Impact of Pretransplant Conditioning and Donor T Cells on Chimerism, Graft Versus Host Disease, Graft Versus Leukemia Reactivity, and Tolerance After Bone Marrow Transplantation

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Graft rejection, mixed chimerism, graft-versus-host disease (GVHD), leukemia relapse, and tolerance are interrelated manifestations of immunologic reactivity between donor and host cells that significantly affect survival after allogeneic bone marrow transplantation (BMT). In this report, a mouse model of BMT, in which the donor and host were compatible at the major histocompatibility complex (MHC), was used (1) to examine the interrelationship of pretransplant conditioning and T-cell content of donor BM with respect to lymphoid chimerism and GVHD and (2) to determine how these factors affected graft-versus-leukemia (GVL) reactivity and donor-host tolerance. AKR (H-2k) host mice were administered optimal or suboptimal total body irradiation (TBI) as pretransplant conditioning followed by administration of BM cells from B10.BR (H-2b) donor mice with or without added spleen cells as a source of T lymphocytes. Transplanted mice were injected with a supralethal dose of AKR leukemia cells 20 and 45 days post-BMT to assess GVL reactivity in vivo. The pretransplant conditioning of the host and T-cell content of the donor marrow affected the extent of donor T-cell chimerism and the severity of GVHD disease. GVL reactivity was dependent on transplantation of mature donor T cells and occurred only in complete chimeras. Transplantation of T-cell-deficient BM resulted in the persistence of host T cells, ie, incomplete donor T-cell chimerism, even when lethal TBI was used. Mixed chimerism was associated with a lack of GVL reactivity, despite the fact that similar numbers of donor T cells were present in the spleens of mixed and complete chimeras. In this model, moderate numbers of donor T cells facilitated complete donor T-cell engraftment, caused only mild GVHD, and provided a significant GVL effect without preventing the subsequent development of tolerance after conditioning with suboptimal TBI. In contrast, severe, often lethal, GVHD developed when the dose of TBI was increased, whereas tolerance and no GVH/GVL reactivity developed when the T-cell content of the marrow was decreased.

Allogeneic bone marrow transplantation (BMT) is being used with increased frequency in the treatment of individuals with leukemia. The success of BMT is limited by posttransplant complications such as graft rejection, graft-versus-host disease (GVHD), and leukemia relapse. Graft rejection is the response of residual host T and non-T immune cells against donor tissues, while GVHD is an immune reaction of donor cells against host tissues. Molecules encoded by the major histocompatibility complex (MHC; HLA in man, H-2 in mouse) are primary targets of T-cell-mediated alloreactivity, but graft rejection and GVHD can occur even when the donor and host are matched at the MHC. Relapse occurs when residual leukemia cells survive the cytoreductive and pretransplant conditioning therapy and proliferate in the transplant recipient.

Animal studies have shown that T cells, which are the major cause of GVHD, also facilitate donor engraftment and contribute to a graft-versus-leukemia (GVL) effect. In clinical settings, T-cell depletion of donor BM has decreased the risk of GVHD, but this has been associated with higher rates of graft failure and leukemia relapse. Donor T lymphocytes facilitate engraftment of allogeneic marrow and appear to suppress host immunity and maintain function of the graft. Depletion of T cells may also increase the risk of incomplete donor engraftment (mixed chimerism). To overcome the problems of graft failure and incomplete engraftment, more intense pretransplant conditioning regimens are often used to achieve greater immunosuppression of the host.

The antileukemic or GVL effect of allogeneic BMT has been studied extensively in animal models. In humans, the GVL effect is not as well understood and conflicting views exist regarding the relative relationship between the GVL reaction and clinical GVHD. While GVHD has been shown to be associated with an antileukemic effect, there is also clinical and experimental evidence for a GVL reaction that is independent of GVHD. Conversely, GVHD is not always accompanied by a reduction in the rate of leukemia relapse.

Although a relationship between mixed chimerism and higher leukemia relapse rates has been suggested, the effect of incomplete donor T-cell engraftment on GVH and GVL reactivity has not been studied. In the present report, a murine model of MHC-compatible BMT was used to explore the relationships between pretransplant conditioning (immunosuppression) and T-cell content of donor BM with regard to GVHD and T-cell chimerism and to determine how chimerism and GVHD affected GVL reactivity. The results demonstrate that both the pretransplant conditioning regimen and the presence of alloreactive cells in the marrow inoculum affected the extent of donor T-cell chimerism and, consequently, GVHD. Complete donor tolerance was not achieved in this study, even when the marrow was mixed, and the GVL effect was significant without GVHD.

