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What Role Is There for Antithymocyte Globulin in Allogeneic Nonmyeloablative Canine Hematopoietic Cell Transplantation?

Razvan Diaconescu,[1] Marie-Térèse Little,[1] Wendy Leisenring,[1,2,3] Murad Yunusov,[1] William J. Hogan,[1] Mohamed L. Sorror,[1] Frédéric Baron,[1] Rainer Storb[1,4]

[1]Clinical Research, and [2]Public Health Sciences Divisions, Fred Hutchinson Cancer Research Center, Seattle, Washington; Departments of [3]Biostatistics and [4]Medicine, University of Washington, Seattle, Washington

Correspondence and reprint requests: Rainer Storb, MD, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., D1-100, P.O. Box 19024, Seattle, WA 98109-1024 (e-mail: rstorb@fhcrc.org).

ABSTRACT

We investigated whether pretransplantation immunosuppression with canine-specific rabbit antithymocyte globulin (ATG), combined with a suboptimal dose of 1 Gy of total body irradiation (TBI), would permit engraftment of canine dog leukocyte antigen–identical marrow. Cumulative ATG doses of 2 to 5 mg/kg produced a T-cell depletion of 1 log in the peripheral blood and 50% in the lymph nodes. Serum levels of ATG peaked on days 4 to 6 after initiation of therapy and became undetectable by day 13 as a result of canine antibody responses to ATG. ATG prolonged allogeneic skin graft survival to 14 days (n = 5), compared with 8 days in control dogs (P = .0003). Five dogs were given marrow transplants after ATG (3.5-5 mg/kg) and 1 Gy of TBI. Posttransplantation immunosuppression consisted of mycophenolate mofetil and cyclosporine. All dogs showed initial engraftment, with maximum donor chimerism levels of 25%. However, only 1 dog achieved sustained engraftment, and 4 rejected their grafts. The duration of engraftment ranged from 8 to ≥36 weeks (median, 11 weeks), and this is comparable to that in 6 historical controls not given ATG (range, 3-12 weeks; median, 10 weeks; P = .20). The total nucleated cell doses in the marrow grafts had the highest correlation coefficient with the duration of engraftment: 0.82 (P = .09). We concluded that administering ATG before an otherwise suboptimal conditioning dose of 1 Gy of TBI failed to secure uniform stable hematopoietic engraftment.
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INTRODUCTION

We have developed a nonmyeloablative regimen for hematopoietic cell transplantation (HCT) between dog leukocyte antigen (DLA)–identical canine littermates. It consists of conditioning with 2 Gy of total body irradiation (TBI) and a short course of postgrafting immunosuppression with mycophenolate mofetil (MMF) and cyclosporine (CSP) [1]. This regimen has been successfully translated into clinical trials for patients thought to be at high risk for toxicities from conventional myeloablative regimens [2-4].

The major role of pretransplantation TBI in this model seemed to be host immunosuppression, rather than creation of "marrow space" for grafts to home,

because successful stable allografts could also be achieved when 4.5 Gy of irradiation to the cervical, thoracic, and upper abdominal lymph node chains was substituted for 2 Gy of TBI [5]. Prompt and stable engraftment of donor cells was documented in nonirradiated marrow and lymph node spaces of recipient dogs.

When the TBI conditioning dose was decreased to 1 Gy, initial uniform allogeneic engraftment was seen, but all dogs eventually lost their grafts, despite MMF and CSP; this shows a delicate immunologic balance between host and donor [1]. Attempts at establishing stable allografts after 1 Gy of TBI have met with mixed success. Substituting the immunosuppressive agent sirolimus for MMF postgrafting yielded only

transient engraftment, [6] as did the pretransplantation use of the novel immunosuppressive agent FTY720, even though it caused profound peripheral blood lymphocytopenia [7].

However, stable grafts after conditioning with 1 Gy of TBI were seen in most recipient dogs after they were "sensitized" against their marrow donors by intravenous (IV) injections of peripheral blood mononuclear cells (PBMCs), with concurrent blockage of costimulatory signals from B7 to CD28 by injections of the fusion peptide CTLA4Ig [8]. This result validated the continued search for immunosuppressive agents that were less toxic than, and could be substituted for, TBI.

Antithymocyte globulin (ATG) is a mixture of polyclonal antibodies with potent immunosuppressive effects [9]. ATG has been used in combination with reduced-intensity [10-12] and nonmyeloablative [13,14] conditioning regimens for allogeneic HCT.

In this study, we asked whether rabbit anti-canine ATG, given before 1 Gy of TBI, would facilitate stable engraftment of marrow from DLA-identical donors in recipients administered a brief course of postgrafting MMF and CSP. The marrow graft studies were preceded by pharmacokinetic and skin graft studies; ATG dosing was aimed at reducing peripheral blood CD3 T-cell counts to ≤10% of the baseline values.

