Stable Mixed Hematopoietic Chimerism in Dogs Given Donor Antigen, CTLA4Ig, and 100 cGy Total Body Irradiation Before and Pharmacologic Immunosuppression After Marrow Transplant

By Rainer Storb, Cong Yu, J. Maciej Zaucha, H. Joachim Deeg, George Georges, Hans-Peter Kiem, Richard A. Nash, Peter A. McSweeney, and John L. Wagner

Stable mixed chimerism can be established in dogs given a sublethal dose of 200 cGy total body irradiation (TBI) before and immunosuppression with mycophenolate mofetil (MMF) and cyclosporine (CSP) for 28 and 35 days, respectively, after dog leukocyte antigen-identical marrow transplantation. Most likely, the role of pretransplant TBI was to provide host immunosuppression, since stable mixed chimerism was also achieved in MMF/CSP-treated dogs when 450 cGy irradiation, targeted to cervical, thoracic, and upper abdominal lymph nodes, was substituted for TBI. When TBI was reduced from 200 to 100 cGy, all grafts were rejected within 3 to 12 weeks. Here, we asked whether stable engraftment after 100 cGy TBI could be accomplished by first reducing the intensity of host immune responsiveness with help of the fusion peptide CTLA4Ig, which blocks T-cell costimulation through the B7-CD28 signal pathway. Accordingly, recipient T cells were activated with intravenous (IV) injections of 10^6 donor peripheral blood mononuclear cells (PBMC)/kg per day on days −7 to −1 before 100 cGy TBI, with concurrent administration of CTLA4Ig 4 mg/kg/d IV. All 7 dogs so treated showed initial mixed chimerism. Two rejected their allografts after 8 and 20 weeks, respectively, and survived with autologous marrow recovery; 1 mixed chimera was unevaleuable because of death at 3 weeks from intussusception; and 4 showed persisting mixed chimerism, including unirradiated marrow and lymph node spaces, for now more than 46 to 70 weeks after transplant. Data support the hypothesis that stable allograft engraftment can be established by combining nonmyeloablative pretransplant host immunosuppression with posttransplant host and donor cell immunosuppression using MMF/CSP.

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pretransplant CTLA4Ig was administered,3 most current dogs achieved sustained engraftment of dog leukocyte antigen (DLA)-identical littermate marrow.

MATERIALS AND METHODS

Litters of beagles, harriers, pit bull-beagle crossbreeds, and other mixed breeds were either raised at the Fred Hutchinson Cancer Research Center (Seattle, WA) or purchased from commercial kennels. The dogs weighed from 7.2 to 11.1 (median, 10.5) kg and were 7 to 14 (median, 8) months old. They were observed for disease for at least 2 months before study. All were immunized for papillomavirus, leptospirosis, distemper, hepatitis, and parvovirus. The research protocols were approved by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center. Research was conducted 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 kennels were certified by the American Association for Accreditation of Laboratory Animal Care.

DLA-identical littermate donor/recipient pairs were chosen based on the identity for highly polymorphic MHC class I and class II microsatellite marker polymorphisms.25 Specific DLA-DRB1 allelic identity was determined by direct sequencing.26

The day of marrow grafting was designated as day 0. Recipients were injected IV with 10⁶ marrow donor PBMC/kg/d on days −7 to −1. Additionally, they received IV CTLA4Ig 4 mg/kg/d on days −7 to −1. On day 0, they were given a single dose of TBI 100 cGy delivered at 7 cGy/min from 2 opposing cobalt-60 sources.27 Marrow was harvested from the donors under general anesthesia through needles inserted into humeri and femora,27 and infused IV into the recipients at doses of 3.8 to 4.7 (median, 4.0) × 10⁸ nucleated cells/kg within 4 hours of TBI. Recipients were given postgrafting immunosuppression that consisted of CSP 15 mg/kg twice daily orally from day −1 to day 35, and MMF 10 mg/kg twice daily subcutaneously from day 0 to day 27.2 Standard postgrafting care included twice-daily oral nonabsorbable antibiotics (neomycin sulfate and polymyxin sulfate) beginning on day 1 to day 35, and MMF 10 mg/kg twice daily subcutaneously from day 0 to day 27.2

