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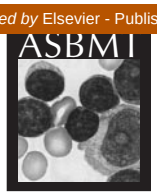

Posttransplantation Cyclophosphamide Facilitates Engraftment of Major Histocompatibility Complex–Identical Allogeneic Marrow in Mice Conditioned With Low-Dose Total Body Irradiation

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ABSTRACT

Cyclophosphamide (Cy) has been studied extensively for its immunosuppressive properties and is frequently combined with total body irradiation (TBI) as conditioning prior to HLA-identical allogeneic blood or marrow transplantation (alloBMT) in humans. Because Cy is most effective at suppressing host-versus-graft reactions when the drug is given after the transplantation (Mayumi H et al. *Transplant Proc*. 1986;18:363-369), we investigated whether posttransplantation Cy could prevent rejection of allogeneic marrow in mice conditioned with low-dose TBI. In a mouse model, posttransplantation Cy reduced the dose of TBI required from 500 cGy to ≤200 cGy for the engraftment of 10 million major histocompatibility complex (MHC)-identical marrow cells in 100% of recipients. In animals conditioned with low-dose TBI and posttransplantation Cy, donor chimerism was proportional to the dose of TBI, was present in multiple hematopoietic lineages, and was associated with the indefinite survival of donor-strain skin grafts. In contrast, animals conditioned with either TBI alone or posttransplantation Cy alone failed to achieve engraftment after alloBMT and contained antidonor cytotoxic T-cells. Although <5% donor chimerism could be induced without TBI by transplanting ≥50 million MHC-identical cells and administering posttransplantation Cy, the addition of low-dose TBI reduced the dose of donor cells required for alloengraftment and increased long-term donor chimerism to >50%. These data demonstrate that low-dose TBI and posttransplantation Cy cooperate to prevent graft rejection following the transplantation of standard doses of MHC-identical marrow cells.

KEY WORDS

Nonmyeloablative conditioning • Low-dose TBI • Allogeneic BMT • Posttransplantation cyclophosphamide • Major histocompatibility complex • Minor histocompatibility antigens • Mixed chimerism

INTRODUCTION

To achieve stable donor hematopoietic cell engraftment with standard doses of allogeneic marrow, conditioning must prevent rejection of the graft by the host's immune system [1] and kill some, but not all, host stem cells to permit the relatively small numbers of donor stem cells to compete effectively for hematopoiesis [2]. Although total body irradiation (TBI) is both immunosuppressive and myelotoxic, comparisons of syngeneic bone marrow transplantation (BMT) to allogeneic BMT (alloBMT) show that host immunity is the primary barrier to engraftment of allogeneic marrow in mice conditioned with low-dose TBI. For example, stable mixed chimerism following the transplantation of 10 to 20 million mouse marrow cells generally requires ≥700 cGy TBI conditioning in major histocompatibility complex (MHC)-incompatible recipients [3], ≥500 cGy TBI in MHC-compatible allogeneic recipients [4], but only ≥200 cGy TBI in syngeneic recipients [2,5]. These results have motivated the development of strategies that combine immunosuppressive drugs with TBI to suppress host-versus-graft (HVG) responses and reduce the total TBI dose required for allogeneic engraftment and mixed chimerism [1,6-8].

Myeloablative doses of TBI are associated with significant acute and delayed toxicities. However, efforts to reduce

the dose of TBI in HLA-identical alloBMT have been complicated by an increased risk of graft rejection, which is likely mediated by radioresistant T-cells specific for non-MHC, minor histocompatibility antigens (mHAs). By exerting toxicity to recently activated T-cells, cyclophosphamide (Cy) promotes alloengraftment in both MHC-incompatible and MHC-compatible donor-recipient pairs of mice. In MHC-incompatible donor-recipient pairs, posttransplantation Cy reduces the TBI dose required for induction of mixed chimerism from 700 cGy to 500 cGy [3]. Chimerism can even be induced in the xenogeneic rat→mouse combination using 500 cGy pretransplantation TBI and posttransplantation Cy [9]. When donor and host differ only in the expression of non-MHC mHAs, posttransplantation Cy can induce mutual donor-host tolerance, even in the absence of TBI [10]. However, the method of inducing bidirectional tolerance with posttransplantation Cy alone may not be feasible in humans for 2 reasons. First, in the mouse model, ≥50 million donor cells (equal to 2.5×10^9 cells/kg) must be infused prior to administration of Cy for tolerance to ensue [11]. An equivalent cell dose in humans could be obtained only by repeated pheresis of the donor and not by a bone marrow harvest. Second, because Cy at limiting doses does not destroy host stem cells [12], long-term chimerism is usually <5% and wanes over time. These observations prompted us to examine whether a higher level of mixed chimerism could be achieved following the administration of fewer marrow cells into MHC-compatible recipients conditioned with the combination of low-dose TBI and posttransplantation Cy.

MATERIALS AND METHODS

Mice

BALB/cAnNCr (H-2^d, CD5.1$^-$, Ly9.1$^+$), DBA/2Cr (H-2^d, CD5.1$^+$), and B6.SJL (H-2^b, CD45.1$^+$) mice were all obtained from the National Cancer Institute (Frederick, MD). C3H.SW-H-2^bSn/J (H-2^b, CD45.1$^-$), B10.BR (H-2^k), and B10.D2oSn/J (H-2^d, Ly9.1$^-$) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in microisolator cages and were fed autoclaved laboratory chow and acidified water, ad libitum. All mice were approximately 6 to 12 weeks old at the time of transplantation.

