Treatment of Established Graft-Versus-Host Disease in Dogs by Antithymocyte Serum or Prednisone

By R. Storb, H. J. Kolb, T. C. Graham, H. Kolb, P. L. Weiden, and E. D. Thomas

Marrow grafts after 1200 R of whole-body irradiation were carried out between pairs of unrelated dogs of different breeds that were incompatible for dog histocompatibility antigens. Three groups of recipients were studied. Recipients in all groups were treated with the immunosuppressive agent methotrexate (MTX), 0.4 mg/kg, on days 1, 3, 6, and 11, and then once weekly until day 102 after grafting. Dogs in group 1 were given MTX only. Thirty of 35 recipients died between 11 and 51 days and five survived more than 102 days. Median survival time was 20 days. Dogs in group 2 were treated with rabbit antidog antithymocyte serum (ATS) when graft-versus-host (GVH) disease became established. Eight of ten recipients died between 19 and 76 days and two survived more than 102 days. Median survival time was 45 days. Dogs in group 3 were treated with daily injections of prednisone when GVH disease became apparent. Seven of nine recipients died between 11 and 34 days and two survived more than 102 days. Median survival time was 24 days.

It was concluded that established GVH disease in dogs with histoincompatible marrow grafts can be favorably influenced by ATS with significant \( p < 0.01 \) prolongation of survival. However, there was no evidence that treatment with ATS increased the fraction of long-term survivors in this histoincompatible setting. Prednisone was ineffective and did not prolong survival \( (p > 0.1) \).

Previous studies in this laboratory have shown uniformly fatal graft-versus-host (GVH) disease within 14 days of grafting in lethally irradiated dogs given marrow from histoincompatible donors. Similarly, acute GVH disease was seen in another randomly bred species, the rhesus monkey, in which death occurs within 25 days of grafting. Efforts to overcome GVH disease as a major barrier to successful marrow engraftment have been directed to matching of donor and recipient by in vitro methods of histocompatibility typing and to preventing or delaying GVH disease by the use of immunosuppressive agents.
immediately before or after marrow grafting, or to separating immuno-
logically active cells from the marrow inoculum before infusion. None of these
approaches has proved to be entirely satisfactory. Fatal GVH disease has been
reported in some patients despite the use of marrow from an HL-A matched
sibling and postgrafting treatment with the immunosuppressive agents metho-
trexate (MTX) or cyclophosphamide (CY). Alternative ways of overcoming
GVH disease, therefore, must be sought and evaluated.

The treatment of established GVH disease following hemopoietic grafting
has been evaluable in only a few studies in rodents. The present study
evaluated the effectiveness of rabbit antidog antithymocyte serum (ATS) or
prednisone for modifying established GVH disease in dogs following hemo-
poietic grafts from histoincompatible unrelated donors.

MATERIALS AND METHODS

All donor-recipient pairs were unrelated and of different breeds. To exclude the remote possi-
Bility of random selection of a compatible pair, donor and recipient were typed for dog leukocyte
antigens (DL-A) as determined by serologic histocompatibility typing. Details on the preparation
of the dogs, the development of the initial DL-A typing sera and technique, and the usefulness of
these sera in selecting donor-recipient combinations for grafts of marrow and of other organs have
been described. Presently, more than 150 DL-A typing sera detecting 23 antigenic groups
are used in this laboratory. Each donor-recipient pair in the present study differed by at least
two of the 23 groups.

Recipients were conditioned for hemopoietic grafting by exposure to 1200 R of whole-body ir-
radiation (midline air exposure) given at a rate of 9.3 R/min from two opposing 60Co sources.
The midpoint body exposure was approximately 1000 rads. Hemopoietic grafts were carried out
using combined infusions of donor marrow and peripheral blood leukocytes. Donor marrow cells,
were infused within 4 hr of irradiation of the recipient. The postgrafting care of marrow re-
cipients has been described.