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T-cell engraftment was necessary for an effective GVL reaction, but also resulted in an increased risk of GVHD. Under certain experimental conditions, tolerance to host alloantigens developed without compromising GVL reactivity during the early period after BMT. Leukemia-cured chimeras that subsequently became tolerant to the host were no longer able to mount a GVL reaction. Mature donor T cells in the marrow inoculum were the most important single factor in determining the extent of donor T-cell chimerism and subsequent development of GVHD, GVL reactivity, and tolerance.

MATERIALS AND METHODS

Mice. AKR/J (H-2'), B1O.BR (H-2'), and CBA/J (H-2') mice (6 to 20 weeks old) were used in these experiments. All mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in the American Association for Laboratory Animal Care (AALAC)-accredited Animal Resource Center of the Medical College of Wisconsin. Mice were given regular mouse chow and acidified tap water ad libitum.

Irradiation and BMT. AKR mice were administered 900 or 1,200 cGy total body irradiation (TBI) within 24 hours of BMT as sublethal and lethal immunosuppression, respectively. TBI was administered in a single dose using a Shepherd Mark 1 Cesium irradiator (J.L. Shepherd and Associates, San Fernando, CA). The dose rate was 89 cGy/min.

BM was flushed from donor femurs with Dulbecco's modified Eagles' medium (DMEM; Gibco, Grand Island, NY) plus 5% fetal bovine serum (FBS; Gibco). The marrow plugs were disrupted, washed, and resuspended in fresh medium, and counted. Donor spleens were removed and gently triturated using a sterile wire mesh screen to obtain a single cell suspension. Erythrocytes were lysed using a warm (37°C) Tris-buffered ammonium chloride acidified tap water ad libitum.

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In one experiment, two cycles of antibody plus complement-mediated lysis were used to deplete BM and spleen cell suspensions of CD4+ and CD8+ T cells ex vivo. Cells were suspended in equal volumes of anti-CD4 and anti-CD8 hybridoma supernatants at a concentration of 1 to 2 x 10^6/mL for 45 minutes on ice. Anti-CD4 hybridoma clone GK1.5 (ATCC TIB 207) and anti-CD8 hybridoma clone 3.155 (ATCC TIB 100) were obtained from American Type Culture Collection (Rockville, MD). After incubation with antibody, the cells were collected, washed, and resuspended in a 1/10 dilution of complement (Low-Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, Ontario) in DMEM and incubated at 37°C for 60 minutes. Cells were treated with antibody plus complement a second time before use in transplant experiments. Two-color immunofluorescence analysis with phycoerythrin (PE)-anti-L3T4 and fluorescein isothiocyanate (FITC)-anti-Lyt2 or FITC-anti-Thy1.2 was used to check the depletion procedure (see below). Greater than 90% depletion was achieved (data not shown).

Determination of chimerism by two-color immunofluorescence. Monoclonal antibodies (MoAbs) labeled with FITC or PE were used to quantitate the number of donor and host cells in the spleens of transplanted mice. FITC-conjugated anti-Thy1.2 and anti-Lyt2 (CD8) as well as PE-conjugated anti-L3T4 (CD4) MoAbs were obtained from Becton Dickinson (Mountain View, CA). Anti-Thy1.1 was obtained from the culture supernatant of hybridoma clone HO-22-1 (ATCC TIB 100) and biotinylated. PE-conjugated streptavidin (Becton Dickinson) or FITC-conjugated goat-antimouse Ig (Tago, Burlingame, CA) was used as a second-step reagent with anti-Thy1.1. Mature T cells of donor (B1O.BR) origin were defined as L3T4+ Thy1.2+ and Lyt2+ Thy1.1-. Mature T cells of host (AKR) origin were defined as L3T4+ Thy1.2- and Lyt2- Thy1.1-. PE-anti-L3T4 and FITC-anti-Lyt2 were used in combination to confirm the number of mature T cells present in the spleens of chimeras. B cells were estimated on the basis of positive staining with FITC-conjugated antimouse IgG1 plus IgM (Tago). "Other" cells were calculated by subtracting the percentage of T and B cells from 100%. Spleens from chimeras as well as normal donor and host mice were processed into single cell suspensions as described above and stained with MoAbs as recommended by the manufacturer for two-color analysis. The cells were analyzed in a fluorescence-activated cell sorter (FACS) Analyzer (Becton Dickinson) equipped with a FACSLite laser and Consort 30 computer support.