MATERIALS AND METHODS

Dogs

Litters of beagles and mini–mongrel crossbreeds were either raised at the Fred Hutchinson Cancer Research Center (FHCRC; Seattle, WA) or purchased from commercial kennels. Dogs were observed for disease for at least 60 days before study. All dogs were enrolled in a veterinary preventive medicine program that included routine anthelmintics and a standard immunization series against canine distemper, parvovirus, adenovirus type 2, parainfluenza virus, coronavirus, and rabies. In addition, 2 intradermal doses of a custom-prepared formalin-inactivated canine papilloma virus vaccine were administered. Dogs weighed from 9.0 to 15.8 kg (median, 12.0 kg). Elevated enclosed runs were used for housing, and dogs were maintained in social groups wherever possible. This study was approved by the Institutional Animal Care and Use Committee at FHCRC, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. DLA matching was performed by using highly polymorphic major histocompatibility complex–associated class I and class II microsatellite markers [15,16] and DLA-DRB1 sequencing [17].

Antithymocyte Globulin

Canine-specific rabbit ATG was provided by SangStat Medical Corporation (Fremont, CA). It was produced by inoculating rabbits with canine thymocytes obtained from neonates. The immunoglobulin G(Ig) fraction from the rabbit serum was isolated by adsorption on a protein A column and cryopreserved at −70°C in a concentration of 8.39 mg/mL with a pH of 8. ATG was thawed before use, filtered through a 0.2-μm-pore membrane, and administered undiluted to the study dogs, subcutaneously (SQ) in 14 and IV in 1. No premedication was given. Endotoxin measurements showed 41 endotoxin units per milliliter.

Monoclonal Antibodies

Monoclonal antibodies (mAbs) specific for canine CD3ε (CA17.6F9; IgG2b), [18] CD4 (CA13.1E4; IgG1), CD8 (CA9.JD3; IgG2a), [19] and CD21 (CA2.1D6; IgG1) were kindly provided by Dr. Peter Moore (School of Veterinary Medicine, University of California, Davis). An antibody against canine CD34 (2E9; IgG1) [20] was kindly provided by Dr. Richard Nash (FHCRC). All mAbs were produced and purified at the Biologics Production Facilities of the FHCRC. The primary mAbs were unconjugated (CD34 and CD21), fluorescein isothiocyanate (FITC) conjugated (CD3), or biotinylated (CD4 and CD8) according to standard protocols. The corresponding isotype control antibodies were used in parallel in each case.

Flow Cytometry

Red blood cells were lysed by using a hemolytic buffer containing ethylenediaminetetraacetic acid. The white blood cells were washed with cold Hanks buffered salt solution (HBSS) supplemented with 2% heat-inactivated horse serum. The primary mAbs were added (4 μg/mL) to 100 μL of a cellular suspension containing approximately 1 million cells per milliliter and incubated for 30 minutes at 4°C in the dark. Secondary antibodies used were streptavidin-phycoerythrin (SAPE; Caltag, Burlingame, CA), goat anti-mouse IgG1-FITC (Southern Biotechnology Associates, Birmingham, AL), and goat anti-rabbit/PE (Caltag). They were added (1 μg/mL) after 1 wash, and the incubation was identical. After a last wash, the cells were resuspended in HBSS/2% heat-inactivated horse serum supplemented with propidium iodide for excluding dead cells and were analyzed on a fluorescence-activated Flow Cytometer (Becton Dickinson, San Jose, CA).

To determine the number of T cells in the circulation (cells per microliter), the white blood cell count was multiplied by the percentage of T cells determined by flow cytometry. The specificity of the ATG batch was assessed by staining blood from healthy donors with ATG itself or with serum from an ATG-

treated dog. T cells were detected with CD3 staining, and cell-bound ATG with goat anti-rabbit-PE. Normal rabbit IgG (DAKO, Glostrup, Denmark) was used as a negative control.

Mixed Leukocyte Reactions

PBMCs from 2 DLA-mismatched unrelated dogs were used as responder and stimulator cells [21]. PBMCs were resuspended in Waymouth-Iscove medium supplemented with 10% heat-inactivated, pooled, normal dog serum, 1% nonessential amino acids, 1% sodium pyruvate, and 2% L-glutamine. Both 1×10^5 responder cells per well and 1×10^5 irradiated (25 Gy) stimulator cells per well were cocultured in quintuplicate in round-bottomed 96-well plates for 6 days at 37°C in a humidified 6% CO_2 air atmosphere. Serial dilutions of ATG or sera from ATG-treated dogs were added to the mixed leukocyte reactions (MLR). Final ATG concentrations ranged from 0 to 11 μg/mL. Normal rabbit IgG was used as a negative control. On day 6, cultures were pulsed with 1 μCi (0.037 MBq) of ^{3}H-thymidine for 18 hours before harvesting. ^{3}H-Thymidine uptake was measured as the mean counts per minute of the 5 replicates by using a β-scintillation counter (Packard BioScience, Meriden, CT).