All recipients were given MTX, 0.4 mg/kg intravenously, on days 1, 3, 6, and 11 after allogeneic
hemopoietic grafting and then once weekly until day 102. The MTX dose was adjusted to the
weight of the dogs following transplantation. Three groups of recipients were studied. Group 1:
Dogs given MTX only. Group 2: Dogs given ATS within 2 days of the time at which GVH disease
became evident. ATS was started 10–18 days postgrafting. The schedule was ATS, 0.2 ml/kg sub-
cutaneously daily for 10 days and then every other day until days 29–31. Group 3: Dogs given
prednisone starting within 2 days of the time at which GVH disease became apparent. Prednisone,
2 mg/kg intravenously, was given daily for 5 days and then 1 mg/kg daily for 10 days.

Marrow engraftment was assessed by promptly rising white blood cell and platelet counts follow-
ing the postirradiation decline, histologic features of the marrow, demonstration of donor sex
karyotypes in cells from marrow and peripheral blood, a change to donor red blood cell antigens
A or B, a change to donor red blood cell glutamic oxaloacetic acid transaminase (GOT), or
the development of GVH disease. Clinical findings of GVH disease consisted of anorexia, diarrhea,
scleral injection, erythematous skin eruptions, and icterus. Histologic findings included regener-
ating marrow, necrosis or degeneration of ileal and colonic mucosa, dyskeratotic and necrotic skin
lesions, focal liver cell necrosis, and bile duct necrosis. GVH changes in the dog have been
described previously and are similar to those described in other species.4529 Marrow graft
rejection was defined by a decline of white blood cell counts to less than 1000/cu mm and of plate-
let counts to less than 10,000/cu mm with extreme marrow hypocellularity following initial evidence
of engraftment.

ATS was produced in 40 New Zealand white rabbits. Thymocytes from newborn puppies
were emulsified in complete Freund’s adjuvant and injected into all four foot pads. After 3 wk, thymocytes in saline without adjuvant were injected intravenously on each of 3 successive days into
each rabbit. Seven days after the last intravenous injection, the rabbits were bled. Sera of all 40
rabbits were pooled. The lymphocytotoxic antibody titer of the pool was 1:1000, the hemagglutinin
The titer was 1:2000. Absorptions on dog red blood cells were not carried out. The pooled ATS was inactivated for 30 min at 56°C and stored in 3-ml plastic syringes at −20°C before use. Skin grafts were carried out between five unrelated pairs of DL-A incompatible dogs to test the immunosuppressive properties of the ATS. ATS, 0.2 ml/kg body weight subcutaneously, was given daily for 23 days beginning 2 days before skin grafting. Four-by-four-centimeter full-thickness grafts were removed from the flank of the donor and sutured into 4 × 4-cm beds on the flank of the recipient. The grafts were bandaged using Teflon-coated material and inspected daily. The day of rejection was determined from gross examination of the graft when more than 75% induration and failure of blanching on pressure occurred. The mean graft survival time in 20 untreated recipients of DL-A incompatible skin was 10.3 ± 4.6 (S.D.) days, and 52 days (18, 26, 28, 90, 98 days) in ATS-treated dogs. There were no major ATS toxicities in the five dogs during ATS administration except peripheral lymphocytopenia. There was no evidence of hemolytic anemia. The platelet counts in the five dogs ranged from 151,000 to 505,000/cu mm during ATS therapy. Two dogs showed on one occasion a platelet count of 117,000 and 138,000/cu mm, respectively.

**RESULTS**

Table I shows the survival of dogs in groups 1-3. All 35 dogs in group 1 treated with postgrafting MTX only had prompt hemopoietic engraftment. Twenty-five of the 35 died between 15 and 51 days with GVH disease. Five rejected the marrow graft and died between 11 and 18 days with marrow hypoplasia. Five survived beyond 102 days. The median survival time of dogs in group 1 was 20 days. One of the five surviving dogs was severely icteric with elevated serum bilirubin and GOT levels between days 16 and 72 and then recovered. The four others showed erythematous skin lesions beginning between days 11 and 17 and lasting between 26 and more than 100 days. One of the surviving dogs differed from its donor with respect to canine red cell antigen A. When tested on repeated occasions more than 102 days after grafting, its red cells were of donor type. Two surviving dogs had donors of opposite sex. All 73 cells in marrow, peripheral blood, and lymph node subjected to cytogenetic analysis between day 56 and more than 1 yr after grafting showed the donor sex karyotype pattern. One dog differed from its donor with respect to red blood cell GOT. When tested on repeated occasions more than 70 days after grafting, the enzyme pattern was of donor type. No marker existed in one of the sur-