Tumor system. AKR mice spontaneously develop acute T-cell leukemia/lymphoma at approximately 8 months of age. The leukemia cells used in these experiments were obtained by pooling the spleens of five AKR mice with advanced spontaneous leukemia. A frozen stock of tumor cells was prepared after expanding the leukemia pool by passage through healthy, young AKR mice. Use of a single frozen stock of leukemia minimized experimental variability sometimes observed when different pools of spontaneous leukemias are used. AKR leukemia cells are L3T4+ Thy1.1+ with variable expression of Lyt2. To simulate leukemia relapse and assess the GVL reactivity of chimeras post-BMT, selected mice from experimental groups were injected IV with 5 x 10^6 leukemia cells. In each experiment, untransplanted young AKR hosts were also injected with leukemia to confirm tumorigenicity. Persistence of leukemia in chimeras was confirmed by two-color flow cytometric analysis, bioassays, or both. In the bioassay, an inoculum of 5 to 10 x 10^6 spleen cells from each chimera was injected IV into a healthy, young AKR host. Leukemia in the spleen of individual chimeras was indicated by death of the "secondary" AKR recipient within 60 days.13

Statistical analysis. Survival results were analyzed using Kaplan-Meier product limit life table statistics and compared using the log rank test. The extent of engraftment (chimerism) was compared using analysis of variance.

Experimental design. AKR host mice were administered 900 cGy or 1,200 cGy TBI as pretransplantation conditioning (Fig 1). Based on control mice administered TBI without marrow transplantation, 900 cGy from the cesium irradiator used in these experiments achieved a lethal dose in 49.2% (LD_{49}) of the mice (n = 28; median survival time [MST] for mice that died = 15 days; range = 13 to 20 days). Anti-Thy1.1 was obtained from the culture supernatant of hybridoma clone HO-22-1 (ATCC TIB 100) and biotinylated. PE-conjugated streptavidin (Becton Dickinson) or FITC-conjugated goat-antimouse Ig (Tago, Burlingame, CA) was used as a second-step reagent with anti-Thy1.1.
were killed for flow cytometric analysis or bioassay after challenge presented in Fig 4. Groups of irradiated AKR hosts were given B10.BR BM alone or admixed with B10.BR spleen cells. B10.BR BM usually contained less than 3% mature T cells (CD4⁺ and CD8⁺ cells) based on flow cytometry studies and limiting dilution analysis of T-cell proliferation (data not shown). Thus, murine BM was considered equivalent to human marrow that is partially depleted of T cells. To mimic the T-cell content of nondepleted human BM, it was necessary to add spleen cells as a source of alloreactive T cells capable of causing GVHD. The average B10.BR spleen consisted of 26% CD4⁺ and 14% CD8⁺ T cells. Mice conditioned with 900 cGy TBI and administered BM or BM plus spleen cells were designated as 900 cGyiBM and 900 cGyiBMS chimeras, respectively. Mice conditioned with 1,200 cGy TBI and administered BM or BM plus spleen cells were designated as 1,200 cGyiBM and 1,200 cGyiBMS chimeras, respectively.

At various times after transplantation, mice were randomly selected for analysis of chimerism or injected with leukemia to assess GVL activity (“GVL Assays” in Fig 1). The remaining mice were followed for clinical evidence of GVHD and survival (“GVH Assays” in Fig 1). Mice that were killed during the study and mice that were injected with leukemia were censored from the survival analysis at the time of death or leukemia challenge. For analysis of leukemia-free survival in leukemia-injected mice, only those that were killed for flow cytometric analysis or bioassay after challenge were censored from the data. Some leukemia-free survivors from the 900 cGyiBMS experimental group were used as spleen cell donors in secondary GVH assays to determine whether donor-host tolerance had developed (“Tolerance” in Fig 1). Details are provided in Results.

**RESULTS**

**Donor T-cell chimerism.** A pilot study was conducted to determine the kinetics of donor T-cell engraftment following lethal irradiation of the host. CBA (H-2b, Thy 1.2⁺) and AKR (H-2b, Thy 1.1⁺) mice were used in this experiment; however, subsequent experiments were performed with B10.BR (H-2b, Thy 1.2⁺) and AKR mice. Both combinations were MHC-compatible. The kinetics of donor T-cell engraftment after transplantation of CBA BM into irradiated autologous (CBA) and allogeneic (AKR) recipients was similar (Fig 2). The addition of spleen cells (5 x 10⁶) to the marrow inoculum resulted in a more rapid appearance of donor T cells in the spleens of allogeneic chimeras, but the proportion of donor T cells in chimeras administered BM alone reached the same level within 30 days posttransplant. Data from AKR/AKR chimeras, shown in Fig 2 for comparison, demonstrate background levels associated with the antibody used to detect engraftment of donor (Thy 1.2⁺) CBA and B10.BR T cells.