ATG Serum Levels, Anti-rabbit Antibody, and Anti-dog IgG Titers

An enzyme-linked immunosorbent assay was used to measure serum ATG levels [22]. Briefly, 96-well polyvinyl plates were coated with goat anti-rabbit IgG (DAKO), blocked with 5% nonfat milk in phosphate-buffered saline, and incubated with serum from the treated dogs, diluted 1:500. Bound ATG was detected by horseradish peroxidase–conjugated goat anti-rabbit IgG (H+L; Pierce, Rockford, IL) as the secondary antibody. The color reagent was 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO), and plates were read with a Vmax microtiter plate reader (Molecular Devices, Menlo Park, CA) at 405 nm. Standard curves were established with known concentrations of ATG, and sera from dogs before infusion served as controls. A similar procedure was used to detect anti-rabbit antibodies, except that plates were coated with diluted ATG, and bound antibodies were detected by horseradish peroxidase–conjugated goat anti-dog IgM (μ chain) or IgG (H+L; ICN, Aurora, OH) to distinguish between the 2 isotypes. Titers of anti-dog IgG within the ATG preparation were determined as follows: plates were coated with normal dog IgG (Jackson Immuno Research, West Grove, PA), and ATG was added in serial 1:2 dilutions; bound antibodies were revealed with horseradish peroxidase–conjugated goat anti-rabbit IgG, and normal rabbit IgG was used as a negative control.

Assessment of Lymph Node Cellularity

Lymph node T cells were quantified in 2 dogs. In each, 1 popliteal lymph node was surgically sampled before and the contralateral lymph node was sampled after ATG administration. Half of the specimen was fixed and embedded in paraffin. Sections were stained with hematoxylin-eosin or anti-human CD3 antibody (DAKO) specific to the intracytoplasmic portion of the CD3ε chain and highly cross-reactive with the canine molecule. The remaining half of the node was weighed, minced in RPMI 1640 with 2% fetal calf serum, and filtered to eliminate macroscopic debris. The total number of cells present in the final suspension was determined and, by using CD3 staining and flow cytometry analysis, the number of T cells per gram of lymph node tissue was calculated.

Immune Function Study

In 2 additional dogs, we assessed the immune responses to rabbit ATG, as well as to sheep red blood cells (SRBCs; Research Diagnostics Inc., Flanders, NJ) as a control immunogen. Methotrexate (MTX) was given for additional immunosuppression to inhibit the production of canine anti-rabbit antibodies. Two dogs received ATG (1 mg/kg/d) from days 0 to 4 and 1 dose of MTX (0.4 mg/kg IV) on day 2 or day 9. SRBCs, 1 mL of a 10% suspension, were given IV on day 0. Sera were collected for up to 6 weeks, and hemagglutinin titers, [23] as well as dog anti-rabbit antibody titers, were measured.

Skin Grafting

The surgical and biopsy protocols have been previously described [24]. Skin grafts were performed between DLA-mismatched unrelated dogs. ATG was given to recipients at 1 mg/kg on day 2 and 0.5 mg/kg/d from day −1 to day +20 after skin grafting. Unrelated and autologous control skin grafts were transplanted simultaneously. After removal of dressings on day 7, skin grafts were examined daily for signs of inflammation. The day of rejection was determined by the occurrence of erythema, edema, and epidermal reactions and was confirmed in all cases by histology, with particular attention to cellular infiltrates and distorted epidermal architecture.

Hematopoietic Cell Transplantation

Recipients were administered 1 Gy of TBI at 7 cGy/min from a high-energy linear accelerator (CLINAC 4; Varian, Palo Alto, CA). Marrow was aspirated from donors under general anesthesia through long needles inserted into the humeri and were infused IV within hours of TBI. Marrow grafts contained 175 to 453×10^6 total nucleated cells per kilogram (median, 263×10^6 total nucleated cells per

kilogram), 4.1 to 11.3 × 10^6 CD34 cells per kilogram (median, 6.6 × 10^6 CD34 cells per kilogram), and 6.9 to 38.5 × 10^6 CD3 cells per kilogram (median, 17.8 × 10^6 CD3 cells per kilogram; Table 1). All dogs received standard postgrafting care. Immunosuppression consisted of CSP 15 mg/kg twice a day orally from day −1 to day 35, combined with MMF 10 mg/kg twice a day SQ from day 0 to day 27. CSP levels were measured at least weekly, and results were used for CSP dose adjustments; MMF doses were adjusted according to clinical toxicity.

Assessment of Chimerism

Hematopoietic engraftment was assessed by documentation of donor dinucleotide and tetranucleotide variable number tandem repeat (VNTR) polymorphisms in cells from peripheral blood and marrow by using a polymerase chain reaction (PCR)–based assay as previously described [25]. Samples were drawn weekly for 3 months and then every 2 weeks. Granulocyte and mononuclear cell (MNC) fractions were isolated from blood by Ficoll gradient centrifugation (specific gravity, 1.074), and DNA was extracted. Chimerism was also assessed at least once in unfractionated marrow samples and among circulating T cells, sorted by fluorescence-activated cell sorting. PCR amplification with informative radioactively labeled VNTR primers was performed [1]. PCR products were analyzed by gel electrophoresis, and percentages of donor chimerism in the recipients were determined by visual approximation after autoradiography.

