DLA-Identical Bone Marrow Grafts After Low-Dose Total Body Irradiation: Effects of High-Dose Corticosteroids and Cyclosporine on Engraftment

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Previous studies found that marrow allografts from DLA-identical littermates resulted in survival of 60% of recipient dogs after an otherwise lethal dose of 450 cGy of total body irradiation (TBI), either because of successful allografts or autologous recovery after rejection of the allografts. Forty percent of dogs died with marrow aplasia after allograft rejection. The current study asked whether allogeneic engraftment could be enhanced and survival improved by treating allograft recipients with high doses of corticosteroids or with cyclosporine (CSP), administered either before or after transplantation. Five dogs in group 1 received corticosteroids beginning on day -5 and ending on day 32 after transplant. The starting dose was 12.5 mg of prednisone per kilogram orally twice daily. All five dogs rejected their allografts; three died early with marrow aplasia and two showed endogenous marrow recovery. Nine dogs received CSP from day -6 to day -1 before transplantation at a dose of 20 mg/kg/d intravenously administered in divided doses. All nine dogs rejected the marrow allograft; six died with marrow aplasia and three survived with endogenous marrow recovery. Seven dogs received CSP after transplantation at a dose of 30 mg/kg/d orally from day -1 to day 35. All seven had sustained allografts (two mixed chimeras and five complete donor-type chimeras) and became healthy long-term survivors without graft-versus-host disease. These results extend previous observations and confirm that grafts of marrow from DLA-identical littermates improved survival of dogs exposed to low but otherwise lethal doses of TBI. Additional therapy with high-dose corticosteroids administered peritransplantation and posttransplantation or CSP administered before transplantation neither enhanced the rate of allogeneic engraftment nor improved survival; however, CSP administered after transplantation resulted in successful allografts and event-free survival in all cases. © 1995 by The American Society of Hematology.

WE HAVE DEVELOPED a canine model of low-dose, but nonetheless lethal total body irradiation (TBI) in which treatment approaches can be studied that may be useful in the management of radiation accident victims. In addition, results from these studies may have implications for the development of nontoxic conditioning programs for allogeneic marrow grafting in patients with nonmalignant hematologic diseases. Specifically, TBI doses of ≥400 cGy delivered at 7 cGy/min were uniformly marrow lethal in dogs when administered without subsequent marrow rescue.

However, when dogs received a supralethal dose of 450 cGy TBI followed by marrow allografts from DLA-identical littermates, 60% of dogs survived, some with successful and complete allografts, some with persistent mixtures of donor and host hematopoietic cells, and some with endogenous marrow recovery after rejection of the transient allografts. Forty percent of dogs so treated died from infections due to the marrow aplasia that developed after rejection of the allograft and before endogenous marrow recovery. A further improvement in survival could be achieved when marrow-grafted dogs were treated by hematopoietic canine growth factors, granulocyte colony-stimulating factor, and stem cell factor, alone or combined. That achievement was due to high levels of granulocyte counts throughout the posttransplantation course seen among dogs receiving growth factors, which resulted in a lessened incidence of fatal infections, even though the proportion of dogs with successful allografts remained unchanged.

The current study investigated whether both the incidence of successful allogeneic engraftment and of survival could be improved by treatment of recipients with additional immunosuppression, either corticosteroids or cyclosporine (CSP). The peritransplantation and posttransplantation schedule of extremely high doses of corticosteroids to enhance engraftment has been reported by Kernan et al with T-depleted grafts in human patients, and the potential usefulness of pretransplantation and posttransplantation CSP to prevent rejection of grafts has been described in both experimental and clinical marrow transplantation settings. Accordingly, three groups of dogs were conditioned by 450 cGy TBI before transplantation of marrow from DLA-identical littermates. Dogs in the first group received high doses of corticosteroids, as described by Kernan et al. Dogs in the second group received CSP before transplantation, and dogs in the third group received CSP after transplantation. Only CSP after transplantation promoted allogeneic engraftment; all dogs so treated survived.