Cell Preparations

Donor spleens were removed aseptically and pressed through a nylon mesh to obtain single-cell suspensions. Bone marrow cell suspensions were prepared by flushing sterile media through the femoral and tibial marrow canals. Cell suspensions were vigorously pipetted, counted, and washed into sterile phosphate buffered saline (PBS) prior to injection.

Hematopoietic Cell Transplantation

The method of Cy-induced tolerance [11,13] entails administration of Cy (Bristol-Myers, Evansville, IN), 50 to 200 mg/kg intraperitoneally (IP), 48 to 72 hours after the intravenous (IV) administration of MHC-compatible cells differing in the expression of multiple mHAs. Cy solution was freshly prepared for each experiment by dissolving the drug in PBS at a final concentration of 20 mg/mL. Cells were injected in a final volume of 0.5 mL PBS. Some animals were irradiated by a dual source ^{137}Cs irradiator (Gammacell 40; Atomic Energy of Canada, Ottawa, ON, Canada) at an exposure rate of approximately 82 cGy/min.

Analysis of Leukocyte Chimerism

At designated times after transplantation, blood was obtained from the lateral tail vein, or animals were killed and suspensions of spleen and/or bone marrow were prepared. Erythrocytes from the peripheral blood were lysed using ammonium chloride lysis buffer prior to initiation of staining. For determination of lineage-specific chimerism, 1 million cells were stained with fluorescein-conjugated antibody to Ly9.1, CD5.1, or CD45.1 (all from BD Pharmingen, San Diego, CA) in conjunction with phycoerythrin (PE)-conjugated antibodies to CD4 and CD8 (Caltag Laboratories, Burlingame, CA) or either biotinylated antibody to B220 or CD11b (both from BD Pharmingen), followed by PE-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). For staining of granulocytes, cells were incubated with unconjugated antibody to Gr-1 (gift of Dr. I. L. Weissman, Stanford University, Palo Alto, CA) followed by fluorescein isothiocyanate (FITC)-conjugated F(ab)′$_2$ fragments of mouse antibody to rat IgG (Jackson ImmunoResearch). After staining, 5000 to 10,000 live cells were analyzed by flow cytometry using a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Franklin Lakes, NJ). In each experiment, samples of spleen or peripheral blood from at least 3 host-strain mice that had not undergone transplantation were stained for donor- or host-specific antigens. The mean + (3 × SEM) of the percentage of FITC$^+$ donor cells (or FITC$^-$ donor cells in BALB/c recipients of B10.D2 cells) in mice that had not undergone transplantation was calculated (and was <1% for every experiment), and any transplant recipient containing a greater percentage of FITC$^+$ cells than this value was considered to have donor cell engraftment.

Analysis of Erythroid Chimerism

Hemoglobin patterns were delineated by cellulose acetate electrophoresis after cystamine modification according to the method described by Whitney [14]. Four microliters of heparinized whole blood were incubated for 15 minutes with 7 μL of cystamine solution, a 1:3 dilution of stock containing 112 mg cystamine dihydrochloride, 0.9 mL distilled water, 0.1 mL of 0.1-mol/L dithiothreitol, and 0.5 mL of 3% ammonium hydroxide solution. Samples were loaded onto commercially prepared cellulose acetate plates (Titan III-H Cellulose Acetate; Helena Labs, Beaumont, TX) and run in an electrophoresis chamber at 300 volts for 20 minutes. Following electrophoresis, the samples were stained with Ponceau S and decolorized 3 times in 5% acetic acid for 5 minutes each and twice in methanol for 2 minutes each. Plates were washed in Clear Aid solution (Helena Labs) for 10 minutes for long-term preservation and densitometric analysis.

Cytotoxic T-Cell Assays

Four million responder splenocytes and 2 million irradiated (3000 cGy) stimulator spleen cells were added to individual wells of a 24-well plate, each well containing 2 mL of EHAA medium (Biofluids, Rockville, MD), 10% fetal calf

