Induction of Early Post-Transplant Graft-versus-Leukemia Effects Using Intentionally Mismatched Donor Lymphocytes and Elimination of Alloantigen-Primed Donor Lymphocytes for Prevention of Graft-versus-Host Disease

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Abstract

Graft-versus-leukemia (GVL) effects can be induced in tolerant mixed chimeras prepared with nonmyeloablative conditioning. GVL effects can be amplified by post-grafting donor lymphocyte infusion (DLI). Unfortunately, DLI is frequently associated with graft-versus-host disease (GVHD). We investigated the feasibility of induction of potent GVL effects by DLI using intentionally mismatched lymphocytes followed by elimination of alloreactive donor T cells by cyclophosphamide for prevention of lethal GVHD following induction of very short yet most potent GVL effects. Mice inoculated with B-cell leukemia (BCL_1) and mismatched donor lymphocytes were treated 2 weeks later with low-dose or high-dose cyclophosphamide. All mice receiving cyclophosphamide 2 weeks after DLI survived GVHD, and no residual disease was detected by PCR; all control mice receiving DLI alone died of GVHD. Analysis of host (female) and donor (male) DNA showed that cyclophosphamide treatment eradicated most alloreactive donor cells, yet mixed chimerism was converted to full donor chimerism following transient self-limited GVHD. Our working hypothesis suggests that short-term yet effective and safe adoptive immunotherapy of leukemia may be accomplished early post-transplantation using alloreactive donor lymphocytes, with prevention of GVHD by elimination of GVL effector cells. (Cancer Res 2005; 65(21): 9735-40)

Introduction

It is by now well established that the main benefits of allogeneic bone marrow or blood stem cell transplantation (BMT) can be ascribed to the antileukemia effect exerted by donor alloreactive T cells (1, 2) and partially, especially following haploidentically mismatched transplants, also by alloreactive natural killer cells (3), rather than to the effects of myeloablative conditioning per se. Therefore, adoptive immunotherapy mediated by infusion of alloreactive lymphocytes after BMT, also referred to as donor lymphocyte infusion (DLI), has been used as an effective clinical tool following BMT for treatment (4–6) or for prevention (7) of recurrent disease. Consequently, reduced intensity conditioning (RIC) or nonmyeloablative stem cell transplantation (NST) became alternative treatment strategies, for a broader spectrum of patients in need, thus minimizing transplant-related toxicity and mortality. Using NST, the primary goal is to achieve engraftment of donor stem cells thus establishing host-versus-graft unresponsiveness, and consequently, durable engraftment of donor immune system cells (8–10). However, in the absence of T-cell depletion, severe acute and especially extensive chronic graft-versus-host disease (GVHD) caused by alloreactive T lymphocytes still remain major obstacles. Over the years, several approaches have been proposed to prevent or at least control GVHD. Slavin et al. (11–13) and Sykes and Sachs (14, 15) have documented in preclinical animal models that establishing a state of mixed chimerism was associated with a reduced risk of GVHD. Slavin et al. (12) and Weiss et al. (16) reported that as the time interval between BMT and DLI increased, resistance to GVHD increased. The latter observation led to the recommendation to use escalating doses of DLI for better control of GVHD (6, 7, 17). Indeed, it was confirmed in clinical studies, as predicted in mice (16), that graded increments of DLI starting with 10^5 or 10^7 (18) T lymphocytes/kg, respectively, may significantly reduce the incidence and severity of early GVHD without loss of graft-versus-leukemia (GVL) effects. Bonini et al. (19) showed that one could use large inocula of donor T cells transduced with herpes simplex virus thymidine kinase suicide gene for depletion of alloreactive donor cells causing severe GVHD following administration of ganciclovir. However, none of these techniques is satisfactory at the patient's bedside because of the unavoidable risk of uncontrolled GVHD on one hand, and the limited efficacy of DLI mediated by compatible T cells in patients with aggressive and rapidly developing leukemia, on the other hand. Hence, increasing the efficacy of GVL effects while reducing the risk of uncontrolled GVHD remains a challenge for future studies.