In subsequent studies, AKR hosts were conditioned with either 900 cGy or 1,200 cGy TBI to determine the effect of suboptimal (LD₉₀) and optimal (LD₅₀) pretreatment conditioning on donor (B10.BR) T-cell chimerism in the presence and absence of alloreactive splenic T cells. The results, summarized in Fig 3, are reported as the percentage of all mature T cells in the spleen (CD4⁺ and CD8⁺) that are donor-derived (Thy 1.2⁺). The average number of donor T, host T, B cells, and other unidentified cells in the spleen is presented in Fig 4. The spleens of AKR mice conditioned for transplantation with 900 cGy TBI and administered BM alone (900 cGy/BM) consisted of approximately equal proportions of T cells derived from the donor (mean ± 1SD = 55% ± 19%; Fig 3) and host (45% ± 20%). The proportion of donor-derived T cells (71% ± 14%) was enhanced (P < .001) by using more intense conditioning (1,200 cGy...
TB1), but a significant proportion of host T cells still remained (29% ± 15% in 1,200 cGy/BM chimeras). The increased proportion of donor T cells was attributed to elimination of residual host T cells by the TBI, because the absolute number of donor T cells in the spleens of these chimeras did not change significantly (Fig 4). Both 900 cGy/BM and 1,200 cGy/BM chimeras were designated as incomplete or mixed chimeras because the proportion of donor T cells in their spleens was significantly lower than normal B10.BR control mice (P < .006). Mixed T-cell chimerism (<80% donor-origin) persisted in mice tested 70 to 80 days post-BMT (68% and 77% for 900 cGy/BM and 1,200 cGy/BM, respectively) (data not shown).

Spleens (5 x 10⁸) were added to the BM inoculum (10 x 10⁶) so that the content of CD4⁺ and CD8⁺ T cells (13% to 15%) was approximately equal to the frequency of T cells found in nondepleted (T-replete) human BM (mean = 10.5% ± 6.6%). Transplantation of T-cell–supplemented BM into AKR mice conditioned with 900 cGy TBI resulted in a significant increase (P < .0001) in the average proportion of donor T cells in the spleen (from 55% ± 19% for 900 cGy/BM chimeras to 83% ± 11% for 900 cGy/BMS chimeras; Fig 3). However, the consequent GVH reaction resulted in significant spleen atrophy that affected the absolute number of cells in all subpopulations (Fig 4). A similar but less dramatic effect was observed when the host mice were conditioned with 1,200 cGy TBI and administered BM plus spleen cells (1,200 cGy/BMS; Figs 3 and 4). The percentage of donor-derived T cells in 1,200 cGy/BMS chimeras (84% ± 9%) and 900 cGy/BMS chimeras (83% ± 11%) was similar (Fig 3), but the absolute number of T cells was significantly lower in 900 cGy/BMS chimeras (P = .005, Fig 4). The 900 cGy/BMS and 1,200 cGy/BMS chimeras were considered complete and stable chimeras because the proportion of Thy1.2⁺ T cells in the spleen was not significantly different (P ≥ .38) from normal B10.BR mice (Fig 3) and remained greater than 80% of donor origin in mice tested 70 to 150 days post-BMT (data not shown).

In one experiment, B10.BR spleen cells were depleted of CD4⁺ and CD8⁺ cells ex vivo and 5 x 10⁶ cells were added to nondepleted BM (10 x 10⁶ cells) to assess the contribution of splenic T cells to the elimination of host cells in these chimeras. In addition, B10.BR BM that was depleted of CD4⁺ and CD8⁺ T cells was tested (10 x 10⁶) without any added spleen cells. These cell suspensions were injected into AKR hosts conditioned with 900 cGy TBI, and the extent of donor chimerism was tested 21 to 40 days later (data included in Fig 3). T-cell depletion of the BM (□ in Fig 3) did not prevent engraftment of donor cells in four mice tested; however, the proportion of T cells present in the spleen was skewed in favor of the host rather than the donor (mean = 61% T cells of host origin v 45% with unmanipulated donor BM). T-cell depletion of the donor spleen cells compromised the ability to achieve complete chimerism in two of four mice tested ( □ in Fig 3). These results demonstrate that mature T cells are not necessary for engraftment of the donor marrow in this model, but T cells are necessary for elimination of residual host cells in mice administered suboptimal conditioning.