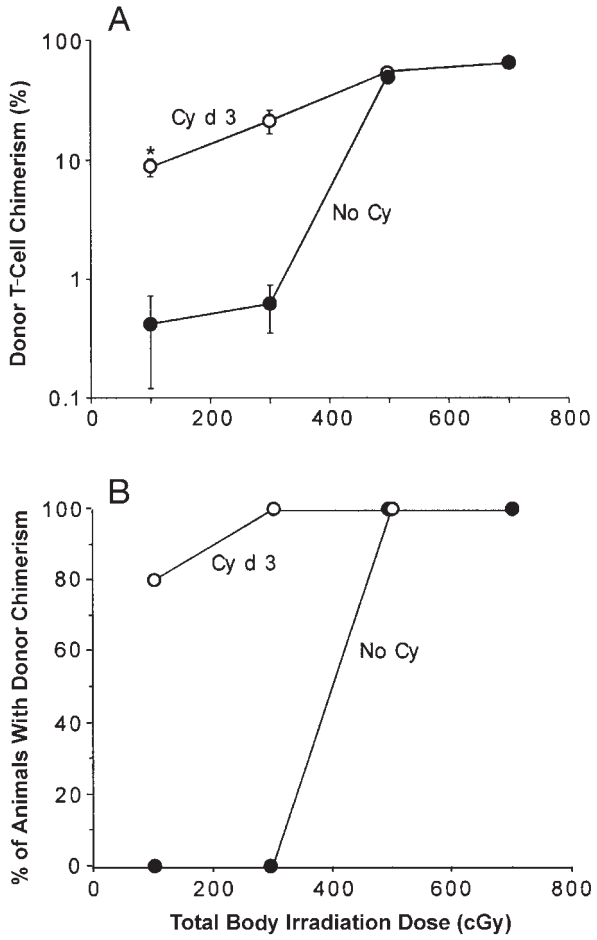

Figure 1. Posttransplantation Cy reduces the dose of TBI required for the engraftment of MHC-compatible cells. BALB/c mice (H-2^d, Ly9.1^+) were conditioned with graded doses of irradiation on day –1 and received transplants of 10 million MHC-compatible B10.D2 (H-2^d, Ly9.1^-) bone marrow cells on day 0. Seventy-two hours later, animals received no further treatment (●; n = 5 mice per TBI dose) or Cy 200 mg/kg IP (○; n = 5-8 mice per TBI dose). Four weeks after transplantation, peripheral blood was stained with fluorescein-conjugated antibody to Ly9.1 and PE-conjugated antibody to either CD4 or CD8. A, Mean donor chimerism ±SEM. *Data point represents the mean chimerism of only the 4 animals with donor engraftment. B, Percentage of animals with donor engraftment. Donor engraftment was defined as >3 × SEM above the mean percentage of T-cells that were Ly9.1^- in BALB/c mice that did not receive transplants (0.38% + [3 × 0.20%] = 0.98%).

serum (GIBCO BRL, Gaithersburg, MD), 5×10^{-5}M 2-mercaptoethanol, glutamine, and antibiotics (complete medium; CM). After 5 days, cells were harvested and plated at various responder-to-target ratios in 96-well U-bottom plates containing 10,000 ^{3}H-labeled target cells (the JAM Test [15]). Target cells were spleen cells (2×10^6/well) cultured for 48 hours in CM containing 2 μg/mL concanavalin A (Sigma Chemical Co., St. Louis, MO) and were pulsed for the last 16 hours of culture with 5 μCi ^{3}H-thymidine. After 4 to 5 hours of coculture of responders and targets, cells were harvested onto glass fiber filters using an automated cell harvester (Brandel, Gaithersburg, MD), and samples were counted in a β-scintillation counter.

Skin Grafting

Tail-to-trunk skin grafting was performed as described [16]. Briefly, recipient mice were anesthetized with a combination of ketamine and xylazine. A graft bed site over the left hemithorax was prepared by removing a piece of skin that was approximately 5 × 5 mm, leaving the panniculus carnosus and associated blood vessels intact. Tail skin of similar size was placed onto the graft bed, covered with Vaseline-impregnated gauze, and the thorax was wrapped circumferentially with Vet-Wrap (A.J. Buck and Co, Owings Mills, MD). One week after graft placement, the bandage was removed and the site was inspected to verify initial acceptance of the graft. Grafts were inspected at least 3 times per week thereafter, and rejection was considered to have occurred when no viable graft tissue was present.

RESULTS

Cy Reduces the Dose of TBI Required for the Induction of Tolerance to mHAs

BALB/c mice (H-2^d, Ly9.1^+; 5-8 animals per group) were conditioned with a single dose of TBI, ranging from 100 cGy to 700 cGy, and received transplants of 10 million MHC-compatible B10.D2 (H-2^d, Ly9.1^-) marrow cells the following day. Three days after BMT, animals were left untreated or received an IP injection of Cy 200 mg/kg. Four weeks after transplantation, donor lymphoid chimerism in the peripheral blood was analyzed by flow cytometry (Figure 1). In animals conditioned with TBI alone, donor T-cell chimerism was >50% in all animals conditioned with ≥500 cGy but was <1%, not significantly different from nontransplantation control mice, in all animals receiving ≤300 cGy (Figure 1A). In animals treated with Cy on day 3, donor chimerism was >5% in all animals conditioned with ≥300 cGy and in 4 of 5 animals conditioned with 100 cGy (Figure 1), indicating that Cy lowers the TBI threshold for the induction of donor chimerism by 200 to 400 cGy. Donor chimerism was not detected in mice if Cy was given less than 48 hours or more than 72 hours after marrow transplantation (data not shown), as observed previously in nonirradiated mice [11]. Similar results were obtained in other MHC-compatible donor-recipient combinations, including DBA/2→BALB/c, BALB/c→B10.D2, B6.SJL (H-2^b)→C3H.SW, and C3H.SW→B6.SJL (data not shown).