Our previous data in a murine model of B-cell leukemia (BCL_1) indicated that GVL effects can be potentiated by using intentionally mismatched donors (20), donor lymphocytes activated with recombinant interleukin 2 (rIL-2; refs. 21, 22), or specifically immune donor lymphocytes (23). We have reported similar observations in patients resistant to DLI successfully treated with donor lymphocytes activated nonspecifically with rIL-2 *in vivo* or *in vitro* (6) or donor lymphocytes alloactivated *in vitro* (24). Furthermore, we have recently documented that in mice with minimal number of tumor cells, GVL effects can be induced faster than the anticipated onset of overt clinical manifestations of acute GVHD (22). Hence, GVL effects induced with DLI in C57BL/6 → (BALB/c × C57BL/6) F_1 chimeras could result in successful elimination of 10^6 BCL_1 cells within 2 to 3 weeks (10-100 cells are sufficient to cause lethal leukemia), whereas death from GVHD in this combination occurs at ≥4 weeks. Prigozhina et al. (25, 26) showed that nonmyeloablative doses of cyclophosphamide can selectively eliminate host alloreactive T cells responding against donor lymphocytes administered 1 to 2 days earlier thus resulting in alloantigen-primed lymphocyte depletion

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thus avoiding subsequent rejection of allografts of donor origin. Hence, it seemed reasonable to assume that similar administration of cyclophosphamide early after DLI may also result in selective depletion of donor anti-host alloreactive cells thus resulting in prevention of GVHD by a similar mechanism responsible for prevention of rejection by elimination of alloreactive donor T cells by cyclophosphamide. Based on these assumptions, the objectives of the current study were to determine whether short-term engraftment of intentionally mismatched donor lymphocytes can effectively eliminate leukemia, convert mixed chimerism to full donor chimerism, and avoid lethal GVHD in recipients conditioned with well-tolerated nonmyeloablative regimen. Our goal was based on two working hypotheses: (a) transient engraftment of mismatched, strongly alloreactive donor lymphocytes may be sufficient for induction of GVL effects against minimal residual disease (MRD); (b) alloactivated donor lymphocytes may be much more susceptible to cyclophosphamide for prevention of lethal GVHD. Accordingly, in the present experiments, we have investigated the feasibility of induction of early post-transplant GVL effects for more effective yet safer immunotherapy of MRD.

Materials and Methods

Mice. Eight- to 12-week-old C57BL/6, H-$2^{b/b}$ (C57), BALB/c, H-$2^{d/d}$ (BALB), and (BALB/c × C57BL/6) F_1, H-$2^{d/b}$ (F_1) mice were purchased from the Hadassah Medical School Animal Facility, Hebrew University, Jerusalem, Israel. All mice were kept in a standard animal house and fed with commercial animal chow *ad libitum*. Cages, sawdust, and water bottles were autoclaved every week. Water was acidified to pH 2.5 to 2.7.

Murine B-cell leukemia. Spontaneous B-cell leukemia/lymphoma of female BALB origin (BCL_1) described by Slavin and Strober (27) was maintained *in vivo* by serial passages. Injection of 10 to 100 BCL_1 cells into BALB mice results in typical leukemia characterized by extreme splenomegaly (up to 50-fold increase compared with normal spleen) with subsequent peripheral blood lymphocytosis (up to 200,000/mm^3) and death in 100% of untreated recipients. Experimental mice were inoculated with 10^6 BCL_1.

Sublethal total body irradiation. F_1 recipients were given a single dose of nonmyeloablative total body irradiation (TBI) 400 cGy, delivered from a linear accelerator (Varian Clinac 6X) at a dose rate of 170 cGy/min, at a source-to-skin distance of 80 cm.

Bone marrow transplantation. Bone marrow cells obtained by flushing the long bones of C57 donor mice with RPMI (Biological Industries, Beit Haemek, Israel) supplemented with 10% bovine calf serum (BCS, Biological Industries) were injected into the lateral tail vein of F_1 recipients 24 hours after TBI.

Donor lymphocyte infusion. Spleen cells from C57 donors were suspended in RPMI 1640 supplemented with 10% BCS washed twice, resuspended in the same medium, and injected into the lateral tail vein of recipient mice 14 days after allogeneic bone marrow transplantation.

Elimination of alloreactive cells with cyclophosphamide. A single dose of cyclophosphamide (200 mg/kg) after BMT, inoculation of BCL_1, and treatment with DLI resulted in significant toxicity (data not shown); therefore, four doses of cyclophosphamide (50 mg/kg each) were administered to F_1 recipients at 2-day intervals, starting 14 days after DLI.

Monitoring graft-versus-host disease. Mice were observed daily for clinical signs of GVHD (weight loss, diarrhea, dermatitis, and hunched posture) and survival.