Statistical Analysis

Responses between ATG-treated MLR cultures and controls were compared by using a Wilcoxon rank-sum test. The median duration of mixed chimerism was estimated with the Kaplan-Meier method. The duration of mixed chimerism was compared with that of 6 historical controls [1] by using the log-rank test. The association between cell doses in the grafts and the mixed chimerism duration was evaluated with the Spearman rank correlation coefficient. All reported P values were 2 sided, and those <.05 were considered significant.

RESULTS

In Vitro Studies, Pharmacokinetics, Lymphocyte Changes, and Skin Grafts in Healthy Dogs

ATG specificity. White blood cells from a healthy dog were incubated with the ATG preparation at a concentration of 4 μg/mL (Figure 1) and with sera from a dog that had received a cumulative dose of 4 mg/kg ATG SQ. Sera were drawn before ATG and on days 5 and 8 after the first ATG injection. Serum ATG levels at those time points were 0, 37, and 17 μg/mL, respectively. Figure 1 shows that ATG bound to all white blood cells. However, ATG had more binding affinity for $CD3^+$ cells, as the fluorescence intensity of their staining (double-positive cells) was approximately 1 log higher than that of $CD3^-$ cells (PE-positive, FITC-negative cells). The ATG preparation also contained antibodies that cross-reacted with canine IgG at a titer of 1:4096.

Mixed leukocyte reactivity. ATG at 4 different concentrations ranging from 0.5 to 10 μg/mL or normal rabbit IgG (negative control) was added to the MLR without complement (Figure 2). Thymidine uptake was significantly decreased at low ATG concentrations (0.5-5 μg/mL; $P < .01$) but was unchanged at the highest concentration of 10 μg/mL ($P = .92$).

Pharmacokinetic studies. Five dogs were administered ATG in a dose-finding approach; they did not receive marrow grafts. The first dog received ATG 1 mg/kg IV once a day for 2 consecutive days; this was associated with severe side effects that resembled septic shock. Thereafter, the SQ route was used in 4 additional dogs. They received ATG 0.5 to 1 mg/kg once a day for cumulative doses ranging from 2 to 5 mg/kg. No premedication was used, and no side effects were seen.

Serum levels of ATG in the 5 dogs peaked at 6 to 42 μg/mL on days 4 to 6, proportional to the total doses administered, and became undetectable by day 13 in all cases. Figure 3 shows the ATG serum levels and the total peripheral blood lymphocyte and CD3 T-cell counts for 2 dogs given cumulative doses of 3.5 and 5 mg/kg.

Table 1. *Marrow Grafts from DLA-Identical Donors after ATG and 1 Gy of TBI Delivered at 7 cGy/min*

Dog No.	ATG (mg/kg)	Marrow Cells (million/kg)			Maximum Donor MNC Chimerism (%)	Graft Rejection	Engraftment Duration (wk)
		TNC	CD34	CD3			
G200	3.5	263	4.2	17.8	5	Yes	8
G198	4	345	4.1	25.1	25	Yes	18
G166	5	206	7.5	6.9	25	Yes	11
G208	4	453	11.3	38.5	40	No	≥36
G252	4	175	6.6	13.2	5	Yes	8

DLA indicates dog leukocyte antigen; ATG, antithymocyte globulin; TBI, total body irradiation; TNC, total nucleated cells; MNC, mononuclear cells.
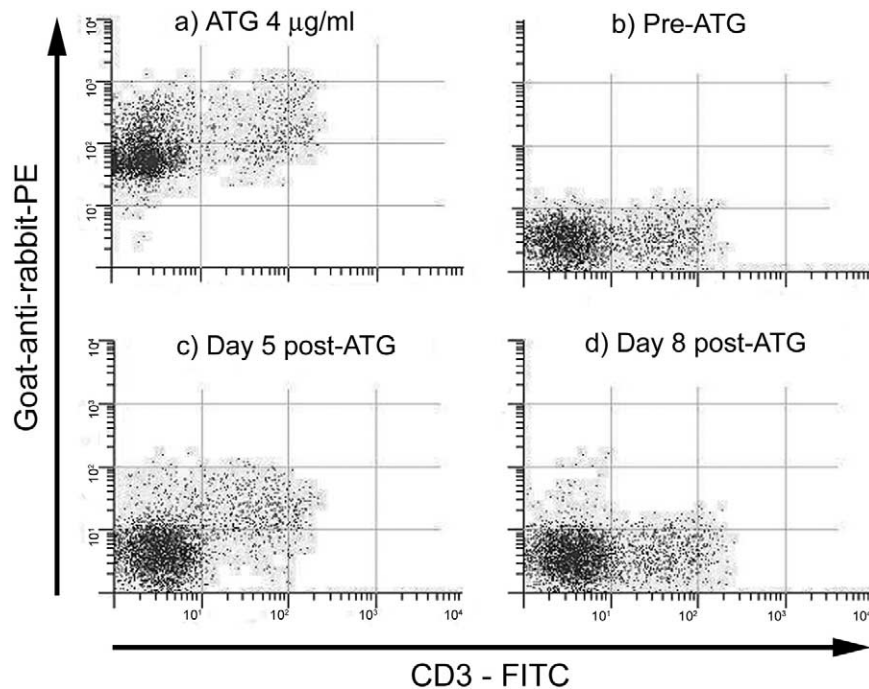
Figure 1. Staining of normal white blood cells with (a) ATG diluted at 4 µg/mL; and (b-d) sera from a dog treated with ATG 4 mg/kg, drawn at baseline and on days 5 and 8 after ATG administration.

