

Use of multigeneration-family molecular dog leukocyte antigen typing to select a hematopoietic cell transplant donor for a dog with T-cell lymphoma

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Case Description—A 7-year-old Golden Retriever was examined because of anorexia, lethargy, vomiting, and gradual weight loss.

Clinical Findings—Splenomegaly, pancytopenia, high serum calcium concentration, and alkaline phosphatase activity were detected. Magnetic resonance imaging revealed an enlarged mesenteric lymph node and increased signals from the bone marrow of the ilium and vertebral bodies. Histologic examination and immunophenotyping of biopsy specimens confirmed a stage V (b) T-cell malignant lymphoma.

Treatment and Outcome—Clinical remission was attained by use of 2 chemotherapy cycles, followed by an allogeneic hematopoietic cell transplant performed at 18 weeks after diagnosis. A donor was identified by molecular dog leukocyte antigen typing methods. The patient was conditioned with 2 fractions of 4 Gy total body irradiation delivered 3 hours apart at 7 cGy/min, followed by an IV infusion of recombinant canine granulocyte colony-stimulating factor mobilized leukapheresis product and postgrafting immunosuppression with cyclosporine. Chimerism analyses revealed full donor engraftment that has been maintained for at least 58 weeks after transplant. Remission has been confirmed by normal results of serum thymidine kinase assays and the absence of peripheral blood clonal T-cell receptor gene rearrangements.

Clinical Relevance—Systemic chemotherapy induces remissions; however, most dogs succumb to disease recurrence because of multidrug resistance. Outcome of allogeneic hematopoietic cell transplantation in dogs can be excellent because of improved donor-recipient selection by use of molecular dog leukocyte antigen typing, compared with early attempts, and better prevention of graft versus host disease, better supportive care, and substitution of peripheral blood mononuclear cells for bone marrow. (*J Am Vet Med Assoc* 2006;228:728–732)

A 7-year-old neutered male Golden Retriever that weighed 37.5 kg (82.5 lb) was clinically evaluated at Bellingham Veterinary & Critical Care in February

2004. The dog had a 20-day history of anorexia, lethargy, and vomiting and had 10% weight loss during the preceding 2 months.

Clinical examination revealed an enlarged spleen without peripheral lymphadenopathy. Hematologic examination revealed pancytopenia and mild anemia. No atypical cells were identified in a blood specimen. Ionized and total serum calcium concentrations were high (1.82 mmol/L and 14.9 mg/dL, respectively), and parathyroid hormone concentration (1.5 pmol/L) was low. In addition, parathyroid hormone-related protein concentration was slightly high (1.2 pmol/L). Serum alkaline phosphatase activity (205 U/L) was high, as was BUN concentration (32.1 mg/dL). Thoracic and abdominal radiography confirmed splenomegaly. Magnetic resonance imaging^a revealed an enlarged mesenteric lymph node and generalized homogeneously increased signals from the bone marrow of the ilium and vertebral bodies. On the basis of these findings, splenectomy was performed, followed by mesenteric lymph node, liver, kidney, duodenum, and skin biopsies. In addition, bone marrow biopsy specimens were obtained from the iliac crest.

Histologic examination of the spleen and biopsy specimens confirmed multicentric lymphoma in the spleen, mesenteric lymph node, and bone marrow. Monomorphic, medium to large lymphocytes with irregular nuclear membrane contours expanded the periarterial lymphoid sheaths of the splenic white pulp and encroached on the follicular areas. The atypical cells invaded the splenic red pulp and trabeculae multifocally. Extramedullary hemopoiesis was also observed in the splenic red pulp. Infiltration of similar monomorphic lymphocytes expanded the paracortical regions of the

COAP	Cyclophosphamide, vincristine, cytosine arabinoside, prednisone
DLA	Dog leukocyte antigen
SSCP	Single-stranded conformational polymorphism
PBMC	Peripheral blood mononuclear cell
HCT	Hematopoietic cell transplantation

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mesenteric lymph node, compressed lymphoid follicular regions, and invaded lymph node trabeculae, but spared the capsule. In this location, the atypical lymphocytes included up to 6 mitotic figures/hpf (40X objective). The bone marrow contained isolated nests of atypical lymphocytes. Histologic examination of liver, kidney, duodenum, and skin did not reveal involvement by lymphoma.