<table>
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*MTX, 0.4 mg/kg intravenously, on days 1, 3, 6, and 11 and once weekly thereafter until day 102.
†ATS treatment was begun between days 10 and 18 postgrafting. ATS, 0.2 ml/kg subcutaneously, was given daily for 10 days and then every other day until day 29–31 postgrafting.
†Prednisone treatment was begun between days 10 and 41 postgrafting. Prednisone, 2 mg/kg intravenously, was given daily for 5 days and then 1 mg/kg daily for 10 days.
viving dogs. The clinical and histologic changes in dogs dying with GVH disease in this group were severe and generally involved all three target organs: skin, intestine, and liver. Lymphoid hypoplasia was prominent. The marrow in these dogs showed good repopulation.

All ten dogs in group 2 showed good evidence of initial marrow engraftment. All had rising white blood cell and platelet counts following the postirradiation decline. Seven of the ten dogs had marrow donors of opposite sex. Marrow for cytogenetic studies was obtained in these seven between days 10 and 20. One hundred and twenty-eight out of 130 cells analyzed showed the donor karyotype. Two other dogs differed from their donor with respect to canine red cell antigen B. When tested repeatedly more than 70 days postgrafting the red cells were of donor type. In one dog no marker existed. Nine of the ten dogs in this group showed GVH disease between days 9 and 16 postgrafting. The diagnosis was based on the development of erythematous skin lesions in all nine and of diarrhea in five. None of the dogs showed icterus. ATS treatment was started between days 11 and 18, 0.2 ml/kg subcutaneously daily for 10 days and then every other day until days 29-31. Under ATS therapy clinically evident GVH lesions disappeared dramatically over a period of 3 to 14 days. The ATS injections were well tolerated with the exception of some local induration. Of the nine dogs treated with ATS, three lost the marrow graft and died between 20 and 46 days with marrow hypoplasia. The other six showed sustained hematopoietic engraftment. Two of these survived more than 102 days. The cause of death in one dog dying on day 33 remained unknown despite extensive gross and histologic autopsy examination and bacterial cultures. There was no GVH disease nor infection. Two dogs dying with septicemia on days 44 and 76 also did not show evidence of GVH disease. One dog died on day 57 with hepatocyte necrosis but no skin nor intestinal GVH lesions. The liver lesions were not diagnostic for GVH disease and may constitute evidence for liver toxic effects of ATS. The resolution of clinical signs and the absence of autopsy evidence of GVH disease in dogs of group 2 contrasted sharply with the findings in dogs of group 1. One dog in group 2 did not develop clinical evidence of GVH disease and was not treated with ATS. After good initial engraftment the dog rejected the marrow graft and died on day 20 with marrow hypoplasia.

All nine dogs in group 3 had good evidence of marrow engraftment. One of the dogs did not develop GVH disease, rejected the marrow graft and died on day 11 with marrow hypoplasia. Of the remaining eight dogs, seven showed clinical evidence of GVH disease between days 10 and 18 and one on day 40 postgrafting. Prednisone therapy, 2 mg/kg intravenously daily for 5 days and then 1 mg/kg daily for 10 days was started between days 11 and 41. In two dogs the GVH lesions disappeared over a period of 10-20 days. These survived more than 102 days without clinical evidence of persistent GVH disease. The remaining six dogs died with rapidly progressing GVH disease between 14 and 34 days. Clinically and histologically, the severity of GVH lesions encountered in dogs of group 3 was not distinguishable from that of dogs in group 1. One of the surviving dogs had a donor of opposite sex. Chromosome analysis of marrow cells on day 53 showed all 20 cells studied to be of donor karyotype.