Failure of Allogeneic Cells to Engraft After Low-Dose TBI Is Associated With the Presence of Antidonor Cytotoxic T-Lymphocytes

Graft rejection following lethal irradiation and MHC-identical BMT in humans is associated with the presence of recipient-derived cytotoxic T-lymphocytes (CTLs) specific for donor mHAs [17]. We therefore tested whether the absence of donor chimerism in mice that had received transplants and were conditioned with low-dose TBI alone is associated with the presence of antidonor CTLs. BALB/c mice received 10 million B10.D2 marrow cells after having been conditioned with 200 cGy TBI alone on day –1, having been treated with Cy 200 mg/kg IP alone on day 3, or having

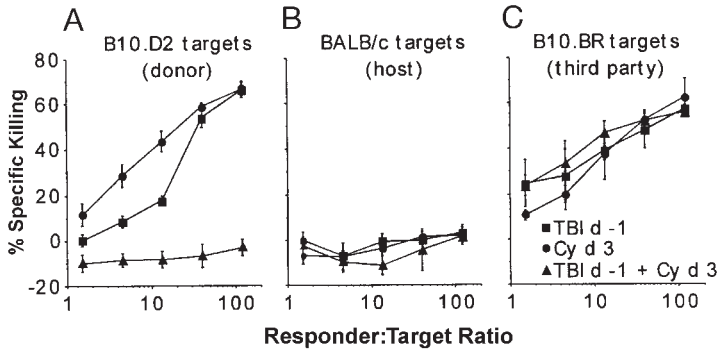

Figure 2. Failure of allogeneic marrow to engraft is associated with the presence of antidonor CTLs. BALB/c mice were conditioned with 200 cGy TBI alone on day –1 (■; n = 3), Cy 200 mg/kg IP alone on day 3 (●; n = 3), or both (▲; n = 3). All conditioned mice received 10 million B10.D2 bone marrow cells IV on day 0. Four months after transplantation, spleen cells from the recipients were cultured for 5 days with irradiated BALB/c, B10.D2, or MHC-incompatible B10.BR stimulators and tested for killing of ^{3}H-labeled host BALB/c (A), donor B10.D2 (B), or third-party B10.BR targets (C). Each data point represents the mean (±SEM) killing from 3 animals tested individually.

received the combination of 200 cGy TBI and posttransplantation Cy. Donor chimerism was >50% at 4 months after transplantation in all animals both conditioned with low-dose TBI and given posttransplantation Cy (data not shown). These chimeric animals failed to generate CTLs against donor-strain B10.D2 cells (Figure 2A). In contrast, animals that were conditioned with low-dose TBI alone or treated with posttransplantation Cy alone had <1% donor chimerism after transplantation and made vigorous CTL responses in vitro against B10.D2 targets. All animals that received transplants demonstrated CTL tolerance of host BALB/c cells (Figure 2B) and reactivity against third-party BALB.B (H-2^{b}) targets (Figure 2C). Cy-induced CTL tolerance displayed rapid kinetics of onset. In a separate experiment, C3H mice (H-2^{k}) were rendered tolerant of MHC-compatible AKR cells using posttransplantation Cy and were challenged intraperitoneally with both AKR and B10.BR (H-2^{k}) cells 1 day after Cy administration. Three weeks after this challenge, spleen cells from the C3H mice generated CTLs against B10.BR but not against AKR cells (data not shown), demonstrating that specific tolerance induction had occurred as early as 1 day after Cy administration. Taken together, the results indicate that the mHA-specific CTL precursors that survive conditioning with 200 cGy TBI are selectively eliminated by the combination of antigen exposure followed by posttransplantation Cy.

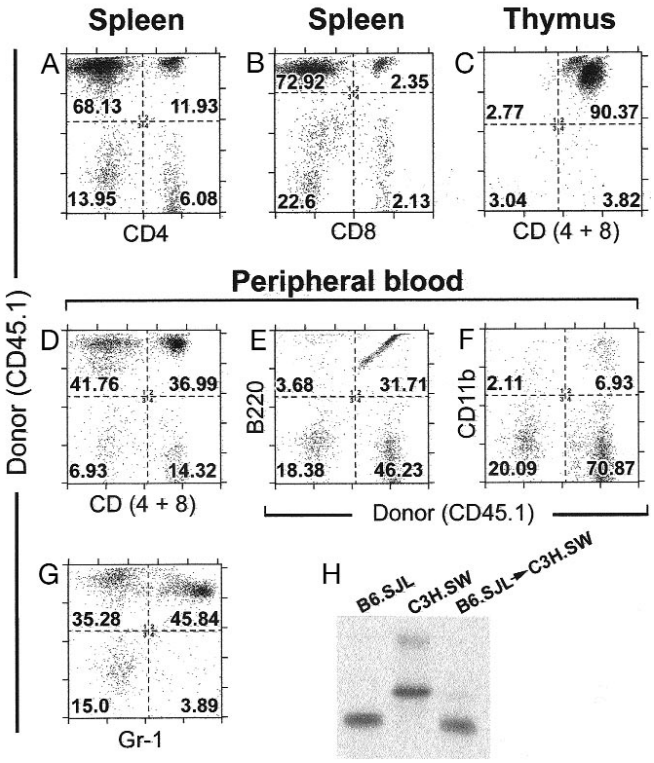

Figure 3. Transplantation of MHC-compatible cells after conditioning C3H.SW mice with low-dose TBI and posttransplantation Cy results in multilineage engraftment. C3H.SW mice (H-2^{b}, CD45.2^{+}) received 200 cGy TBI on day –1, 10 million B6.SJL marrow cells (H-2^{b}, CD45.1^{+}) on day 0, and Cy 200 mg/kg IP on day 3. A-G, Six months after transplantation, splenocytes, thymocytes, or peripheral blood cells were stained with FITC-conjugated (E and F) or PE-conjugated (A-D and G) antibody against CD45.1 (donor) and fluorochrome-conjugated antibodies against lineage-specific markers: CD4 (T-cell; A, C, and D), CD8 (T-cell; B, C, and D), B-220 (B-cell, E), CD11b (granulocyte/macrophage, F), or Gr-1 (granulocyte, G). Numbers in each quadrant represent the percentage of total cells. H, Erythrocyte chimerism in the peripheral blood was analyzed by cellulose acetate electrophoresis after cystamine modification [14]. Five mice were analyzed; typical results from an individual mouse are shown.