Effect of ATG on lymphocytes. The baseline peripheral blood CD3 T-cell counts were 1240 to 2100/µL. After ATG administration, T-cell counts declined in all 5 dogs, reaching nadirs of 90 to 220/µL, concurrent with the peak of serum ATG (Figure 3). CD4$^+$ and CD8$^+$ T cells were reduced equally. Peripheral blood B cells were reduced to a lesser extent. Neutrophils, platelets, and red blood cells were not affected (data not shown).

We observed a dose-dependent effect of ATG on T cells. Lymph node biopsies in the dogs that received ATG 2 mg/kg showed approximately 50% T-cell depletion on days 5 and 9 compared with baseline, as assessed by flow cytometry and morphology (Figure 4). Their peripheral blood showed T-cell nadirs of 160 and 220 cells per microliter, or 9% and 18% of baseline levels, respectively. On the basis of these findings, ATG doses of 3.5 to 5 mg/kg were used for HCT, and they resulted in T-cell nadirs of approximately 5% of baseline, or 100 cells per microliter.

Skin grafts. Five dogs were given ATG and skin transplants from DLA-mismatched unrelated donors to assess the immunosuppressive properties of the ATG batch. They rejected the grafts after a median of 14 days (range, 13-17 days). This was significantly longer than historical and concurrent controls not given ATG (n = 28), which rejected unrelated skin grafts after a median of 8 days (range, 7-9 days) [23,24] ($P = .003$; Wilcoxon rank-sum test). Autologous skin grafts survived indefinitely.

Hematopoietic Cell Transplantation

Five dogs were given HCTs from DLA-identical donors, which were either littermates (n = 4) or siblings from another litter (n = 1). TBI 1 Gy and marrow infusion were given on day 0. ATG was administered between days −12 and −7 at cumulative doses of 3.5 to 5 mg/kg (median, 4 mg/kg; Table 1). Serum ATG levels peaked at 31 to 46 µg/mL (median, 37 µg/mL) and became undetectable by day 0. CD3 cells reached pretransplantation nadirs of 24 to 205 cells per microliter (median, 134 cells per microliter).
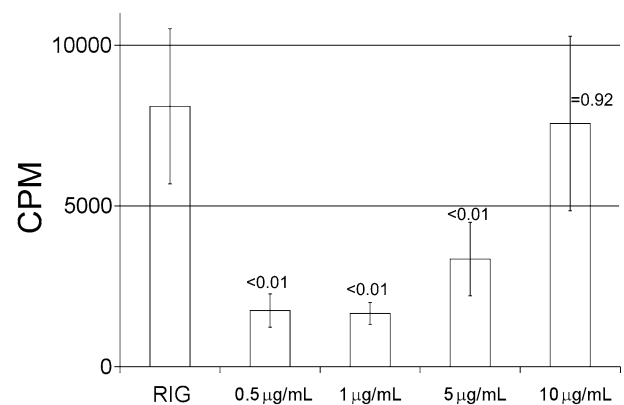

Figure 2. MLR assays using MNCs from DLA-mismatched unrelated dogs. ATG at concentrations from 0.5 to 10 µg/mL or normal rabbit IgG (RIG), as a negative control, was added to the reactions. ^{3}H-Thymidine uptake is shown as average counts per minute from quintuplicate assays. *P* values are in reference to rabbit IgG.
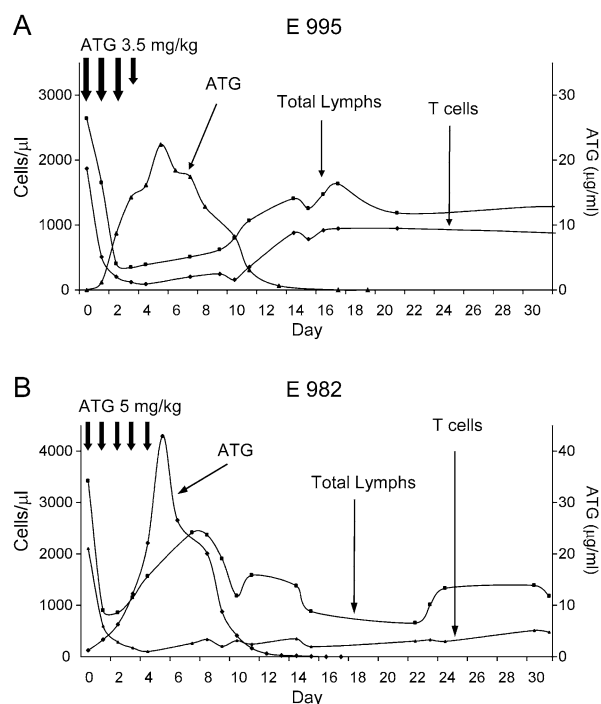
Figure 3. Pharmacokinetic studies in nontransplanted dogs: ATG administration, peripheral blood ATG levels, and total lymphocyte and CD3 T-cell counts in 2 healthy dogs given cumulative ATG doses of 3.5 mg/kg (A) and 5 mg/kg (B).