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Hematopoietic engraftment was assessed by sustained recoveries of peripheral blood granulocyte and platelet counts after the postirradiation nadirs, histologic features of the marrow from biopsy or autopsy specimens, and documentation of donor microsatellite marker polymorphisms in nucleated cells from blood and marrow. Conversely, graft rejection was defined as complete repopulation of the hematopoietic system with cells of host type. Posttransplant marrow aspirates from the humoral head were done under general anesthesia. Donor and host hematopoietic cells were distinguished by microsatellite marker polymorphisms as assessed in a polymerase chain reaction (PCR)-based assay.28 The technique detected between 2.5% and 97.5% mixtures of donor and host cells. Mixed hematopoietic chimerism was quantified by estimating the proportion of donor-specific DNA among host DNA using the storage phosphorimaging technique.29

Results in current dogs were compared with those in 6 previously reported dogs given the same treatment except for omission of pretransplant donor PBMC and CTLA4Ig injections.3 Additionally, peripheral blood count changes in transplanted dogs were compared to those of 12 dogs given 100 cGy TBI and no subsequent marrow transplant (unpublished observations, 1999).

Mixed leukocyte cultures (MLC)30 were performed to assess the immunosuppressive effectiveness of CTLA4Ig on dog cells in vitro. To this purpose, PBMC from healthy dogs were separated from heparinized whole blood using a Ficoll-Hypaque gradient (density = 1.074). Cells were washed and then resuspended in Waymouth’s medium supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 1% L-glutamine, and 20% heat-inactivated pooled, normal dog serum. A total of 10⁵ responder cells/well and 10⁵ irradiated (2,200 cGy in vitro irradiation from a cesium source) stimulator cells/well obtained from DLA-nonidentical unrelated donors were cultured together in the presence of increasing concentrations of CTLA4Ig or a control peptide, L-6, in round-bottom, 96-well plates for 6 days at 37°C in a humidified 5% CO² air atmosphere. On day 6, cultures were pulsed with 1 µCi of ³H-thymidine (³H-Td) for 18 hours before harvest. ³H-Td incorporation was determined using a β-scintillation counter (Beckman Instruments, Fullerton, CA). Data were analyzed as mean counts per minute (cpm) of 3 replicates.

Samples for serum levels of CTLA4Ig were collected from 3 dogs before and at various time intervals after a single IV administration of CTLA4Ig 4 mg/kg/d. Serum CTLA4Ig levels were measured using a sandwich enzyme-linked immunoadsorbent assay (ELISA).31 All tests were performed in triplicate.

RESULTS

Table 1 lists data on the immunosuppressive effectiveness of CTLA4Ig in an in vitro MLC assay. At CTLA4Ig concentrations ranging from 0.625 to 10 µg/mL, 90% to 95% inhibition of MLC reactivities was seen. At 0.3 µg/mL, inhibition ranged from 55% to 90%, and at 0.1 µg/mL, it ranged from 35% to 60%. No significant suppression of MLC reactivity was seen with comparable concentrations of the control peptide, L-6.

CTLA4Ig serum levels in 3 dogs given 7 daily injections of CTLA4Ig 4 mg/kg IV each are shown in Fig 1. Within 1 minute of the first injection, CTLA4Ig levels up to 200 µg/mL were observed, with subsequent declines over 2 hours to levels ranging from 60 to 70 µg/mL. Subsequent daily trough levels ranged from 25 to 80 µg/mL. These levels were well above the range at which virtually complete suppression of MLC reactivity was observed in vitro.

Table 2 summarizes the results of the transplant studies. The results of transplant studies. The 6 previously reported dogs not given pretransplant donor

<table>
<thead>
<tr>
<th>Protein (µg/mL)</th>
<th>0</th>
<th>0.15</th>
<th>0.31</th>
<th>0.625</th>
<th>1.25</th>
<th>2.50</th>
<th>5.00</th>
<th>10.00</th>
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<tr>
<td>CTLA4-Ig (cpm)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Experiment 1</td>
<td>25,346</td>
<td>10,254</td>
<td>2,536</td>
<td>1,181</td>
<td>1,333</td>
<td>1,193</td>
<td>1,126</td>
<td>700</td>
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<tr>
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<td>8,091</td>
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<td>1,182</td>
<td>473</td>
<td>328</td>
<td>179</td>
<td>334</td>
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<tr>
<td>L-6 (cpm)</td>
<td>14,607</td>
<td>20,956</td>
<td>19,360</td>
<td>14,265</td>
<td>19,568</td>
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</table>