MATERIALS AND METHODS

Litters of beagles, barhiers, Walker hounds, and pit bull/beagle crossbreeds were either raised at the Fred Hutchinson Cancer Research Center (Seattle, WA) or purchased from commercial kennels in the state of Washington. The dogs weighed from 6.7 to 16.4 kg (median, 11.4 kg) and were 6 to 13 months old (median, 8 months old). They were observed for disease for at least 2 months before entering the study. All were immunized for distemper, leptospirosis, hepatitis, and parvovirus. Research was performed according to the
principles outlined in the Guide for Laboratory Animal Facilities and Care prepared by the National Academy of Sciences, National Research Council. The research protocol was approved by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center. The kennels are certified by the American Association for Accreditation of Laboratory Animal Care.

Twenty-one littermate donor/recipient pairs were chosen on the basis of identity for the serologically detectable canine histocompatibility antigens DLA-A and -B,' complemented by restriction fragment length polymorphism assays as well as gene sequencing for canine major histocompatibility complex II genes (unpublished observations). Donor/recipient pairs were sex mismatched in 13 cases, although cytogenetic evaluation of marrow and peripheral blood cells for chimerism after transplantation succeeded in only five cases owing to the pancytopenia. In addition, in 16 pairs, a polymerase chain reaction (PCR)-based assay for polymorphic (CA), repeats was used to assess success or failure of the allograft.

Marrow for transplantation was aspirated under general anesthesia through needles inserted into humeri and femora. Recipients were administered TBI at a dose of 450 cGy delivered at 7 cGy/min from two opposing cobalt-60 sources.7 Marrow was infused intravenously at doses of 1.1 to 4.5 × 10^6 cells/kg (median, 3.6 × 10^6 cells/kg) within 4 hours of TBI. The day of marrow grafting was designated as day 0. Postgrafting care included twice daily oral nonabsorbable antibiotics, neomycin sulfate, and polymyxin sulfate, which were administered from day 1 until the day of recovery of white blood cell counts to more than 1,000/µL. Prophylactic systemic antibiotics twice daily (usually ceftazidime unless sensitivity tests indicated otherwise) were begun on the day of TBI and continued until the white blood cell count reached 1,000/µL. In addition, dogs received red blood cell and platelet transfusion support based on the results of the daily blood cell counts. All transfusions were irradiated in vitro with 2,000 cGy from 60 cobalt sources. The dogs' clinical status was assessed twice daily. Acute and chronic graft-versus-host disease (GVHD) were diagnosed using standard criteria.3-13 All dogs underwent complete autopsies, including histopathologic examinations at the conclusion of the study.

Three groups of recipients were studied. The five dogs in group 1 received corticosteroids as outlined in Table 1. Three of the five dogs received oral prednisone throughout the study while two dogs received equivalent doses of intravenous methylprednisolone sodium after transplantation to control for possible differences in enteric absorption of the drug by different dogs. Nine recipients in group 2 received CSP intravenously (Sandimmune ampules; Sandoz Pharma Ltd, Basel, Switzerland) at 20 mg/kg/d in divided doses from days -6 through -1. Seven recipients in group 3 received CSP orally (Sandimmune, oral solution; Sandoz Pharma Ltd) at 30 mg/kg/d in divided doses from day -1 to day 35.

Table 1. Dose Schedule of Corticosteroids in Dogs of Group 1

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<tr>
<th>DOG #</th>
<th>BID</th>
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Values shown are in milligrams per kilogram. Day 0 is the day of TBI and marrow transplantation.

Abbreviation: P, prednisone (Deltasone tablets; The Upjohn Co, Kalamazoo, MI) administered orally; M, methylprednisolone sodium (Solu-Medrol; Upjohn) administered intravenously; BID, twice daily.