Under ATG therapy, T-lymphocyte counts declined from a median of approximately 3000/μL to <200/μL, and there was also a decline in median platelet counts from 300 000/μL to 120 000/μL. After TBI, T-lymphocyte counts remained <1000/μL for 5 weeks before returning to pre-ATG levels. The median absolute neutrophil count ranged from 2000/μL to 6000/μL throughout the first 4 weeks after transplantation before returning to the reference range. Platelet counts nadired on day 14 at a level of approximately 20 000/μL before full recovery.

Engraftment. All 5 dogs showed prompt initial engraftment. The donor chimerism levels reached peaks of 10% to 75% (median, 25%) for granulocytes and 5% to 40% (median, 25%) for MNCs just after postgrafting immunosuppression was discontinued (week 5; Figure 5, A and B). Four of the 5 dogs rejected their grafts 8 to 18 weeks after HCT without evidence of cytopenias. The fifth dog has remained a mixed chimera at >36 weeks of follow-up, with donor contributions of 1% for granulocytes and 25% for MNCs. The median durations of engraftment in the 5 dogs (11 weeks) were similar to those among 6 historical controls [1] not given ATG (median, 10 weeks; P = .20; log-rank test).

Transplant-related toxicities. Hematologic toxicity was generally mild (Figure 5C). There were no neutropenias and no episodes of infections. Thrombocytopenias were moderate, with median platelet nadirs

of 33 × 10⁹/L on day 13. Only 1 dog (G200) experienced severe thrombocytopenia (nadir, 8 × 10⁹/L) and required transfusions. T cells reached a first nadir on day −7 and a second nadir around day +10. Their recoveries started after discontinuation of immunosuppression (week 5) and reached pre-ATG values around week 7 after HCT. The T-cell changes in the 4 dogs with rejection were similar to those in dog G208, which had sustained engraftment. The main nonhematologic toxicity was gastrointestinal and included transient anorexia, weight loss, and diarrhea, most likely due to MMF. Graft-versus-host disease was not observed.

Marrow cell doses and chimerism. Table 1 shows that the dog with sustained engraftment (G208) had received the largest marrow cell dose. Analysis of total nucleated cells and CD34 and CD3 cell doses administered showed that the total nucleated cell dose did not have a statistically significant Spearman correlation coefficient with the duration of engraftment (0.82; P = 0.09).
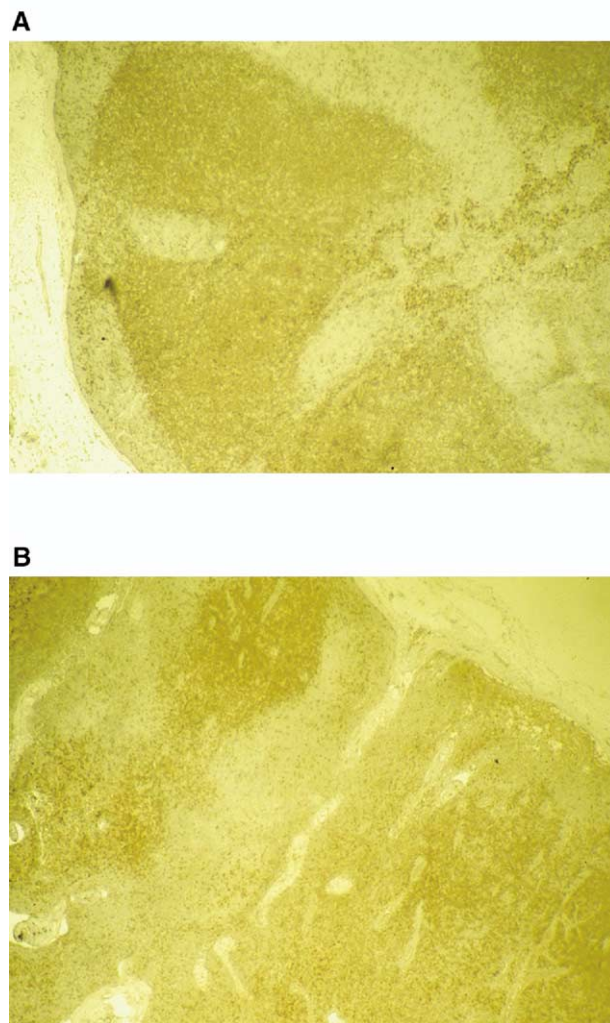