Flow cytometric analysis for detection of chimerism. Blood samples collected in heparin (50 μL) were incubated on ice for 15 minutes with mouse anti-mouse MHC class I H-$2K^b$ R-phycoerythrin (Serotec, Raleigh, NC) and R-phycoerythrin–conjugated mouse anti-mouse H-$2K^d$ monoclonal antibody (PharMingen, San Diego, CA). The samples were washed with PBS (Biological Industries) containing 0.05% sodium azide and hemolized with ammonium chloride potassium carbonate to remove red cells. All the samples were analyzed using a Becton Dickinson (Mountain View, CA) FACSan flow cytometer. Normal F_1 and BALB cells were all stained positive for H-$2K^d$, in contrast to all normal C57 cells staining positive for H-$2K^b$. The percentage of circulating C57 cells in F_1 recipients was determined by calculating the number of cells negative for H-$2K^d$ using the following formula: 100% (percent cells staining positive for H-$2K^b$) − % (tested cells staining positive for H-$2K^d$).

DNA Preparation and PCR analysis. Genomic DNA samples were prepared from fresh peripheral mice blood by the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Genomic DNA amplification for detection of minimal residual BCL_1 or normal host cells was done as described by Pugatsch et al. (28).

Statistics. Statistical evaluation was done by using a two-tailed Fisher's exact test.

Results

Leukemia eradication and event-free survival without graft-versus-host disease in chimeras prepared with nonmyeloablative conditioning following donor lymphocyte infusion treatment and rescue with cyclophosphamide. A total of 24 sublethally irradiated (TBI = 400 cGy) F_1 mice were reconstituted with 10^7 C57 bone marrow cells. All mice were found to be chimeric (Table 1) and seemed healthy, with no clinical signs of early GVHD. As shown in Fig. 1, which represents one of two similar experiments, at 14 days following BMT, all mice were inoculated i.v. with 10^6 BCL_1 cells, to mimic a state of minimal residual disease following BMT. A total of 16 mice received DLI (30×10^6 C57 spleen cells) for induction of GVL effects (groups A and B). Another group of eight control mice (group C) received no additional treatment. After additional 14 days, with the goal in mind to allow sufficient time for GVL effects for eradication of BCL_1, eight experimental mice (group A) and controls (group C) were given a total of four injections of 50 mg/kg cyclophosphamide every other day (on days 28, 30, 32, and 34; total dose of 200 mg/kg). Another control group received saline injections instead of cyclophosphamide with no further treatment and all developed leukemia and died within 4 to 6 weeks (data not shown). All experimental mice receiving DLI (C57 spleen cells) and rescued with cyclophosphamide (group A) survived for >150 days with no signs of GVHD and no evidence of leukemia (Fig. 1). In contrast, all mice receiving similar injections of bone marrow cells and BCL_1 treated with DLI receiving saline instead of cyclophosphamide (group B) died of GVHD within 107 days (median, 76 ± 23 days). The survival advantage of mice treated with cyclophosphamide after DLI was significant by the log-rank test ($P < 0.01$). To exclude the possibility that BCL_1 eradication resulted from cyclophosphamide rather than mediated by DLI, another control group of F_1 mice that received C57 BM and inoculated with BCL_1 (group C) received similar treatment with cyclophosphamide without DLI and all mice died of leukemia by day 66 (median, 46 ± 13 days).

Prevention of clinical signs of graft-versus-host disease induced by donor lymphocyte infusion by elimination of alloreactive donor lymphocytes with cyclophosphamide. All eight F_1 mice receiving 30×10^6 C57 spleen cells 14 days following nonmyeloablative conditioning with TBI 400 cGy that received C57 bone marrow developed GVHD, as manifested by skin ruffling and considerable weight loss at 3 weeks after DLI. Control mice ($n = 8$) receiving no cyclophosphamide treatment continued to lose weight due to progressive GVHD until death, whereas the few surviving mice featured persistent signs and symptoms of chronic GVHD with a syndrome compatible with runting. In sharp contrast, as can be seen in Fig. 2, experimental mice ($n = 8$) receiving the same GVHD challenge following identical conditioning that were rescued with

Table 1. Persistence of circulating donor cells (by fluorescence-activated cell sorting analysis) in C57BL/6 → (BALB/c × C57BL/6) F_1 bone marrow chimeras prepared with nonmyeloablative conditioning inoculated with BCL_1 and treated with DLI with or without elimination of alloreactive donor lymphocytes with cyclophosphamide