**GVHD and survival.** Life table survival curves for the chimeras described above are shown in Fig 5. The combined results of six to eight experiments are presented. In addition, the results from two experiments are shown for which mice were conditioned with 900 cGy and administered BM plus 20 x 10⁶ spleen cells to mimic the higher levels of T-cell content found in human BM (ie, 27% T cells). The latter group of chimeras (900 cGy/BMS-20 in Fig 5A) developed lethal GVHD characterized by wasting, diarrhea, ruffled and dull fur, dyspnea and kyphosis, and “scaling” of the tail and ears. Similarly conditioned mice administered BM plus 5 x 10⁶ spleen cells (900 cGy/BMS-5) had less intense GVHD and significantly improved survival (77% actuarial survival at 60 days; P = .0003; Fig 5A); however, their survival rate was significantly lower (P = .025) than the 95% 60-day survival rate for chimeras administered BM alone (900 cGy/BM in Fig 5A). No clinical signs of GVHD developed in 900 cGy/BM chimeras. They appeared healthy and their average body weight 4 to 5 weeks after transplant (27.2 ± 3.4 g) was not different from surviving TBI controls (27.4 ± 3.7 g) or normal AKR mice (26.7 ± 2.9 g). In contrast, 900 cGy/BMS-5 and 900 cGy/BMS-20 chimeras had lower body weights (23.8 ± 3.3 g and 19.1 ± 3.6 g, respectively). Body weight loss can be a sensitive indicator of GVHD. These results demonstrated that MHC-compatible B10.BR donor cells were capable of causing lethal GVHD in AKR hosts, even when the pretransplant conditioning regimen was suboptimal (LD₅₀). The intensity of the GVH reaction was dependent on the number of spleen cells added to the BM inoculum.

The addition of spleen cells to BM administered to mice conditioned with lethal TBI (1,200 cGy) also resulted in a significant decrease (P = .035) in 60-day survival (from 79% to 61% for 1,200 cGy/BM and 1,200 cGy/BMS, respectively; Fig 5B). Moderate to severe clinical GVHD was shown in 1,200 cGy/BMS chimeras, while those admini-
Averaged body weight of chimeras 4 to 5 weeks after transplantation was 20% to 25% below that of control mice (21.4 ± 3.7 and 19.7 ± 2.4 g for 1,200 cGy/BM and 1,200 cGy/BMS chimeras, respectively). In comparison with mice prepared with 900 cGy, the survival of 1,200 cGy-chimeras administered BM alone (95% vs 79%; \( P = .13 \)) or BM plus spleen cells (77% vs 61%; \( P = .26 \)) was not significantly different (Fig 5A and B). Thus, pretransplant conditioning affected the severity of the GVH reaction when the donor cell inoculum was constant, but overall survival was not significantly reduced. On the other hand, when the conditioning regimen was kept constant, an increase in the T-cell content of the BM resulted in significantly higher GVH-related mortality.

**GVL reactivity.** Chimeras were injected with a supralethal dose of AKR leukemia cells (5 × 10⁶ cells IV) at 20 (±2) and 45 days posttransplant to simulate leukemia relapse and assess GVL reactivity. This experimental approach was chosen for two reasons. First, preliminary studies showed that when leukemia was present during the early transplant period, chimeras usually died before significant engraftment was achieved in the absence of added T cells. Second, this approach ensured that the leukemia burden was the same for each group of chimeras regardless of the pretransplant conditioning regimen because the leukemia was administered after immunosuppression. It is important to bear in mind that the model was designed to test resistance to relapse post-BMT and not the antileukemic effect of pretransplant conditioning (although that is an important clinical variable). Therefore, an identical minimal residual disease state was created in all chimeras by injection of a constant leukemia dose.

The combined results from three GVL experiments are presented in Fig 6. Chimeras administered BM alone were unable to resist leukemia injected 20 days posttransplant, indicating that they did not have significant GVL reactivity (Fig 6). This was true regardless of the conditioning regimen used (900 or 1,200 cGy TBI). Although mice conditioned with 1,200 cGy TBI had a slightly longer MST than mice administered 900 cGy (18.3 vs 15.0 days), the survival curves were not significantly different (\( P = .05 \)). Sixteen mice were challenged with leukemia at 45 days posttransplant (nine administered 900 cGy and seven administered 1,200 cGy) when donor engraftment was complete (see Fig 2), and all died within 21 days of leukemia injection (Fig 6). Thus, the lack of GVL reactivity in mice administered BM alone was not attributed to a slower rate of donor engraftment but to the absence of sufficient mature donor T cells in the marrow inoculum. Normal AKR mice were injected in each experiment as leukemia controls; their median survival time was 12 days (range = 11 to 13 days; \( n = 15 \)) (data not shown).

Addition of spleen cells to the BM inoculum resulted in a significant increase in GVL reactivity for mice conditioned with 900 cGy TBI (900 cGy/BM vs 900 cGy/BMS, \( P < .0001 \); Fig 6A). Assessment of GVL reactivity in 1,200 cGy/BMS chimeras was confounded by deaths related to GVHD. Nevertheless, survival after leukemia challenge approached statistical significance (\( P = .059 \)) in comparison with similarly conditioned mice administered BM alone (1,200 cGy/BM; Fig 6B).