A total of 10⁵ responder cells and 10⁵ irradiated (2,000 cGy) stimulator cells obtained from DLA-nonidentical unrelated dogs were cultured per well either in the absence or the presence of increasing concentrations of CTLA4Ig or the control peptide L-6. ³H-thymidine incorporation was determined after 6 days of culture. Responses are presented as the mean cpm of triplicate experiments. CTLA4Ig was tested in 2 separate experiments.
PBMC and CTLA4Ig showed initial allogeneic engraftment that lasted for 3 to 12 weeks with subsequent graft rejection, and they survived with complete autologous recovery. The 7 dogs given pretransplant PBMC and CTLA4Ig also engrafted. One of the 7 died from an intussusception, a CSP-associated toxicity in dogs, at 3 weeks with mixed donor/host hematopoietic chimeraism present. This dog’s early death precluded final evaluation of the fate of the allograft. Two dogs rejected their grafts after 8 and 20 weeks, respectively, and survived with autologous marrow recovery. Four dogs have remained stable mixed donor/host chimeras for now more than 46 to 70 weeks after transplant.

Figure 2 illustrates the hematologic changes and the results of microsatellite marker studies in one of the current dogs (E519). The granulocyte nadir occurred at approximately day 10 with a count of 3,000/µL followed by rapid recovery. The platelet count nadir occurred at 60,000/µL on day 14 followed by recovery, while the lymphocytopenia was more prolonged, and recovery did not occur until after week 7. The lowest lymphocyte counts were on the order of 600/µL. The microsatellite marker studies performed 41 to 44 weeks after transplant demonstrated the presence of donor cells among all nucleated peripheral blood cells, mononuclear cells, and granulocytes.

Figure 3 illustrates the median peripheral blood granulocyte, lymphocyte and platelet changes in all current dogs. During the first 2 weeks after TBI, no obvious differences were seen between their lymphocyte counts and those of the previously transplanted dogs not given pretransplant CTLA4Ig and of dogs not given marrow grafts after exposure to 100 cGy TBI. The speed of recovery of counts between days 15 and 35 was marginally but not significantly slower in CTLA4Ig-treated dogs compared with dogs of the 2 other groups.

The complete results of microsatellite marker studies in 5 of the evaluable current dogs are shown in Fig 4. Continued allogeneic engraftment was seen in 4 and graft rejection in 1 of the 5 dogs. The degree of stable donor chimerism ranged from 10% to 60%.

Comparing the duration of mixed chimerism in the 6 current evaluable CTLA4Ig-treated dogs (not included was dog E736 that died from intussusception) with that among previously transplanted dogs not given pretransplant CTLA4Ig and of dogs not given marrow grafts after exposure to 100 cGy TBI. The speed of recovery of counts between days 15 and 35 was marginally but not significantly slower in CTLA4Ig-treated dogs compared with dogs of the 2 other groups.

<table>
<thead>
<tr>
<th>Table 2. Marrow Grafts From DLA-Identical Littermates After Conditioning With 100 cGy TBI Delivered as a Single Dose at 7 cGy/min</th>
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<tbody>
<tr>
<td>Pretransplant Donor PBMC + CTLA4Ig*</td>
</tr>
<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>No†</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>E736</td>
</tr>
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<td>E606</td>
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NOTE. All recipients were given MMF/CSP* after transplant: MMF 10 mg/kg/twice daily, subcutaneously days 0 to 27; CSP, 15 mg/kg/twice daily, orally days 1 to 35.

Abbreviations: ET, euthanized at completion of the study; NE, not evaluable.
*106 PBMC/kg/day IV days — 7 to — 1; CTLA4Ig, 4 mg/kg/d IV days — 7 to — 1.
†Assessed by weekly microsatellite marker studies of PBMC or marrow cells.
‡Data in these dogs were previously published.§
§Dog was a mixed chimera at the time of death.
DISCUSSION

