT chimeras without inducing GVHD, but that lymphocytes sensitized against host alloantigens induced GVHD.\textsuperscript{34} Weiden et al\textsuperscript{34} suggested that an active mechanism was present that suppressed recognition of host antigens and subsequent development of GVHD. Our results indicated that the activation of both host-reactive CTL and T\textsubscript{H} cells were suppressed in chimeras given delayed infusions of donor cells, but suggest that the activation of T\textsubscript{H} cells was preferentially suppressed (Table 2). It is important to note that CD4\textsuperscript{+} T cells are required for the induction of GVHD in the B10.BR, AKR transplant model.\textsuperscript{35} Thus, the selective suppression of CD4\textsuperscript{+} T-cell activation may account for the absence of GVHD in these BMT models. Perhaps the suppressive mechanism is an attempt by the newly developing immune system to maintain a state of immune tolerance. It would be interesting to perform the same types of LDA experiments clinically to determine whether suppression of T-cell reactivity occurs in a selective fashion. Another possibility for reduced GVH reactivity is that the cytokine storm, induced via tissue damage from the conditioning regimen, is avoided by delaying the infusion of donor cells.\textsuperscript{27,28}

Although anti-host reactivity was suppressed, it was not completely eliminated. The presence of anti-host-reactive donor T cells could be detected within 1 week after the delayed-infusion of donor spleen cells into chimeras (Table 2), and persisted until day 100 post-BMT (Table 1 and Fig 3) without inducing severe GVHD. It appears that the alloreactive T cells originated from the infused donor cells, because detectable host-reactive T cells were absent in tolerant BM-alone chimeras by 40 days after BMT (Fig 2). Thus, the maintenance of a tolerant state in BM-alone chimeras was broken by delayed infusion of donor cells, and apparently did not become reestablished over time. However, disruption of tolerance was not sufficient to affect the clinical state of the chimeras because the infused chimeras maintained a healthy physical appearance. Mice given donor spleen cells on the day of BMT also had persisting V\textbeta/6-responsive T cells (Fig 2), but in contrast, they developed severe acute GVHD. Surprisingly, spleen cell from these chimeras undergoing GVHD had poor responses to anti-V\textbeta/6 during the early post-BMT period. We have attributed the low responsiveness in part to GVH-associated immunodeficiency because positively selected Thy 1.2\textsuperscript{+} donor T cells were able to respond in the cross-linking assays (R. Truitt, manuscript in preparation). Isolation and functional characterization of the persisting antihost-reactive cells in delayed-infused chimeras are necessary to explain why they do not induce clinically evident GVHD. Interestingly, the MLR responses in the long-term chimeras given delayed infusion of donor cells were as strong as those of normal B10.BR mice (Fig 3). Results from LDA assays suggested that there might be some degree of selectivity in the T cells that persist long-term, which may help to explain the absence of GVHD. Because the activation of alloreactive T\textsubscript{H} cells appeared to be selectively inhibited (Table 2), one might hypothesize that the persistent antihost-reactive cells are primarily of the CD8\textsuperscript{+} T-cell phenotype. We are currently addressing this hypothesis. Another possible explanation for the absence of overt GVHD in these chimeras is that GVH-reactivity was reduced to a level where GVHD was subclinical.

Increasing the immunogenetic disparity, by using a haplo-mismatched BMT model (P, F1) did not increase the risk for developing GVHD in chimeras infused with donor cells post-BMT (Fig 5). In fact, the antileukemic effect of infused haplotype-mismatched SJL cells (Fig 6) was more potent than that previously observed in MHC-matched B10.BR/AKR chimeras, in which the same dose of leukemia was tested at the same time after donor cell infusion.\textsuperscript{16} The stronger antileukemic effect in SJL, F1 chimeras may be caused by the greater level of donor-host immunogenetic disparity. A long-term antileukemic effect of post-BMT-infused donor cells was also observed in haplo-mismatched chimeras (Fig 8). The donor cell-infused F1 chimeras were challenged with a supralethal dose of leukemia (5 x 10\textsuperscript{4}) on day 102 after BMT; the leukemia-free survival rate may have been greater if a minimal lethal dose had been given. The results of these experiments also indicate that multiple infusions of donor cells provide a better long-term antileukemic effect than a single infusion.