Low-Dose TBI and Posttransplantation Cy Induce Multilineage Donor Chimerism

To characterize donor chimerism in distinct hematopoietic lineages, transplantation was performed between mHA-incompatible strains of mice that also differed in the expression of alleles of CD45, the common leukocyte antigen, and in hemoglobin electrophoretic patterns. C3H.SW (H-2^{b}, CD45.2^{+}) mice received 200 cGy TBI on day –1, 10 million MHC-compatible B6.SJL (H-2^{b}, CD45.1^{+}) marrow cells on day 0, and Cy 200 mg/kg IP on day 3. Substantial donor chimerism at 6 months after transplantation was detected in splenic CD4^{+} and CD8^{+} T-cells (Figure 3, A and B), thymocytes (Figure 3C), peripheral blood T-cells (Figure 3D), B220^{+} B-cells (Figure 3E), CD11b^{+} monocyte/macrophages (Figure 3F), and peripheral blood granulocytes (Figure 3G).

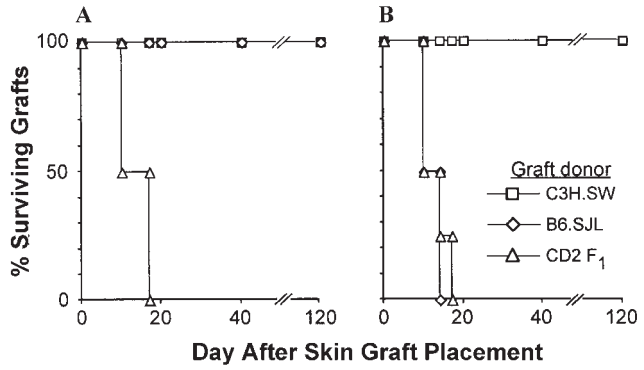

Figure 4. Engraftment of allogeneic marrow cells after low-dose TBI and posttransplantation Cy is associated with donor strain–specific skin graft acceptance. C3H.SW mice (n = 4 per group) were conditioned with 200 cGy TBI on day –1 and treated with Cy 200 mg/kg IP on day 3. On day 0, conditioned mice received 10 million B6.SJL marrow cells (A) or nothing (B). Six to 8 weeks later, mice received grafts of either host C3H.SW (□), donor B6.SJL (◇), or third-party allogeneic BALB/c × DBA/2 (CD2) F_1 skin (△). Rejection was deemed to have occurred when no visible grafted tissue remained.

Hemoglobin electrophoresis demonstrated that red blood cells were derived almost exclusively from the donor (Figure 3H, right lane). A detailed kinetic analysis of the evolution of multilineage engraftment was not performed, although peripheral blood T-cell chimerism was lower at earlier time points (eg, compare Figure 1A with Figure 3D), consistent with the paucity of mature T-cells in the marrow graft and eventual repopulation of the peripheral T-cell pool with donor-derived thymocytes (Figure 3C). Mixed chimerism in animals prepared with 200 cGy pretransplantation TBI and treated with posttransplantation Cy was stable for more than 1 year. Consistent with the absence of GVHD in mixed hematopoietic chimeras [18,19], animals that had undergone transplantations did not lose weight [20] or show clinical signs of acute or chronic GVHD, such as ruffled fur, hunched posture, diarrhea, or dermatitis for more than 1 year.

Tolerance of Allogeneic Marrow Induced by Low-Dose TBI and Posttransplantation Cy Leads to Donor-Specific Skin Graft Acceptance

Skin grafting was performed to test for the presence of functional, in vivo tolerance in animals receiving nonmyeloablative conditioning. C3H.SW mice were conditioned with 200 cGy TBI on day –1 and Cy 200 mg/kg IP on day 3, and received either no donor marrow or 10^7 B6.SJL marrow cells on day 0. Six to 8 weeks after transplantation, mice received on the trunk grafts of tail skin from B6.SJL, C3H.SW, or third-party BALB/c x DBA/2 F_1 (H-2^d) donors. C3H.SW recipients of TBI, B6.SJL marrow cells, and posttransplantation Cy accepted skin grafts from either C3H.SW or B6.SJL donors but promptly rejected grafts from third-party CD2 F_1 mice (Figure 4A). In contrast, C3H.SW mice that were conditioned but did not receive B6.SJL marrow accepted only C3H.SW skin grafts and rejected both B6.SJL and CD2 F_1 skin (Figure 4B). Thus, the level of donor chimerism that is induced by transplantation of MHC-identical marrow in mice that received low-dose TBI and posttransplantation Cy is sufficient for acceptance of parenchymal tissue grafts from the same donor.