Treatment of F_1 recipients	Time following BMT (wk)	% Donor type (C57BL/6) cells	
		Range	Median
TBI*, BMC †, BCL_1 ‡, and DLI§ (treated with cyclophosphamide)‖	6	1-2	2
TBI, BMC, BCL_1, and DLI (treated with saline)	6	29-68	49
TBI, BMC, and BCL_1	6	2-30	22
TBI, BMC, BCL_1, and DLI (treated with cyclophosphamide)	17	86-88	87

*TBI, 400 cGy (day 0).
†BMC, 10^7 C57BL/6 bone marrow cells (day 1).
‡BCL_1, 10^6 B-cell leukemia (day 14).
§DLI, 30×10^6 C57BL/6 spleen cells (day 14).
‖Cyclophosphamide, four courses of 50 mg/kg/d on days 28, 30, 32, and 34.

cyclophosphamide seemed healthy, with no clinical signs of progressive GVHD. After a mild initial weight loss, probably due to nonspecific cytotoxic effects of cyclophosphamide or due to early GVHD that may have been suppressed, treated mice gained weight and maintained stable clinical condition with no clinical evidence of acute or chronic GVHD.

Engraftment and assays for chimerism. Irradiated hosts inoculated with BCL_1 and C57 cells were examined for the presence of donor cells at 6 weeks and again at 17 weeks after BMT, using fluorescence-activated cell sorting analysis (Table 1). During the first 6 weeks after allogeneic BMT alone, 2% to 30% peripheral blood mononuclear cells (median, 22%) were donor type, with an increase to a median of 49% (range, 29-68%) donor cells after additional treatment with DLI. Initially, early post BMT, experimental mice treated with cyclophosphamide had substantially less donor cells compared with mice treated with saline (Table 1). However, as shown in Table 1, at 17 weeks after BMT, a repeated analysis for chimerism revealed recovery of chimerism in the experimental group with 86% to 88% donor cells (median, 87%) of donor origin in the blood in all mice (Table 1).

Minimal residual disease. Blood cells from all mice were tested for MRD by BCL_1-specific PCR for detection of dormant BCL1 at 4 months after BMT, to assure complete elimination of all BCL_1, as previously described (28); yet, all of them were found negative (Fig. 3). The data indicate that BCL_1 were indeed eliminated by DLI.