The present study is based on the assumption that allografts can create their own space in the host’s marrow through GVH reactions and, consequently, that intensive cyto-reductive and toxic conditioning regimens conventionally used for hematopoietic stem-cell transplants can be replaced by nonmyelotoxic immunosuppression capable of promoting acceptance of grafts. Specifically, immunosuppressive agents delivered before transplant would be applied to blunt host T-cell responses, while immunosuppression delivered after transplant would serve to modify both host and donor immune reactivities, with resulting mutual graft/host tolerance in the form of stable mixed donor/host hematopoietic chimerism. Ideally, agents administered before transplant would suppress only host cells with the potential to react to donor antigens while other, say anti-viral, immune responses would be left intact. Experimental evidence in support of this novel transplant approach was provided by a previous canine study in which pretransplant TBI was replaced by 450 cGy irradiation targeted to cervical, thoracic, and upper abdominal lymph nodes. When combined with posttransplant immunosuppression by MMF/CSP, pretransplant lymph node radiation resulted in stable, long-term mixed chimerism also in those marrow and lymph node spaces that were shielded from irradiation. Further evidence has come from successful clinical studies in 2 patients with inherited T-cell deficiencies other than severe combined immunodeficiency disease (SCID), in whom pretransplant immunosuppression was omitted and stable grafts were established solely with posttransplant MMF/CSP.

The current study was a first step toward the ideal transplant program in patients with malignant and nonmalignant hematologic diseases that would rely entirely on nonmyelotoxic immunosuppression. The dose of TBI used here, 100 cGy delivered at 7 cGy/min, was small and resulted only in moderate and transient declines of peripheral blood cell counts, even in dogs not rescued by subsequent marrow transplants. Previous studies had shown 100 cGy TBI alone to be insufficient to assure stable allografts in this model. Adding pretransplant activation of host T-cell receptors through daily injections of...
donor PBMC along with blockade of the second T-cell activation signal through B7—CD28 allowed stable engraftment in 4 of 6 evaluable dogs conditioned with 100 cGy TBI. The success with this approach is all the more impressive since extensive previous studies had shown exposure of canine recipients to donor PBMC or whole blood transfusions before high-dose TBI (920 cGy single dose) to result in a high incidence of graft rejection in the absence of concurrently administered CTLA4Ig.33 Current CTLA4Ig-treated marrow transplant dogs had slower peripheral blood count recoveries than either radiation controls or marrow grafted dogs not given CTLA4Ig. The marginal differences in blood count recoveries could be explained as follows. Recoveries in radiation controls and in previous marrow transplant recipients were largely or entirely derived from the considerable numbers of autologous progenitors which had survived 100 cGy TBI—the donor contributions in the transplanted dogs were not only transient, but also weak and barely above the levels of detection by PCR (data not shown). In contrast, the donor contribution in current CTLA4Ig-treated dogs was strong in at least 4 of the 6 evaluable recipients. This would imply the elimination of a proportion of host progenitors via the graft and, accordingly, a shift in the dependence of blood count recoveries from the relatively large autologous toward the smaller pool of transplanted allogeneic progenitors. Given the differences in stem-cell pool sizes, slower recoveries would be anticipated in the allogeneically engrafted dogs.

A study by Wekerle et al34 in mice conditioned with 300 cGy TBI (dose rate not given) has explored blockade of costimulatory signals to establish mixed hematopoietic chimerism. They found that monotherapy with either an injection of CTLA4Ig on day 2 or a monoclonal antibody to CD40L on day 0 failed to induce stable mixed chimerism. However, combining injections of CTLA4Ig and antibody to CD40L and, thereby, blocking 2 costimulatory signals, resulted in “high levels (>40%) of stable (>8 months) multilineage donor hematopoiesis.” Improved cardiac and skin xenograft survivals were also observed when both CTLA4Ig and antibody to CD40L were combined in murine hosts.18 The combination of the 2 agents also prolonged kidney grafts in primates better than either agent alone.22

There are at least 2 reasons why pretransplant CTLA4Ig was not uniformly successful in the current model. First, the fusion peptide not only blocks the positive signal from B7—CD28, but
it also interrupts the desirable negative signal from B7—CTLA4Ig, which serves to turn off T-cell activation. Also, one study has shown the development of acute GVHD in TBI-treated mice given hematopoietic grafts from CD28− donors. Another study, however, failed to confirm that result. These contrasting results were explained by the differences in the mouse strain combinations used.

Nevertheless, taken together, these studies suggest that either selective blockade of the B7−CD28 pathway with intact signaling through CTLA4 or the additional blockade of other costimulatory signals, may permit to lower the TBI dose further or even completely eliminate TBI in the current canine model of stem-cell transplantation.

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