Marrow engraftment was assessed by sustained increases in granulocyte and platelet counts after the postirradiation nadir, by histologic features of the marrow from biopsy or autopsy specimens, by documentation of cells with donor karyotype or with donor (CA), repeat polymorphisms in specimens from peripheral blood and marrow, and by the development of GVHD. Graft rejection was defined as failure of sustained recovery of granulocyte or platelet counts of donor origin after the postirradiation nadir, along with extreme marrow hypocellularity at autopsy or decreasing counts after initial engraftment, followed by the reappearance of cells with host karyotype or host (CA), repeat polymorphisms, and the absence of clinical and histologic features of GVHD. In five dogs, the two marker methods, cytogenetics and (CA), repeat polymorphisms, were used simultaneously. In three instances, results were identical. In two cases, discrepancies were noted, with cytogenetics showing mixtures of predominantly donor but also rare host cells and (CA). dinucleotide repeats showing only donor cells. These discrepancies may be because of the differences in cells examined by the two techniques, with cytogenetics restricted to karyotypes from dividing cells and with the PCR-based technique looking at DNA from both dividing and nondividing cells. The rare dividing host cell detected by cytogenetics may not be detectable by the PCR technique whose sensitivity is limited to detect as little as 2.5% to 97.5% mixtures.19 For the purposes of this study, mixed chimerism was assumed if one of the marker methods showed mixtures of donor and host hematopoietic cells.

RESULTS

Table 2 summarizes the data. One of the five dogs in group 1 receiving high-dose corticosteroids failed to show an increase in granulocyte counts after the postirradiation nadir and died on day 21 with very hypocellular marrow. The other four dogs showed increases in granulocyte counts, consistent with initial allogeneic engraftment. In all four, granulocyte counts began declining in the third week after transplant. In two of the four, death with severe marrow hypoplasia occurred on days 20 and 26, respectively, whereas the remaining two animals showed late endogenous hematopoietic recovery. Marker studies were consistent with graft rejection and reappearance of host cells.

One of the nine dogs in group 2 receiving pretransplantation CSP failed to show an increase in granulocyte counts, and this dog died on day 23 with hypocellular marrow and the demonstration of host cells. The remaining dogs showed initial increases in granulocyte counts consistent with allogeneic engraftment. However, in all cases, the allograft was not sustained and granulocyte counts began declining in the
Table 2. Marrow Grafts From DLA-Identical Littermates After 460 cGy TBI Delivered at 7 cGy/min

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<th>Group</th>
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<th>Recipient No.</th>
<th>Increase in Granulocytes*</th>
<th>Sustained Allograft</th>
<th>GVHD1</th>
<th>Rejection</th>
<th>Autologous Recovery</th>
<th>Survival (d)</th>
<th>Cause of Death</th>
<th>Marrow Cellularity at Autopsy (%)</th>
<th>Cytogenetics (CA)</th>
<th>Repeat Markers</th>
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Abbreviations: ND, not done (donor and host were of same sex); NE, not evaluable; H, host origin; D, donor origin; D/H, mixed chimerism (donor cells predominate); E1, euthanasia because of worsening clinical condition; E2, euthanasia at end of study.

* Increase of peripheral granulocyte count to ≥500/µL after the postirradiation nadir.

† Based on clinical and histopathologic criteria.8-14

‡ On average, 45 metaphases were analyzed per dog; only cells with 78 chromosomes were analyzed.
third week after transplantation. In five of the eight dogs, death occurred with intercurrent infection, and dogs died between days 20 and 28 with severely hypoplastic marrow. In those dogs in which marker studies were successful, only host cells were detected. Three animals showed ultimate endogenous marrow recovery. This was confirmed both by cytogenetic and dinucleotide (CA) repeat markers.