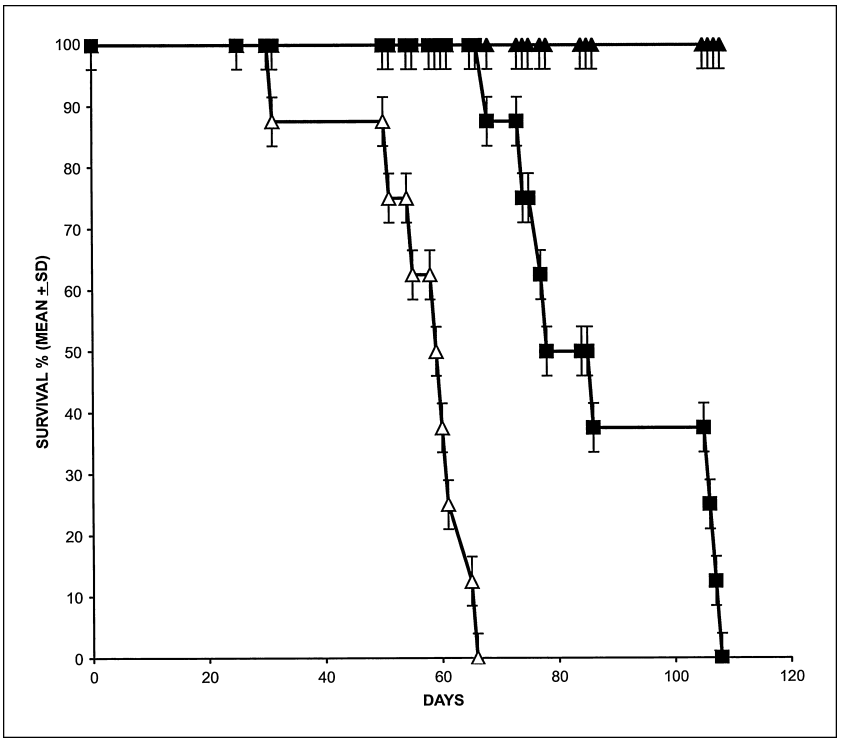

Figure 1. Disease-free survival of mice inoculated with BCL_1 and treated with DLI, 14 days after BMT rescued from GVHD by cyclophosphamide administered 14 days later. Sublethally irradiated (400 cGy) (BALB/c × C57BL/6) F_1 mice were transplanted with C57BL/6 bone marrow cells on day 0 and received 10^6 BCL_1 on day 14. Group A ($n = 8$) received BMT and DLI (30×10^6 C57BL/6 spleen cells) on day 14 and cyclophosphamide (*Cy*, four doses of 50mg/kg) starting 14 days later, on days 28, 30, 32, and 34 after BMT. Group B ($n = 8$ mice) received BMT, DLI, and four saline injections starting 14 days later. Group C ($n = 8$ mice) received a similar course of four cyclophosphamide injections with no DLI.

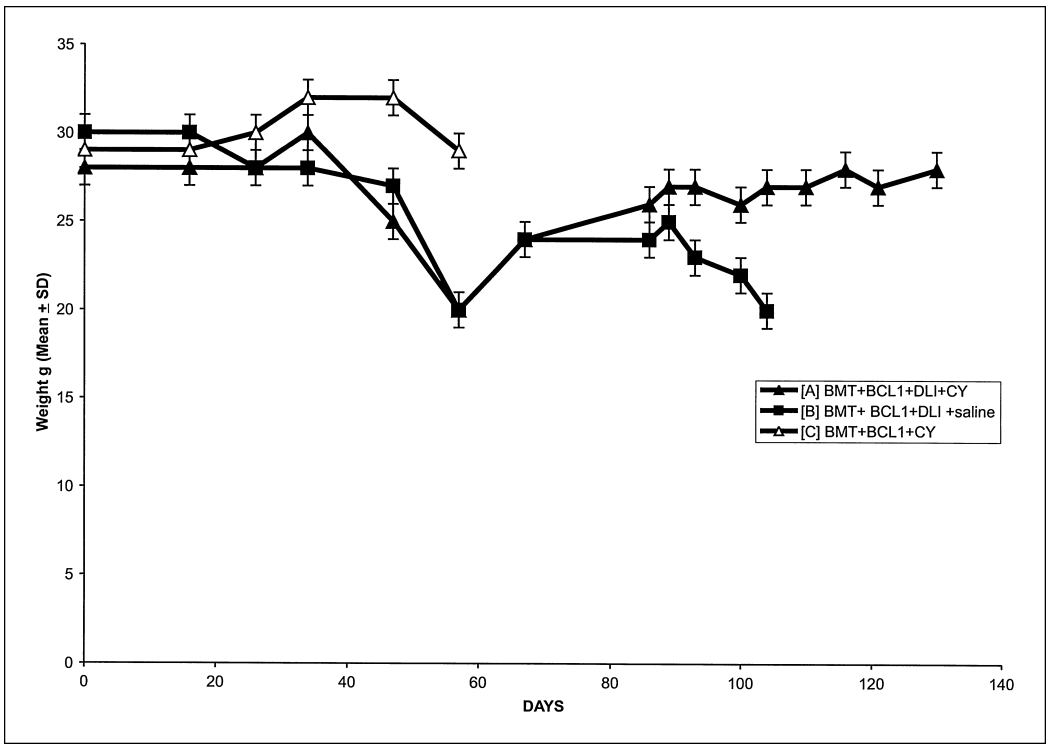

Figure 2. Rescue from GVHD induced by DLI with cyclophosphamide in chimeras inoculated with BCL_1. Sublethally irradiated (400 cGy) (BALB/c × C57BL/6) F_1 mice were transplanted with C57BL/6 bone marrow cells on day 0 and received BCL_1 on day 14. Group A (n = 8) received BMT and DLI (30×10^6 C57BL/6 spleen cells) on day 14 and cyclophosphamide (*Cy*, four doses of 50 mg/kg) starting 14 days later, on days 28, 30, 32, and 34 after BMT. Group B (n = 8) received BMT, DLI, and four saline injections 14 days later instead of cyclophosphamide. Group C (n = 8) received BMT, a similar course of four cyclophosphamide injections with no DLI.