Long-term Donor Chimerism After Pretransplantation TBI and Posttransplantation Cy Is Proportional to the Dose of Irradiation

We next examined the effect of varying the dose of pretransplantation TBI or donor marrow cells on donor chimerism within the spleens of C3H.SW mice at 8 months after induction of tolerance to B6.SJL bone marrow. Donor hematopoietic chimerism was primarily a function of recipient irradiation, whereas increasing the dose of transplanted marrow from 10 million to 20 million cells had little, if any, effect (Figure 5). The percentage of long-term donor chimerism was linearly related to the irradiation dose up to 200 cGy TBI, at which point a plateau was reached. Donor chimerism within the spleen was also observed in 4 of 5 animals receiving 50 cGy TBI and 20 million donor cells, and in all animals receiving ≥100 cGy TBI and ≥10 million donor cells. These results indicate that durable engraftment of mHA-disparate cells can be achieved with as little as 50 cGy TBI if it is combined with posttransplantation Cy.

Pretransplantation TBI Reduces the Dose of Cells Required for Tolerance Induction

Previous studies using MHC-compatible donor-recipient pairs of mice have shown that posttransplantation Cy alone induces tolerance and stable microchimerism (<5%) without the need for pretransplantation TBI, but only if the graft contains ≥50 million cells [11]. When C3H.SW mice (n = 5) received 20 million marrow cells from MHC-compatible B6.SJL donors on day 0 and Cy 200 mg/kg IP on day 3, peripheral blood donor chimerism at 6 months after transplantation was <0.05% in each of the mice, not significantly different than that for nontransplantation C3H.SW control mice. However, donor chimerism at 6 months after transplantation

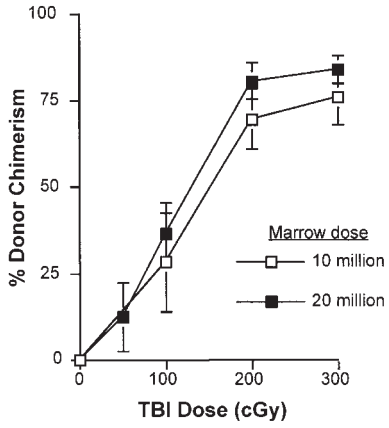

Figure 5. Long-term donor chimerism in mice that underwent transplantation is proportional to the dose of total body irradiation. C3H.SW mice (n = 5 per group) received graded doses of TBI on day –1, 10 million (□) or 20 million (■) B6.SJL marrow cells on day 0, and Cy 200 mg/kg IP on day 3. Eight months after transplantation, splenocytes were stained with FITC-conjugated antibody against CD45.1 (donor) and analyzed for donor chimerism by flow cytometry. Data are plotted as median donor chimerism/group ±SEM.

was 69.9% ±8.7% (mean ±SEM) in 6 C3H.SW recipients of 200 cGy TBI on day –1, 10 million B6.SJL marrow cells on day 0, and Cy 200 mg/kg IP on day 3. These results demonstrate that the cell dose threshold for the induction of stable macrochimerism by posttransplantation Cy is effectively lowered by the addition of pretransplantation low-dose TBI.

DISCUSSION

A major goal of clinical transplantation is to induce allogeneic hematopoietic chimerism with a minimum of acute and long-term procedural toxicities. Donor cell engraftment can be reliably induced by transplantation of allogeneic marrow following conditioning with myeloablative doses of TBI. However, high-dose TBI is associated with significant acute and delayed toxicities, including infection, mucositis, sterility [21], growth and developmental defects in children [22], and secondary malignancies [23]. Moreover, increasing the dose of TBI prior to alloBMT augments the risk of allogeneic GVHD [24,25]. Thus, efforts to reduce the dose of TBI as conditioning for alloBMT without increasing the risk of graft rejection are warranted.

In this report, we show that posttransplantation Cy reduces, by 200 to 400 cGy, the dose of TBI that is required for the engraftment of 10 million MHC-identical bone marrow cells differing in the expression of multiple mHAs. Interestingly, studies have also demonstrated that tolerance of MHC-mismatched cardiac allografts can be induced by donor spleen cell transfusion followed in 2 to 3 days by administration of the soluble fusion protein CTLA-4Ig [26,27], which blocks T-cell costimulatory interactions [28]. As in our approach, animals treated with costimulatory blockade in this sequence remained immunocompetent but exhibited donor-specific unresponsiveness. Recently, new insights into the mechanisms of peripheral tolerance induction and the importance of deletion of activated T-cells through activation-induced cell death have been described [29,30]. These studies provide strong evidence that alloantigen recognition and subsequent apoptosis of mature alloreactive T-cells are essential for the induction of stable peripheral tolerance. Thus, administration of Cy and selective elimination of donor-reactive host T-cells after initial antigenic challenge [31] follow the basic requirements for the induction of peripheral tolerance: (1) antigenic challenge and proliferation of T-cells, and (2) selective elimination of proliferating T-cells [32,33]. Most likely, the specific deletion of alloactivated cells in the Cy-induced tolerance approach occurs because of the high sensitivity of proliferating cells to Cy [34]. In adult mice, the induction of tolerance in mature peripheral T-cells is a prerequisite for the establishment of central alloantigen tolerance, which requires intrathymic mixed chimerism and is mediated by the clonal deletion of alloreactive thymocytes [35].