Bioassays were used to determine whether leukemia was still present in chimeras administered BM plus spleen cells. Leukemia was detected in the spleens of all 900 cGy/BMS chimeras tested (six of six) at 18 days after leukemia injection; however, at 30 days, leukemia was present in only one of eight chimeras (12.5%). In contrast, leukemia could not be detected at either 18 or 30 days in mice conditioned with 1,200 cGy TBI and administered BM plus spleen cells (none of 11 1,200 cGy/BMS chimeras tested). Thus, while both groups of chimeras administered BM plus spleen cells had GVL reactivity, there was a difference in the rate at which leukemia was eliminated from the hosts. Deaths in
was not detected. In addition, autopsies, performed when 900 cGy/BM chimeras were injected at 20 days 900 I-1-I-5 20% for BlO.BR/AKR chimeras injected IV with 5 x 10^6 AKR leukemia analysis at the time of their death. [A] Survival of leukemia-injected was 13 (21) days. Chimeras that were killed were censored from the 5 MST for control AKR mice that were injected with leukemia 4 days later (81 days 1,200 cGy/BM chimeras was attributed to GVHD n = 16). [B] Survival of leukemia-injected 1,200 cGy/BM and 1,200 cGy/BMS chimeras from Fig 5A is shown. The 1,200 cGy/BM chimeras was 60 days earlier (81 days on day 91) showed complete donor T-cell chimerism (ie, 88% of the T cells were donor-origin). Nevertheless, all of the “secondary” chimeras died between 12 and 14 days after injection of leukemia, ie, at the same time as normal AKR control mice. Thus, leukemia-cured chimeras, which had survived a mild GVH reaction, subsequently became tolerant of the host and lost their ability to mount an antileukemic reaction.

In this report, the consequences of incomplete donor T-cell chimerism on the development of GVHD, GVL.

**Fig 6.** GVL analysis. Kaplan-Meier product limit analysis of survival for B10.BR/AKR chimeras injected IV with 5 x 10^6 AKR leukemia cells. GVL studies were usually terminated 30 days after leukemia challenge. MST for control AKR mice that were injected with leukemia was 13 (±1) days. Chimeras that were killed were censored from the analysis at the time of their death. (A) Survival of leukemia-injected 900 cGy/BM and 900 cGy/BMS chimeras from Fig 5A is shown. The 900 cGy/BM chimeras were injected at 20 days ([II], n = 16) and 45 days ([+], n = 9) post-BMT; 900 cGy/BM chimeras at 20 days ([II], n = 16). (B) Survival of leukemia-injected, 1,200 cGy/BM and 1,200 cGy/BMS chimeras from Fig 5B is shown. The 1,200 cGy/BM chimeras were injected at 20 days ([II], n = 16) and 45 days ([+], n = 7) post-BMT; 1,200 cGy/BMS chimeras at 20 days ([II], n = 16).

The 1,200 cGy/BMS chimeras was attributed to GVHD rather than the lack of a GVL reaction, because leukemia was not detected. In addition, autopsies, performed when possible, did not show overt evidence of leukemia.

**Donor-host tolerance in leukemia-cured chimeras.** Spleen cells (20 x 10^6) from 900 cGy/BMS chimeras that had survived a leukemia challenge 60 days earlier (81 days post-BMT) were mixed with fresh B10.BR BM (10 x 10^6) and injected IV into lethally irradiated (1,200 cGy) AKR mice to determine whether they retained GVH reactivity against the host. Survival curves are shown in Fig 7. Control AKR mice were administered BM and 20 x 10^6 spleen cells from normal B10.BR donors or no cells (TBI controls). The average spleen cell recovery in chimeras used as donors (n = 4) was 56 x 10^6 cells/mouse (range 46 to 65 x 10^6). Flow cytometric analysis showed that the mice were completely engrafted with donor T cells (ie, 90% ± 2% of the mature T cells were Thy 1.2+, including 24% ± 3% CD4+ Thy 1.2 and 10.2% ± 1% CD8+ Thy 1.2+ cells). These values were not significantly different from the T-cell content of normal B10.BR spleens (26% CD4+ and 14% CD8+ cells). AKR mice given cells from normal B10.BR donors (n = 9) developed typical clinical symptoms of GVHD and died within 23 days of transplant. In contrast, seven of eight mice administered B10.BR BM plus spleen cells from 900 cGy/BMS chimeras were alive and showed no clinical symptoms of GVHD after more than 70 days. Thus, 900 cGy/BMS chimeras became tolerant of host AKR alloantigens although they had rejected AKR leukemia earlier.