Discussion

In the present study, the goal was to assess the feasibility of a two-step approach for immunotherapy of leukemia by alloreactive, fully mismatched lymphocytes, while avoiding lethal GVHD. First, GVL was induced by treatment of mice inoculated with BCL_1 intentionally mismatched DLI, to maximize the intensity of GVL effects, while in parallel minimizing the period of time needed for elimination of minimal residual disease. At 2 weeks following DLI, a period of time proven to be sufficient for elimination of 10^6 BCL (22), the goal was to eliminate donor anti-host alloreactive T cells that were expected to induce GVHD. Following allogeneic BMT across MHC in mice prepared with nonmyeloablative conditioning and inoculated with BCL_1, all showed partial engraftment of donor bone marrow cells, resulting in mixed chimerism, yet without obvious signs of GVHD. The protective effects induced by mixed chimerism against GVHD are well established (11–15). As shown earlier, the risks of GVHD diminishes as the time interval from BMT to DLI increases in mice (12, 16) and man (17, 18), presumably in part because of persistence of host immunoregulatory cells that may be responsible in part for veto effects (29). Nonetheless, due to the large inoculum of mismatched DLI, all untreated recipients developed lethal GVHD, whereas all the experimental mice that received four courses of cyclophosphamide treatment starting at 2 weeks after DLI survived GVHD. In addition, no residual BCL_1 could be detected in GVHD-free recipients by BCL_1-specific PCR. Elimination of alloactivated T cells by timely administration of high-dose cyclophosphamide, was anticipated based on our previous work using a mirror image experimental design, yet based on a similar principle, for induction of host-versus-graft unresponsiveness by cyclophosphamide (25, 26). When cyclophosphamide was administered 1 or 2 days following stimulation of the recipients with donor bone marrow cells, donor-specific unresponsiveness resulted and recipients accepted permanently a second inoculum of donor bone marrow cells and full thickness skin allografts (>1 year; refs. 25, 26). Interestingly, such a mechanism of induction of unresponsiveness does not interfere with induction of graft-versus-malignancy effects (26).

Interestingly but not surprisingly, during the immediate period following DLI, mice treated with cyclophosphamide featured only a low degree of chimerism compared with control mice treated with DLI and saline following BMT (Table 1). However, subsequently,

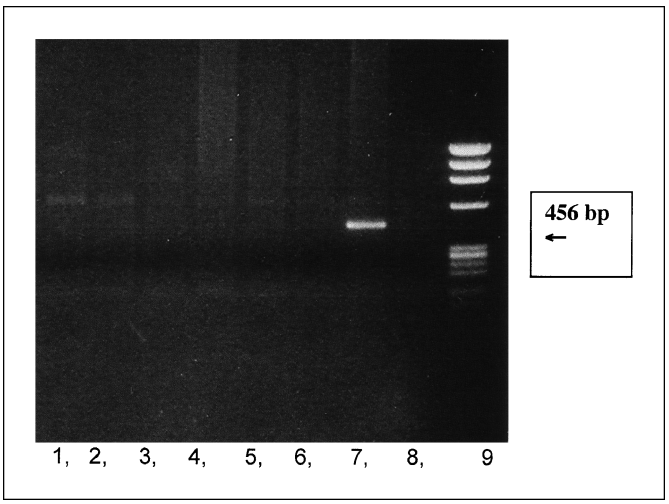

Figure 3. PCR analysis of DNA from peripheral blood samples for deduction of MRD. PCR of individual peripheral blood cells collected from F_1 mice were transplanted with C57BL/6 bone marrow cells on day 0 and received 10^6 BCL_1 cells with 30×10^6 C57BL/6 spleen cells on day 14 (*lanes 1-3*). Another group of mice received the same protocol and was treated with cyclophosphamide (50 mg/kg × 4), 2 weeks after the infusion of DLI (*lanes 4-6*). DNA from BPL of BCL_1-positive mice (*lane 7*). No DNA (*lane 8*). DNA marker (ϕX174 DNA/*Hae*III; *lane 9*).

following rescue from GVHD by cyclophosphamide, all recipients successfully recovered donor chimerism. This observation suggests that DLI using fully mismatched hence strongly alloreactive lymphocytes followed by cyclophosphamide could still result in elimination of residual hematopoietic cells of host origin, tumor cells, and normal hematopoietic cells alike, in parallel with rescue of the recipients from lethal GVHD.