All seven dogs in group 3 receiving CSP after transplantation showed prompt and sustained increases in granulocyte and platelet counts. None of the seven experienced graft rejection. Dinucleotide (CA) repeat markers showed only donor-type cells, whereas cytogenetic studies in two of the dogs showed rare dividing cells with host karyotype. Serum creatinine values, obtained before transplantation and at weekly intervals after transplantation until day 35, were within the normal range in all seven dogs. CSP serum levels were measured weekly by TDx assay (Abbott Laboratories) in five dogs until day 35. The mean level for the five dogs was 950 ng/mL (range, 144 to 3,310 ng/mL). The high mean serum level was in large part due to one dog (D870) that had levels ranging from 1,750 to 3,310 ng/mL. Dogs were euthanized at the completion of the study on days 110 to 345 (median, day 294) because of limitations in kennel space. None of the dogs showed any clinical or histopathologic evidence of GVHD.

Table 3 compares the overall results in the current study with those obtained in 17 historical12,16 and concurrent control dogs not receiving additional immunosuppression. Control dogs had 64% graft rejection, 41% early mortality, 24% survival with autologous marrow recovery, and 35% survival with allogeneic engraftment, either in the form of mixed chimerism or with all donor cells. By comparison, rejection in dogs receiving high-dose corticosteroids was 100%, early mortality 60%, and survival with autologous marrow recovery 40%. Similarly, dogs receiving CSP before transplantation had 100% rejection, 67% early mortality, and 33% autologous marrow recovery and survival. In contrast, none of the dogs receiving CSP postransplantation rejected the transplant and all are surviving with sustained allogeneic engraftment. A statistical comparison of results in control dogs and in dogs receiving CSP postransplantation using a Monte Carlo simulation program showed that the latter dogs had both significantly increased rates of allogeneic engraftment and of survival (P = .01).

**DISCUSSION**

As early as 1959, at the occasion of the Vinca nuclear reactor accident in the former Republic of Yugoslavia, Mathé et al17 hypothesized that transiently engrafting human marrow allografts could be used to protect patients from marrow lethal effects of accidental exposure to radiation. Previous canine studies from this18 and other laboratories19-20 supported Mathé et al’s hypothesis, although it was determined that, to be effective, marrow grafts needed to be from littermate donors that were compatible for the antigens of the major histocompatibility complex. An earlier study in dogs using unrelated DLA-mismatched donors failed to show a protective effect of marrow transplantation after low-dose but lethal TBI.21 In other studies2 using gradually increasing TBI doses ranging from 450 to 920 cGy, we found that between 60% and 70% of animals so treated survived, either because of sustained allogeneic engraftment or because of late endogenous marrow recovery that was made possible by the transient protection provided by the marrow allograft. As the TBI doses used increased, the number of successful allografts increased and the number of animals showing endogenous marrow recovery decreased, commensurate with both increases in immunosuppression and in marrow toxicity. For the current study, we used a TBI dose of 450 cGy that was barely in the supralethal range (400 cGy is uniformly lethal).22 The dose was chosen because previous data showed that only 35% of marrow allografts after 450 cGy were sustained.24 In this way a stringent setting was established to test the effects of additional immunosuppressive agents.

The use of additional immunosuppressive drugs not only might help in improving the results of marrow allografts in victims of radiation accidents, but also might give insight...
into the development of new and perhaps less toxic conditioning programs for patients with nonhematologic malignancies undergoing marrow grafting.

The immunosuppressive regimens were chosen on the basis of past studies. In 1988, Kerman et al. published a regimen consisting of antithymocyte globulin and high-dose corticosteroids that was used to condition patients for second marrow grafts after rejection of the first. The same high-dose corticosteroid schedule was used by Anasetti et al. in conjunction with a nonmitogenic monoclonal antibody to the CD3 complex in preparation of patients for successful second marrow grafts after rejection of the first grafts. The relative contribution made by each of the immunosuppressive agents to the regimen's clinical successes is unknown. However, the complete lack of effectiveness of the high-dose corticosteroid regimen in the present study would suggest that this agent's usefulness for marrow transplantation is limited. A marginal reduction in graft rejection with prednisolone was reported in rats receiving 3 Gy TBI combined with 35 mg busulfan/kg in preparation for semiallogeneic marrow grafts from F1 hybrids.