Five of the seven AKR hosts administered spleen cells from host-tolerized 900 cGy/BMS chimeras were injected with 5 x 10^6 AKR leukemia cells 77 days after “secondary” BMT to determine whether they retained any of the antileukemic reactivity observed in the primary host. Analysis of spleens from the remaining two “secondary” chimeras (on day 91) showed complete donor T-cell chimerism (ie, 88% of the T cells were donor-origin). Nevertheless, all of the “secondary” chimeras died between 12 and 14 days after injection of leukemia, ie, at the same time as normal AKR control mice. Thus, leukemia-cured chimeras, which had survived a mild GVH reaction, subsequently became tolerant of the host and lost their ability to mount an antileukemic reaction.

**DISCUSSION**

In this report, the consequences of incomplete donor T-cell chimerism on the development of GVHD, GVL.
reactivity, and tolerance were examined in a mouse model of MHC-compatible BMT. The kinetics of T-cell engraftment were affected by the T-cell content of the marrow inoculum (Fig 2). In the absence of mature T cells, residual host T cells were not eliminated and incomplete chimerism resulted, even if lethal TBI was used for conditioning (Figs 3 and 4). However, when mature donor T cells were present in the marrow, lethal GVHD developed regardless of whether the pretransplant conditioning was optimal (LD<sub>90</sub> in Fig 7) or suboptimal (LD<sub>50</sub> in Fig 5A). The intensity of the GVH reaction was dependent on the number of T cells present and was more severe when optimal pretransplant immunosuppression was administered. An effective GVL reaction occurred only when mature T cells were transplanted and complete donor T-cell chimerism was achieved (Fig 6). The rate at which the leukemia was eliminated from the hosts correlated with the intensity of the GVH reaction. Donor T cells that differentiated and matured de novo in the MHC-compatible host from lymphopoietic stem cells were incapable of eliminating residual host T cells or mediating GVH/GVL reactivity, i.e., they were tolerant of the host.

In this model, reduction in the number of transplanted T cells resulted in decreased GVH and GVL reactivity, but not in graft rejection, even though mature host T cells and competitive host hematopoietic stem cells were still present (900 cGy/BM chimeras with and without T-cell depletion in Fig 3). Graft rejection in mice is highly dependent on the degree of immunogenetic differences, 22 and host-versus-graft (HVG) reactivity may not be a significant problem in the B10.BR/ AKR donor-host combination because mixed lymphocyte reactivity does not occur in the HVG direction. 23 Although mouse BM contains a small number of mature T cells (less than 3%) that may affect engraftment, their removal by antibody and complement did not result in graft failure. However, the proportion of donor T cells in the spleen decreased (from 55% to 39%) when T-cell-depleted BM was used. Mixed T-cell chimerism consistently developed in AKR hosts administered suboptimal conditioning and BM without added T cells (Fig 3).

In clinical BMT, mixed lymphoid chimerism has become a more frequent occurrence with the increased use of T-cell depletion as GVHD prophylaxis. Roy et al 15 reported that mixed chimerism occurred in 51% of 43 patients with hematologic malignancies who were administered HLA-compatible sibling BM depleted of CD6<sup>+</sup> cells. Schouten et al 16 found incomplete chimerism in 14 of 22 patients (64%) administered T-depleted marrow. Mixed chimerism has been estimated to occur in 5% to 15% of T-replete BM transplants, but may occur in up to 50% depending on the techniques used and whether lymphoid, erythroid, or myeloid cells are evaluated. 11,12 Split lymphoid chimerism in which B and T cells are of different donor-host origin has also been reported after BMT. 24

The clinical and immunologic implications of mixed or split chimerism in leukemia patients are unclear. In the study by Roy et al, 17 early reconstitution with T cells, but apparently not natural killer (NK) cells, appears to play a role in preventing recovery of recipient cells after BMT, and GVHD is associated with maintenance of complete chimerism. However, the probability of relapse, survival, and disease-free survival is identical in patients with mixed and complete donor hematopoiesis. Schouten et al 11 could not establish a relationship between GVHD and complete or incomplete chimerism or between the number of T cells administered and chimerism.

A beneficial effect of GVH reactivity has not been demonstrated in all leukemias 25 or in all clinical studies. 26 This suggests that other factors, at both the host and donor level, impact on the GVL reaction associated with allogeneic BMT. Slow tumor growth rates, for example, or resistance to different immune effector cells may contribute to higher relapse rates by allowing less aggressive or more immunoresistant leukemia cells to survive GVH/GVL reactions, when they develop or if they are transient. In the present study, an identical leukemia-burden was eliminated at a slower rate in 900 cGy/BMS chimeras as compared with 1,200 cGy/BMS chimeras (Fig 6). Thus, controllable factors such as pretransplant conditioning can affect survival of leukemia cells independent of any direct tumor cytoreduction by influencing the rate and extent of donor engraftment. Variable numbers of a less indolent AKR leukemia are being used to determine how tumor burden and growth rate affect outcome in this model.