Figure 4. Lymph node immunohistochemistry for T cells using CD3 staining (dark areas) before (A) and after (B) ATG 2 mg/kg.
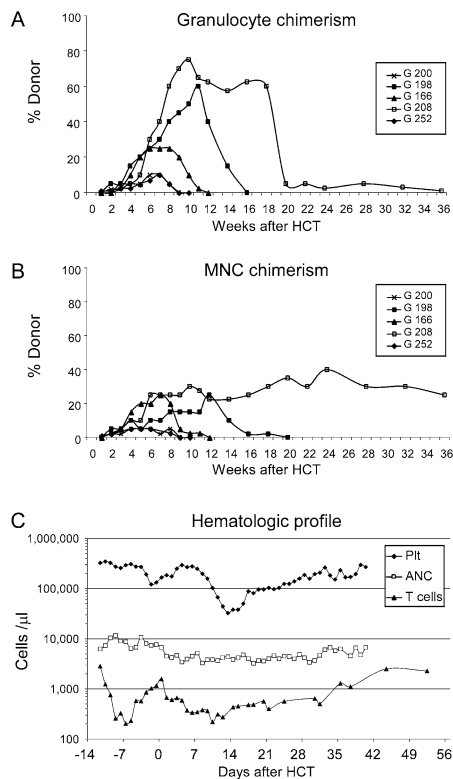
Figure 5. Donor chimerism levels in the granulocyte (A) and MNC (B) fractions and (C) median peripheral blood changes in 5 dogs given ATG and 1 Gy of TBI before and MMF/CSP after marrow grafts from DLA-identical donors.

Canine Anti-ATG Antibody Responses

Prompted by the observation of a shorter-than-anticipated ATG half-life, antibody responses to ATG were measured in dogs given either skin grafts (n = 3) or HCT (n = 5; Figure 6). Antibodies to rabbit ATG began to appear 6 to 7 days after the first ATG dose, and their titers increased as ATG was cleared from the circulation. The hosts' immune responses were predominantly of the IgG isotype, as these titers were significantly higher than the IgM antibodies. Of note, in the skin-grafted animals, the ATG levels peaked on day 8 and decreased afterward through antibody neutralization, becoming almost undetectable after day 14, even though ATG was scheduled to be administered until day 22. In the HCT recipients, the anti-rabbit IgG contributed to ATG clearance by day 0.

This observation prompted us to study immune responses to ATG and SRBCs in 2 additional dogs. Immunosuppression with MTX was administered after ATG to abrogate the production of canine antibodies. MTX did not change the immune response to ATG compared with the previous dogs that received ATG alone; anti-rabbit ATG antibodies appeared 6 to 8 days after the first ATG dose and consisted mostly of IgG antibodies. However, neither of the 2 dogs showed detectable antibody responses to SRBCs,

which were used as a control neoantigen (data not shown).

DISCUSSION

Marrow grafts between DLA-identical canine littermates have been almost uniformly successful after conditioning with the sublethal dose of 2 Gy of TBI when recipients were given postgrafting immunosuppression with MMF/CSP. This regimen has been successfully translated into the clinic to treat human patients with malignant and nonmalignant hematologic diseases [2-4]. However, engraftment in dogs after only 1 Gy of TBI was uniformly transient; this suggests a delicate immunologic balance. Results from previously published TBI dose de-escalation studies [26,27] have allowed us to estimate the residual host T-cell populations in dogs given 2 and 1 Gy of TBI to be 10% and 35%, respectively (Schwartz and Storb, unpublished data, 2005).

This study was undertaken under the assumption that pretransplantation ATG would reduce host T-cell numbers in a manner equivalent to 1 Gy of TBI. We were concerned, however, that residual ATG might adversely affect the grafted donor T cells and, thereby, impair engraftment.

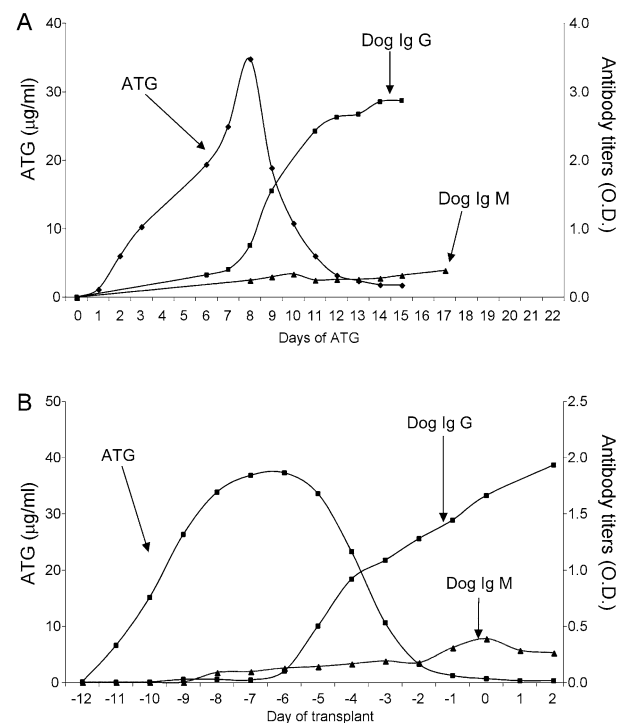That concern turned out to be unfounded because, surprisingly, recipient dogs mounted IgG anti-

Figure 6. ATG levels and canine antibody production to rabbit ATG in recipients of (A) skin grafts (n = 3) and (B) HCT (n = 5). Days of ATG administration were 0 to 22 in (A) and between day −12 and −7 in (B).