Statistical analysis of the data using a two-sided Mann-Whitney U test for
comparison of nonparametric samples showed a significant improvement in survival of dogs in group 2 treated with ATS over that of dogs in group 1 treated with MTX only \( (p < 0.01) \). Dogs in group 3 treated with prednisone showed no improvement in survival \( (p > 0.1) \) over that of dogs in group 1.

**DISCUSSION**

GVH disease is remarkably similar in randomly bred species including the dog, monkey, and man. The clinical and histologic changes are the result of disturbances in target organs, in particular the skin, gut, liver, and the lymphoid tissue. The model system used in the present study, i.e., grafts between unrelated DL-A incompatible canine donor-recipient pairs, is characterized by a rapidly fatal course within 14 days of grafting when no posttransplantation immunosuppression is used. In contrast, GVH disease in rodents generally occurs later and takes a milder course, and a variable proportion of the animals recover.

A variety of chemotherapeutic agents have been used in attempts to ameliorate or prevent GVH disease in radiation chimeras. Most reports indicate that, to be effective, treatment with these agents must be begun well before the GVH disease has become established. MTX is an effective agent to delay or prevent GVH disease in mice, dogs, and monkeys when given in the immediate postgrafting period. Studies in dogs also have shown that MTX is most effective when continued for a prolonged period of time, and stable long-term chimerism for periods of years was achieved in some DL-A incompatible graft recipients with postgrafting MTX only. CY was beneficial in ameliorating GVH disease in mice, rats, and monkeys, and, in various dose schedules used immediately postgrafting, has been found ineffective in preventing lethal GVH disease in dogs. Studies comparing the usefulness of CY and MTX for achieving long-term survival in species other than the dog have not been carried out.

Antilymphocyte serum (ALS) or ATS therapy beginning immediately postgrafting has been used in rodents and dogs and monkeys and has generally been disappointing in that only minimal prolongation of survival was obtained in some animal systems studied. Dogs either died with GVH disease within the range during which untreated animals died, or they died with marrow aplasia due to the hemopoietic stem cell toxicity of the ATS. ALS or ATS pretreatment of recipients of hemopoietic grafts was found to be effective in mice, dogs, and monkeys and more recently in dogs. Merritt et al., using antihuman and antimonkey ALS or ATS prepared in rabbits or horses, extended the survival of six of 11 monkeys beyond the period during which control monkeys died. Van Bekkum et al., using horse antimonkey ALS, prolonged survival of four of 16 monkeys beyond the control range. Dogs given ATS before grafting (0.2 ml/kg daily \( \times 10 \)) and the presently used MTX regimen postgrafting showed significantly prolonged survival \( (p < 0.05) \) over dogs given MTX only. When the ATS dose was raised (1.0 ml/kg daily \( \times 3 \)), most dogs failed to sustain engraftment. This failure of permanent engraftment did not seem to be due to marrow stem cell toxic effects of the ATS but rather was due to marrow rejection by surviving host lymphoid cells as dogs treated with the same combination showed uneventful engraftment of autologous marrow. In view of the un-
certainties related to the use of ALS or ATS in the immediate pre- or postgrafting period, the present study explored the effectiveness of ATS at a later stage postgrafting when the graft was firmly established and GVH disease became apparent.

Effective treatment of established GVH disease would eliminate the necessity of exposing all marrow graft recipients to the potential toxicity of additional immunosuppressive agents in the immediate pre- or postgrafting period. This is particularly relevant since currently available methods of donor selection and postgrafting therapy with MTX permit survival of a number of patients without evident GVH disease. Unfortunately, at present it is not possible to predict which human marrow graft recipient will show no clinical signs of GVH disease and which will develop lethal GVH disease despite HL-A matching with the sibling marrow donor and the use of postgrafting MTX.