Theoretically, the development of donor-host-tolerance following a GVH reaction, as occurred in the 900 cGy/BMS chimeras, has potential for increasing the risk of relapse by allowing leukemia cells to persist. However, the development of donor-host-tolerance is not always associated with loss of GVL reactivity. For example, Sykes et al 25 induced specific transplantation tolerance by infusing T-depleted host BM along with nondepleted MHC-mismatched donor BM into irradiated hosts. In this model, the T-depleted host BM was eliminated by T cells in the nondepleted donor BM, resulting in complete allogeneic reconstitution and immunocompetence without GVHD (tolerance). The chimeras were able to eliminate a small inoculum of EL4 leukemia (200 cells) despite the development of donor-host tolerance. In another experimental model, Slavin et al 26 induced GVL reactivity against BCL1 leukemia without the development of GVHD using total lymphoid irradiation. Differences in the experimental BMT models, immunogenetic relationships, and susceptibility of leukemias to T-cell-mediated and/or non–T-cell-mediated immunity may account for some of the differences between the studies of Sykes et al 25 and Slavin et al 26 and our results in which development of tolerance appears to coincide with loss of GVL reactivity. The consequence of donor-host tolerance in human BMT is unknown.

Tolerance to MHC molecules is the result of clonal deletion of autoreactive immature lymphocytes in the thymus, 27 but extra-thymic tolerance can also develop through mechanisms such as suppression 28 and clonal anergy. 29 The mechanism or mechanisms of tolerance to minor histocompatibility antigens in the context of MHC-compatible BMT, as in the present study, are not well
defined. Perreault et al examined donor-host-tolerance in a murine model of MHC-compatible BMT. Although suppressor cells were thought to establish and maintain tolerance, they could not identify natural suppressor cells, veto cells, or anti-idiotypic T suppressor cells as the responsible cell type. However, these investigators did demonstrate that the number of donor T cells administered to the host determined whether GVHD or tolerance developed after MHC-compatible BMT. Our data are consistent with this observation, but also indicate that the conditioning regimen can affect the balance between development of GVHD or tolerance. For example, chimeras conditioned with 900 cGy TBI and administered BM plus spleen cells developed GVHD followed by donor-host-tolerance. The use of more intense conditioning with the same cell inoculum resulted in acute GVHD without tolerance in 1,200 cGy/BMS chimeras, whereas decreasing the T-cell content resulted in donor-host-tolerance (stable mixed chimerism) without GVHD disease (or GVL reactivity) in 900 cGy/BM chimeras.

Loss of GVL reactivity after transplantation of BM deficient in T cells has been reported in some clinical studies. This result has generally been attributed to a reduction in GVHD, but loss of GVL reactivity with diminished acute GVHD after T-cell depletion is not a universal finding. A recent analysis of data reported to the International Bone Marrow Transplant Registry has identified a number of “controllable” variables that are associated with outcome in T-cell-depleted BMT. These variables include higher doses of radiation, higher dose rates, fractionated radiation, posttransplant immune suppression, and the type of T-cell-depletion technique (MoAbs or physical techniques). Clinical strategies that address the problems and benefits associated with the use of T-cell-depleted BMT may be forthcoming, but it has been estimated that approximately one-half of all HLA-identical transplants performed worldwide on an annual basis would be required to analyze these new strategies.

Animal models can help identify how different transplant variables interrelate and impact on leukemia-free survival. The experimental studies described here demonstrate that manipulation of either the pretransplant conditioning or the T-cell content of the BM can directly affect the extent of donor T-cell engraftment and the subsequent development of GVH disease and GVL reactivity. These variables are controllable, and changes in them can determine whether GVHD or donor-host-tolerance develops after MHC-compatible BMT. In allogeneic BMT, diminished GVL reactivity and development of donor-host-tolerance may be desirable outcomes with regard to survival, but they have the potential to negate any beneficial GVL effect. The temporal relationship between development of GVHD, tolerance, and reemergence of residual leukemia cells may be critical in MHC-compatible BMT. Mature T cells in the donor marrow appear to be the most important factor in determining these relationships and, ultimately, survival in leukemic hosts.

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Impact of pretransplant conditioning and donor T cells on chimerism, graft-versus-host disease, graft-versus-leukemia reactivity, and tolerance after bone marrow transplantation

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