Low-dose TBI regimens for MHC-identical BMT have been developed in dogs and translated to the clinic for the treatment of patients with hematologic cancers. In dogs, engraftment of DLA-identical marrow was achieved in 4 of 5 recipients conditioned with 200 cGy TBI on day –1 and treated with cyclosporin A and mycophenolate mofetil after the transplantation [1]. When this regimen was applied to hematologic-malignancy patients receiving HLA-identical peripheral blood stem cell grafts, a 20% incidence of nonfatal graft rejection was observed [6]. Rejection of HLA-identical–sibling grafts was completely abrogated by the addition of IV fludarabine on days –4 through –2; however, HLA-identical stem cell grafts from unrelated donors were rejected by 12% of patients receiving the same conditioning [36]. The addition of posttransplantation Cy to regimens that contain low-dose TBI, with or without fludarabine, may confer 2 advantages. First, we have found that posttransplantation Cy increases the degree of host immunosuppression in mice conditioned with fludarabine and TBI, and the combination of these 3 agents is sufficient for the stable engraftment of even fully MHC-incompatible marrow [37]. Thus, posttransplantation Cy, when combined with pretransplantation fludarabine and 200 cGy TBI, may reduce the risk of rejection of HLA-identical unrelated-donor grafts. Second, posttransplantation Cy ameliorates GVHD [38,39], a major complication of nonmyeloablative allogeneic stem cell transplantation following low-dose TBI regimens in humans [6]. The effect of posttransplantation Cy on GVH reactions in MHC-identical donor-recipient pairs of mice has been characterized extensively by Eto and colleagues [40]. These investigators found that Cy kills mature, host-reactive T-cells and promotes the establishment of intrathymic mixed chimerism and clonal deletion of developing host-reactive thymocytes. Although engraftment, rather than GVHD, was the focus of the current study, the presence of stable mixed chimerism and the absence of CTLs against host cells (Figure 2) are both consistent with the absence of significant GVH reactions in our model.

Based on studies in animals, low-dose posttransplantation Cy was used to prevent GVHD after lethal conditioning and BMT of HLA-identical–sibling donor cells. However, posttransplantation methotrexate was superior to posttransplantation Cy in the prevention of GVHD following DLA-mismatched BMT in dogs [41], and Cy was found to be inferior to cyclosporin A as a posttransplantation prophylaxis for GVHD in HLA-identical alloBMT [42]. Because of these results, the strategy of administering Cy after myeloablative BMT was abandoned. However, these findings do not rule out a role for posttransplantation Cy in HLA-identical nonmyeloablative alloBMT. The dose and timing of Cy treatment in these studies may not have been optimal for the induction of tolerance, which in mice requires the administration of a single high dose of Cy on day 2 or 3 after transplantation [11]. Concerns about the effect of a high dose of Cy on donor stem cells and the recovery of hematopoiesis are allayed to some extent by the finding of high aldehyde dehydrogenase levels and resistance to Cy among hematopoietic stem cells [43]. Ultimately, however, the efficacy of posttransplantation Cy in promoting engraftment and inhibiting GVHD following HLA-identical nonmyeloablative alloBMT must be assessed in clinical trials.