ATG antibody responses that promptly cleared the agent from the circulation by the time of HCT. Despite this and despite the profound lymphocytopenia and lymph node depletion of T cells, most transplanted dogs eventually rejected their grafts after extended periods of mixed donor/host chimerism. This result was not significantly different from that in dogs conditioned with 1 Gy of TBI but without ATG.

How can we explain the current failure of sustained engraftment in 4 of the 5 dogs studied? In all likelihood, the T-cell depletion achieved with rabbit ATG was insufficient. If we assume the estimates of T-cell depletion by 1 and 2 Gy of TBI, respectively, to be correct, then 10% of residual host T cells favored sustained engraftment in this model, whereas 35% of host T cells did not. Assuming, next, a reduction of host T cells by rabbit ATG of 50%, the combination of pretransplantation ATG and 1 Gy of TBI should result in an estimated residual host T-cell population of approximately 17%. Thus, ATG plus 1 Gy of TBI fell short of the 10% residual host T cells estimated for 2 Gy of TBI, which permitted sustained engraftment in 11 of 12 dogs studied. It was not clear why 1 of the 5 dogs showed sustained engraftment, although a possible explanation might be fortuitous matching for a more important minor histocompatibility locus. At present, we are unable to determine minor histocompatibility antigens by in vitro tests.

Other characteristics of the ATG preparation could have interfered with its immunosuppressive effects. For example, the inhibitory in vitro effect in MLRs was lost at higher ATG concentrations. This might have been due to the presence in ATG of antibodies with mitogenic activity (directed to CD2 and CD3), [9] the relatively high content of endotoxin (4.9 endotoxin units per milligram of IgG), or both.

Besides immunosuppression, other important components of HCT are the quantity and composition of the hematopoietic cells administered. Successful engraftment after 1 Gy of TBI was achieved in this model when marrow transplants were combined with granulocyte colony-stimulating factor–mobilized donor PBMCs [18]. In this analysis, larger total nucleated cell numbers showed a trend toward a longer duration of engraftment, and this suggests that marrow cells might facilitate their own engraftment in a dose-dependent manner and through a similar mechanism. In clinical HCT studies using myeloablative regimens and marrow transplantation from related [28] or unrelated [29] donors, larger total nucleated cell doses were associated with better leukemia-free survival.

The pharmacokinetic studies revealed that ATG was cleared from the circulation by neutralizing antibodies within 13 days. This is very similar to observations in a nonhuman primate model [22]. In clinical studies of HCT, [30-32] ATG persisted for more than 30 to 100 days after transplantation. Human patients given HCT are often immunosuppressed from prior chemotherapy. Therefore, humoral antibody production might be impaired in comparison to healthy experimental animals, and this might explain the longer half-life of ATG in the clinical reports.

The antibody response to ATG had the characteristics of a secondary immune response, not only because of its rapid kinetics, but also because of the production of antibodies of the IgG isotype. The latter observation is corroborated by 2 other studies [22,31] in which the hosts produced anti-ATG antibodies of the IgG isotype. Dogs in the present study were exposed to ATG for the first time; however, they mounted secondary immune responses. To reconcile these 2 facts, we propose the following explanation: as shown, ATG contained rabbit antibodies that cross-reacted with canine IgG. IgG is also found in membrane-bound form in the B-cell antigen receptor (mIgG-BCR). It is possible that ATG cross-reacted with the mIgG-BCR and triggered B cells through this very effective signaling pathway, leading to their activation and proliferation and to efficient production of IgG antibodies [33], which, in turn, might have ATG complementarities and might lead to neutralization and clearance of ATG.

This hypothesis was indirectly supported by the observations made in 2 dogs that received MTX in an attempt to suppress the anti-ATG immune responses. Their antibody responses to the neoantigen, SRBC, were completely suppressed; however, the magnitudes of their antibody responses to ATG were comparable to those seen in the 8 previous dogs. This is consistent with secondary immune responses that were not affected by MTX.

In conclusion, rabbit ATG produced an estimated 50% T-cell depletion in dogs; however, this degree of T-cell depletion was not sufficient to compensate for the deletion of 1 Gy of TBI conditioning, and all dogs but 1 eventually rejected their marrow grafts. ATG triggered a strong immune response in immunocompetent hosts, and this resulted in its rapid clearance from circulation.

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