In support of the proof of concept, our published data using the same murine model indicated that GVL effects against MRD could be induced before overt clinical manifestations of GVHD (22), or even in the absence of clinical signs of GVHD, in stable bone marrow chimeras (20). Indeed, DLI-induced GVL effects against MRD, in mice inoculated with 10^6 BCL_1, could be confirmed within 2 weeks, whereas death from GVHD in this strain combination is usually observed within 3 to 6 weeks (median, 4.5 weeks) after BMT and DLI (22). These observations imply that temporary engraftment of MHC mismatched effector cells for $\geq$2 weeks may be sufficient to induce effective GVL effects against MRD before development of irreversible GVHD. We have also documented that the time required for elimination of MRD following DLI may be shorter if donor cells are activated nonspecifically with rIL-2 (22), or activated specifically against the tumor in mixed lymphocyte-tumor cultures (23). Based on the aforementioned consideration, by extrapolation, successful clinical application of a similar strategy for the treatment of MRD following BMT may be accomplished, depending on a delicate balance between the timing of administration and the intensity of alloreactivity induced by DLI, with the need to assess optimal timing of cyclophosphamide administration to avoid severe and irreversible GVHD. Obviously, such an approach based on short duration of donor lymphocytes is unlikely to be effective for treatment of bulky and rapidly progressing disease. We have already documented that successful elimination of MRD that can be accomplished with low-dose DLI may not be sufficient for treatment of overt relapse in patients with acute leukemia (4–6).

Taken together, we can conclude that elimination of alloreactive lymphocytes may be accomplished by administration of cyclophosphamide following activation of alloreactive cells, both for prevention of allograft rejection (25, 26) as well as for prevention of GVHD. Because alkylating agents like cyclophosphamide are relatively more effective against activated and rapidly dividing T lymphocytes, elimination of alloreactivity is likely to be mediated by a mechanism of alloantigen-primed clonal deletion (25, 26). Interestingly, the use of clonal deletion may serve as a preclinical model to test the feasibility of intentional induction of more effective GVL effects by haploidentically mismatched T cells following induction of host-versus-graft unresponsiveness accomplished by engraftment of donor stem cells. In clinical practice, intentional induction of GVL by DLI and elimination of proliferating T cells before clinical onset of GVHD may represent a nice theoretical option that needs to be investigated.

In routine clinical practice, cyclosporine, as well as other agents used for prevention of GVHD, negate the GVL effects inducible by T lymphocytes infused with the allograft or by DLI given following transplantation, as shown in mice (30) and man (31). In this regard, the advantage of using RIC or NST conditioning before inoculation of GVL effector cells is obvious. First, it will not result in major toxicity (8–10) and thus will not facilitate undesirable cytokine release which can aggravate toxicity induced by GVHD (32). Second, it was already shown that mixed chimerism, which is induced initially following NST, may be associated with improved immunocompetence and reduced susceptibility to GVHD (11–15). Furthermore, GVL effects may be better accomplished in mixed chimeras following RIC compared with chimeras prepared with myeloablative conditioning (20, 33), because following RIC, residual antigen-presenting cells can continue to stimulate allogeneic T cells. Based on the above, NST following lymphoablative conditioning should be considered as a better platform for safer delivery of cell-mediated immunotherapy by DLI, which subsequently results in conversion of mixed chimerism to full donor chimerism.

In conclusion, based on the concept of activation-induced elimination of alloreactive T cells supported by our data, more effective yet safer, adoptive allogeneic cell therapy of malignant hematopoietic cells of host origin may be accomplished by early DLI using mismatched or otherwise activated donor lymphocytes following stem cell transplantation. In principle, such potent GVL effects could be also induced pretransplantation, using the myeloablative conditioning to eliminate residual tumor cells as well as alloreactive donor lymphocytes for prevention of GVHD. The feasibility of clinical applicability of short yet effective peritransplant GVL effects, according to the aforementioned working hypothesis, awaits further confirmation.

Acknowledgments

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Correction

Pretransplant Graft-versus-Leukemia Effects

In the article on pretransplant graft-versus-leukemia effects in the November 1, 2005 issue of *Cancer Research* (1), the correct spelling of the first author's name is Iris Yang.

1. Yang I, Weiss L, Abdul-Hai A, Kasir J, Reich S, Slavin S. Induction of early post-transplant graft-versus-leukemia effects using intentionally mismatched donor lymphocytes and elimination of alloantigen-primed donor lymphocytes for prevention of graft-versus-host disease. Cancer Res 2005;65:9735–40.

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