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REFERENCES

1. Storb R, Yu C, Wagner JL, et al. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood*. 1997;89:3048-3054.
2. Stewart FM, Zhong S, Wuu J, Hsieh C, Nilsson SK, Quesenberry PJ. Lymphohematopoietic engraftment in minimally myeloablated hosts. *Blood*. 1998;91:3681-3687.
3. Colson YL, Wren SM, Schuchert MJ, et al. A nonlethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *J Immunol*. 1995;155:4179-4188.
4. Down JD, Tarbell NJ, Thames HD, Mauch PM. Syngeneic and allogeneic bone marrow engraftment after total body irradiation: dependence on dose, dose rate, and fractionation. *Blood*. 1991;77: 661-669.
5. Tomita Y, Sachs DH, Sykes M. Myelosuppressive conditioning is required to achieve engraftment of pluripotent stem cells contained in moderate doses of syngeneic bone marrow. *Blood*. 1994; 83:939-948.
6. McSweeney PA, Niederwieser D, Shizuru JA, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood*. 2001;97:3390-3400.
7. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. *J Exp Med*. 1989;169:493-502.
8. Vries-van der Zwan A, Besseling AC, de Waal LP, Boog CJ. Specific tolerance induction and transplantation: a single-day protocol. *Blood*. 1997;89:2596-2601.
9. Song X, Yuan X, Xie S. Study on the induction of xenogeneic tolerance of rat to mouse bone marrow transplantation [in Chinese]. *Zhonghua Xue Ye Xue Za Zhi*. 1999;20:409-412.
10. Mayumi H, Himeno K, Shin T, Nomoto K. Drug-induced tolerance to allografts in mice. VI. Tolerance induction in H-2-haplotype-identical strain combinations in mice. *Transplantation*. 1985;40:188-194.
11. Mayumi H, Himeno K, Tokuda N, Nomoto K. Drug-induced tolerance to allografts in mice. VII. Optimal protocol and mechanism of cyclophosphamide-induced tolerance in an H-2 haplotype-identical strain combination. *Transplant Proc*. 1986;18:363-369.
12. Yoshikawa M, Tomita Y, Uchida T, Zhang QW, Nomoto K. Inability of cyclophosphamide-induced tolerance to permit engraftment of pluripotent stem cells contained in a moderate number of syngeneic bone marrow cells. *Immunobiology*. 2000;201: 552-567.
13. Mayumi H, Umesue M, Nomoto K. Cyclophosphamide-induced immunological tolerance: an overview. *Immunobiology*. 1996;195: 129-139.
14. Whitney JB III. Simplified typing of mouse hemoglobin (Hbb) phenotypes using cystamine. *Biochem Genet*. 1978;16:667-672.
15. Matzinger P. The JAM test. A simple assay for DNA fragmentation and cell death. *J Immunol Methods*. 1991;145:185-192.
16. Rosenberg AS, Mizuochi T, Sharrow SO, Singer A. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. *J Exp Med*. 1987;165:1296-1315.
17. Voogt PJ, Fibbe WE, Marijt WA, et al. Rejection of bone-marrow graft by recipient-derived cytotoxic T lymphocytes against minor histocompatibility antigens. *Lancet*. 1990;335:131-134.
18. Sykes M, Eisenthal A, Sachs DH. Mechanism of protection from graft-vs-host disease in murine mixed allogeneic chimeras, I: development of a null cell population suppressive of cell-mediated lympholysis responses and derived from the syngeneic bone marrow component. *J Immunol*. 1988;140:2903-2911.
19. Childs R, Clave E, Contentin N, et al. Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: full donor T-cell chimerism precedes alloimmune responses. *Blood*. 1999;94:3234-3241.
20. Fontaine P, Perreault C. Diagnosis of graft-versus-host disease in mice transplanted across minor histocompatibility barriers. *Transplantation*. 1990;49:1177-1179.
21. Sanders JE, Hawley J, Levy W, et al. Pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. *Blood*. 1996;87:3045-3052.
22. Sanders JE, Pritchard S, Mahoney P, et al. Growth and development following marrow transplantation for leukemia. *Blood*. 1986; 68:1129-1135.
23. Curtis RE, Rowlings PA, Deeg HJ, et al. Solid cancers after bone marrow transplantation. *N Engl J Med*. 1997;336:897-904.
24. Clift RA, Buckner CD, Appelbaum FR, et al. Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. *Blood*. 1990; 76:1867-1871.
25. Truitt RL, Atasoylu AA. Impact of pretransplant conditioning and donor T cells on chimerism, graft-versus-host disease, graft-versus-leukemia reactivity, and tolerance after bone marrow transplantation. *Blood*. 1991;77:2515-2523.
26. Bolling SF, Lin H, Wei RQ, Turka LA. Preventing allograft rejection with CTLA4IG: effect of donor-specific transfusion route or timing. *J Heart Lung Transplant*. 1996;15:928-935.
27. Lin H, Bolling SF, Linsley PS, et al. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J Exp Med*. 1993;178:1801-1806.
28. Linsley PS, Wallace PM, Johnson J, et al. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science*. 1992;257:792-795.
29. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med*. 1999;5:1298-1302.
30. Wells AD, Li XC, Li Y, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med*. 1999;5:1303-1307.
31. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Nomoto K. Sequential mechanisms of cyclophosphamide-induced skin allograft tolerance including the intrathymic clonal deletion followed by late breakdown of the clonal deletion. *J Immunol*. 1990;145: 1303-1310.
32. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nomoto K. Intrathymic clonal deletion of V beta 6+ T cells in cyclophosphamide-induced tolerance to H-2-compatible, Mls-disparate antigens. *J Exp Med*. 1990;171:97-113.
33. Tomita Y, Nishimura Y, Harada N, et al. Evidence for involvement of clonal anergy in MHC class I and class II disparate skin allograft tolerance after the termination of intrathymic clonal deletion. *J Immunol*. 1990;145:4026-4036.
34. Aisenberg AC. Studies on cyclophosphamide-induced tolerance to sheep erythrocytes. *J Exp Med*. 1967;125:833-845.
35. Matsuzaki G, Yoshikai Y, Ogimoto M, Kishihara K, Nomoto K. Clonal deletion of self-reactive T cells at the early stage of T cell

development in thymus of radiation bone marrow chimeras. *J Immunol*. 1990;145:46-51.
36. Maris MB, Sandmaier BM, Niederwieser D, et al. Comparisons of donor chimerism, graft rejection, and GVHD after hematopoietic stem cell transplants (HSCT) from HLA matched siblings and unrelated donors using conditioning with 2 Gy TBI with and without fludarabine [abstract]. *Blood*. 2001;98:858a.
37. Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood*. 2001;98:3456-3464.
38. Santos GW, Owens AH. Production of graft-versus-host disease in the rat and its treatment with cytotoxic agents. *Nature*. 1966; 210:139-140.
39. Glucksberg H, Fefer A. Chemotherapy of established graft-versus-host disease in mice. *Transplantation*. 1972;13:300-305.
40. Eto M, Mayumi H, Tomita Y, et al. Specific destruction of host-reactive mature T cells of donor origin prevents graft-versus-host disease in cyclophosphamide- induced tolerant mice. *J Immunol*. 1991;146:1402-1409.
41. Storb R, Graham TC, Shiurba R, Thomas ED. Treatment of canine graft-versus-host disease with methotrexate and cyclophosphamide following bone marrow transplantation from histoincompatible donors. *Transplantation*. 1970;10:165-172.
42. Santos GW, Tutschka PJ, Brookmeyer R, et al. Cyclosporine plus methylprednisolone versus cyclophosphamide plus methylprednisolone as prophylaxis for graft-versus-host disease: a randomized, double-blind study in patients undergoing allogeneic marrow transplantation. *Clin Transplant*. 1987;1:21-28.
43. Kastan MB, Schlaffer E, Russo JE, Colvin OM, Civin CI, Hilton J. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood*. 1990;75: 1947-1950.