We previously reported that pretransplantation CSP was capable of overcoming transfusion-induced sensitization, thereby enhancing hematopoietic engraftment in recipients of DLA-identical marrow after high-dose (920 cGy) TBI. However, when used before transplantation in nonsensitized recipients of the current study, the regimen failed to enhance allografts. Thus, although suppressing the effect of lymphocytes sensitized to minor histocompatibility antigens in a setting of high-dose (920 cGy) TBI, pretransplantation CSP failed to provide supplemental immunosuppression in a setting of suboptimal (450 cGy) TBI.

Hows et al. postulated previously that CSP enhanced the rate of allogeneic marrow engraftment in patients with severe aplastic anemia, although their study was uncontrolled. Another uncontrolled study from Seattle failed to confirm How's et al.'s observations. It could be argued that, in the case of transplantations for aplastic anemia, the conditioning programs consisting of cyclophosphamide or cyclophosphamide and some form of irradiation are sufficiently immunosuppressive to establish HLA-identical transplants in hosts who are not sensitized against the donors and that, therefore, any added beneficial effect of CSP might be difficult to show. Rejection of grafts in patients with aplastic anemia is most likely due to sensitization of host against donor by previous transfusions, and it has never been studied experimentally whether posttransplantation CSP is capable of overcoming such sensitization. Nevertheless, the fact that posttransplantation CSP, as used here, was uniformly successful in establishing allografts after barely lethal TBI in nonsensitized recipients would lend support to the observations reported by How's et al. Similar uniform engraftment in dogs was previously seen only when the TBI dose was increased from the current 450 cGy to 920 cGy or when the dose rate with which 450 cGy TBI was delivered was increased to 70 cGy/min. Thus, the immunosuppressive effect of CSP in this transplantation setting could be equated to that of 470 cGy of TBI at 7 cGy/min or to that of an increase in radiation dose rate by one log.

Other previously reported animal studies have involved histoincompatible donor-recipient settings. Uharek et al. grafted marrow from F1 hybrids into parental strain rats and achieved improved engraftment with CSP after suboptimal conditioning regimens. In their studies, CSP was administered for 28 days after transplant, and they observed frequent late graft rejections after cessation of CSP therapy. In contrast to these studies, Wagner, using completely allogeneic T-depleted marrow grafts in rats, reported stable donor type engraftment with CSP administered at 10 to 12.5 mg/kg for 28 days, although survival figures in the report end at day 60 after transplantation. Earlier data in DLA-nonidentical unrelated dogs were in agreement with those reported by Uharek et al. and suggested an increased incidence of graft failure with CSP postgrafting. Most likely, CSP blocked the graft-enhancing effect of donor lymphocytes in histoincompatible canine recipients while not interfering with host natural killer cells thought to cause graft rejection. In contrast, the host effectors with genotypically DLA-identical grafts are likely to be T cells.

The lack of GVHD in current dogs was striking, consistent with findings in earlier studies. The absence of GVHD may be attributable in part to the posttransplantation use of CSP and may be related in part to mixed host-donor chimerism, which is known to facilitate graft-host tolerance in studies in mice and perhaps also in patients with aplastic anemia receiving marrow grafts from HLA-identical siblings.

Even though the dose of posttransplantation CSP used here was higher than that used after human allogeneic marrow transplantation (30 mg/kg/d of 12.5 mg/kg/d), none of the current dogs showed side effects from CSP. Whether the dose of CSP could be decreased without losing the graft-enhancing effect is currently unknown. However, it is possible that higher doses of CSP could also be tolerated in human patients with nonmalignant hematopoietic diseases if the intensity of the pretransplantation conditioning program was decreased to a level similar to that used in current dogs. Whether the TBI dose could be decreased even further in the present model without jeopardizing engraftment remains to be established.

In conclusion, high-dose CSP posttransplantation enhances engraftment of DLA-identical marrow in a setting of barely lethal TBI exposure while pregrafting CSP and peritransplantation and posttransplantation high-dose corticosteroids were ineffective.

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