Surprisingly, only a few studies have been reported in which established GVH disease was treated with either cytostatic agents or ALS. All of these studies were carried out in rodent systems. Haskovcova attempted to treat runt disease with ALS in (B 10 LP × A) F₁ hybrids given A cells and found it to be ineffective. Boak et al., using the Simonsen assay, treated (DBA × C57BL) F₁ hybrids with ALS 7 days after injection of C57BL cells and found it to be ineffective. Silvers and Billingham produced lethal runt disease by injecting Lewis rat lymph node cells into neonatal BN hosts. They were able to reverse runt disease by injecting anti-Lewis antiserum or rabbit anti-DA rat ATS between days 10 and 13. The authors did not demonstrate whether the animals remained chimeras. Glucksberg and Fefer inoculated BALB/c mice with C57BL/6 spleen cells after a nonlethal dose of CY and treated the animals between days 6 and 9 with various doses of CY or procarbazine and were able to decrease the incidence of death in these mice. The surviving mice apparently did not remain chimeras as suggested by the absence of detectable donor gammaglobulin allotype in host serum. Owens and Santos in a similar model using (C57BL/6 × DBA/2) F₁ hybrids as hosts and BALB/c mice as donors were able to suppress clinically established GVH disease by CY, and nearly all surviving mice remained chimeras for at least 6 wk as determined by immunoglobulin allotype markers. MTX, 6-mercaptopurine, cortisol, and HN-2 used in a similar way in their model system were not effective in suppressing established GVH disease.

In the current study, established GVH disease in the randomly bred canine model was treated with rabbit antidog ATS. Significant prolongation of survival was demonstrated. The dogs treated with ATS when GVH disease became established showed dramatic changes in their clinical status. In most instances, the skin lesions totally disappeared and the beginning diarrhea was reversed. Only one dog developed liver disease. The relatively high incidence of marrow graft rejection observed in the ATS-treated group is a disturbing finding. It could be related either to toxicity of the ATS or to inactivation of the immunologically competent cells of the graft, thus enabling remaining host lymphoid cells in turn to reject the grafted marrow. Cytogenetic studies of PHA-stimulated peripheral blood lymphocytes in dogs undergoing marrow rejection were carried out but failed to yield evaluable metaphase spreads. In addition, some other dogs in the ATS-treated group died of bacterial infection or for unex-
plained reasons. In the absence of clinical or histologic GVH disease and of evidence for bacterial, fungal, or parasitic infections the cause of death of one of these animals might have been related to infection with a virus. However, inclusion bodies were not observed histologically despite a careful search. A high incidence of virus infections in ATS pretreated primate marrow graft recipients has been reported previously by van Bekkum et al.41

We also studied another immunosuppressive agent, prednisone, which has little or no marrow toxicity,43 to test its ability to reverse established GVH disease. Although the mechanisms of action of corticosteroids in various types of immune responses are poorly understood, it is possible that some of the beneficial effects can be related to their anti-inflammatory properties.41 Based on initial studies by Zukoski et al.44 demonstrating kidney graft prolongation in dogs, prednisone is now widely used clinically in solid organ grafting. In particular, it has been shown to be effective at extremely high doses in reversing pending kidney graft rejection in dogs treated with azathioprine.45 In the present study, prednisone proved to be ineffective in reversing or ameliorating GVH disease. The evolution of GVH disease in the prednisone-treated dogs appeared to be identical to that seen in dogs given MTX only, and survival was not significantly prolonged. Histologic lesions in skin, small and large bowel, and liver of the prednisone-treated dogs were not distinguishable from those observed in dogs given MTX only.

In summary, the present study demonstrates the relative effectiveness of ATS and the ineffectiveness of prednisone in reversing canine GVH disease. Unrelated histoincompatible donor-recipient pairs constitute a severe test model for any immunosuppressive regimen, yet significant prolongation of survival was observed with ATS. The same regimen applied to the more favorable setting of donor-recipient pairs matched at the major histocompatibility locus might result in an increased number of long-term survivors. The finding that severe GVH lesions could be reversed after only a few doses of ATS provides some support for the use of this regimen in human marrow transplant recipients with potentially life-threatening GVH disease. In fact, one human patient with a marrow graft from an HL-A matched sibling developed severe GVH disease and was successfully treated with rabbit antihuman ATS.46 She is clinically well 10 mo later. Nevertheless, ATS should be used judiciously in the clinical situation in view of the potential threat of marrow graft rejection and of increased incidence of infection.

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