Jiang et al\textsuperscript{29} monitored the frequencies of CTL against pre-BMT leukemia and phytohemagglutinin-transformed lymphocytes in two patients at 2 and 8 weeks after donor leukocyte infusion. Remission of leukemia occurred in both patients, and they found that the ratio of CTL frequency against leukemia versus the frequency against transformed lymphocytes in two patients at 2 and 8 weeks after donor leukocyte infusion. Natural killer (NK) cell activity also increased over preinfusion levels, which suggested that both MHC-restricted and MHC-nonrestricted effectors may have contributed to the antileukemic effect. In our mouse BMT models, we have not ruled out a possible antileukemic effect of donor NK cells after donor cell infusions. However, previous work has shown that T cells are the principal GVL effectors in B10.BR/AKR chimeras.\textsuperscript{30}

A major problem with the use of delayed infusions of donor cells to treat relapse leukemia is the aggressiveness of the disease. Patients with accelerated-phase CML have
been more resistant to treatment with donor leukocyte infusions than those with chronic phase disease.10,14 Similarly, acute leukemias have been less responsive to treatment.11 It may be difficult, if not impossible, to use this form of immunotherapy if the tumor burden is high or growth of the leukemia is too rapid. The AKR leukemia used in our studies is highly aggressive, which may account for the relatively weak GVL effect observed when we attempted to treat leukemic chimeras with delayed infusions of donor cells (Fig 7A). An approach that might be more effective clinically is to identify patients at high risk for relapse and administer low doses of donor leukocyte infusions prophylactically to prevent relapse. Prophylactic administration of donor cells at relatively low doses might reduce the risk of developing severe GVHD while avoiding the problems associated with high tumor burden.

An important observation made in these studies was that the persistence of alloreactive T cells in the delayed-infused chimeras correlated with the long-term presence of antileukemic reactivity (Figs 7 and 8). These results have clinical implications. Specifically, if host-reactive donor cells persist in patients given donor leukocytes after BMT, then they may prevent potential reemergence of leukemia from cells that escape the initial chemoradiotherapy. The long-term antileukemic effect may be relatively weak because of suppression of alloreactivity (Table 2), but may be sufficient to prevent the reemergence of disease from a small number of surviving leukemia cells. Data from the International Bone Marrow Transplant Registry indicate that patients have a low but significant risk (≈10%) for late relapse beyond 2 years after BMT, regardless of the disease state or type of leukemia.11 A prophylactic approach for administration of donor leukocyte infusions at relatively low doses might reduce the incidence of late as well as early relapse.

Of note, is the fact that 33% of the BM-alone chimeras survived the initial leukemia inoculum given on day 18, whereas all survivors died upon rechallenge with the same dose of leukemia on day 100 (Fig 7). It appears there was limited antileukemic reactivity in BM-alone chimeras ≈3 weeks after BMT. There was a temporal correlation with the presence of host-reactive Vβ6+ T cells around that time period (Fig 2). Antileukemic reactivity may have been caused by the clonal expansion of the small number of mature T cells (<2%) in the donor BM inoculum. This hypothesis is supported by data from Arase-Fukushi et al,12 who showed that a small number of T cells (<0.5%) present in donor BM accelerated the elimination of host T cells in lymphoid tissues. We could not detect host-reactive donor T cells at 5 weeks after BMT (Fig 2), indicating that a state of immune tolerance had evolved in these chimeras. Acquisition of graft-host tolerance may explain why none of the BM-alone chimeras survived rechallenge with leukemia at day 100 post-BMT (Fig 7A).

In conclusion, large numbers of donor cells were infused into MHC-matched and haplo-mismatched murine BMT chimeras to provide long-term antileukemic reactivity without inducing GVHD. The infused cells were able to break tolerance in the chimeras without inducing lethal GVHD. Importantly, the persistence of anti-host reactive T cells correlated with the long-term presence of antileukemic reactivity. It appeared that activation of alloreactive Tc cells was inhibited and may account for the absence of GVHD in the infused chimeras; however, alloreactive CTL were still present. The cellular nature of the antileukemic effectors and the mechanism of suppressed allograft in the post-BMT donor cell-infused chimeras are still unknown, but are under investigation. These experimental studies may help identify protocols that more effectively use this form of immunotherapy in the treatment of leukemia by BMT.

REFERENCES


Delayed infusion of immunocompetent donor cells after bone marrow transplantation breaks graft-host tolerance allows for persistent antileukemic reactivity without severe graft-versus-host disease

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