Immunophenotyping of the splenic and mesenteric lymph node infiltrates confirmed that the lymphoma was of T-cell origin (CD3+). Genomic DNA was extracted from paraffin-embedded sections of the mesenteric lymph node and spleen for T-cell (T-cell receptor γ locus) and B-cell antigen receptor rearrangement (immunoglobulin heavy chain locus) analysis.¹ Duplicate PCR reactions were performed for each tissue analyzed and revealed consistent clonal T-cell receptor γ rearrangements in the spleen and mesenteric lymph node. Clonal immunoglobulin heavy chain rearrangements were not observed. The morphologic, immunohistochemical, and molecular clonality results confirmed that the atypical lymphoid infiltrates consisted of a clonal T-cell population. These findings, combined with the clinical signs, were consistent with a stage V (b) T-cell malignant lymphoma, according with the World Health Organization Staging System of Canine Lymphoma.²

Induction of remission was undertaken by administration of cyclophosphamide (50 mg/m², PO, q 48 h), vincristine (0.56 mg/m², IV, weekly), cytosine arabinoside (100 mg/m², SC, q 12 h), and prednisone (50 mg/m², PO, q 24 h; COAP protocol).³

After 2 weeks of COAP chemotherapy, the dog developed pancytopenia, anemia, anorexia, and acute bilateral pyoderma on the forelimbs, and transfusion support was initiated by use of dog erythrocyte antigen 1.1-negative compatible packed RBCs. In an attempt to prevent sepsis and bleeding, chemotherapy was discon-

tinued and filgastrim^b (5 μ g/kg [2.3 μ g/lb], SC, q 12 h) was administered for 5 days. Chemotherapy was then reinstated with cyclophosphamide (50 mg/m², PO, once) and prednisone (20 mg/m², PO, q 48 h), and a 2-month remission was achieved.

Relapse was made evident by the appearance of lymphoblasts (2%) with high nuclear-to-cytoplasmic ratio in the blood, accompanied by increased serum calcium concentration (14.1 mg/dL). In addition, the dog developed anemia; lymphocytopenia (280 cells/ μ L); azotemia (BUN, 52 mg/dL); and increased serum activities of alkaline phosphatase (435 U/L), alanine aminotransferase (110 U/L), and aspartate aminotransferase (64 U/L). A second remission induction with cyclophosphamide (50 mg/m², PO) and prednisone (20 mg/m², PO, q 48 h) was attempted; however, after a single dose of cyclophosphamide, whole-blood transfusion was required for severe thrombocytopenia. One week later, a single dose of *Escherichia coli* L-asparaginase (20,000 U/m², IM) was given. A second remission was achieved; atypical lymphocytes disappeared from the blood, and hypercalcemia resolved.

To find a suitable HCT donor, DLA typing of 29 family members from 4 generations of dogs located in 3 countries was performed at the Fred Hutchinson Cancer Research Center. Blood samples from each dog were obtained for DNA extraction via standard protocols.⁴ The DNA samples were tested for highly polymorphic microsatellite markers^{5,6} in the DLA class I (FH 2200) and DLA class II (FH 2202) regions by use of a variable number tandem repeat-PCR method⁵ and specific 2200 and 2202 primer pairs.^{7,8,c} Results were analyzed by use of electrophoresis on 6% polyacrylamide gels prepared with a sequencing system kit.^d Interpretation of the banding patterns suggested that 5 family members were DLA matched for both DLA class I and class II regions with the affected dog. For confirmation, the DLA class II

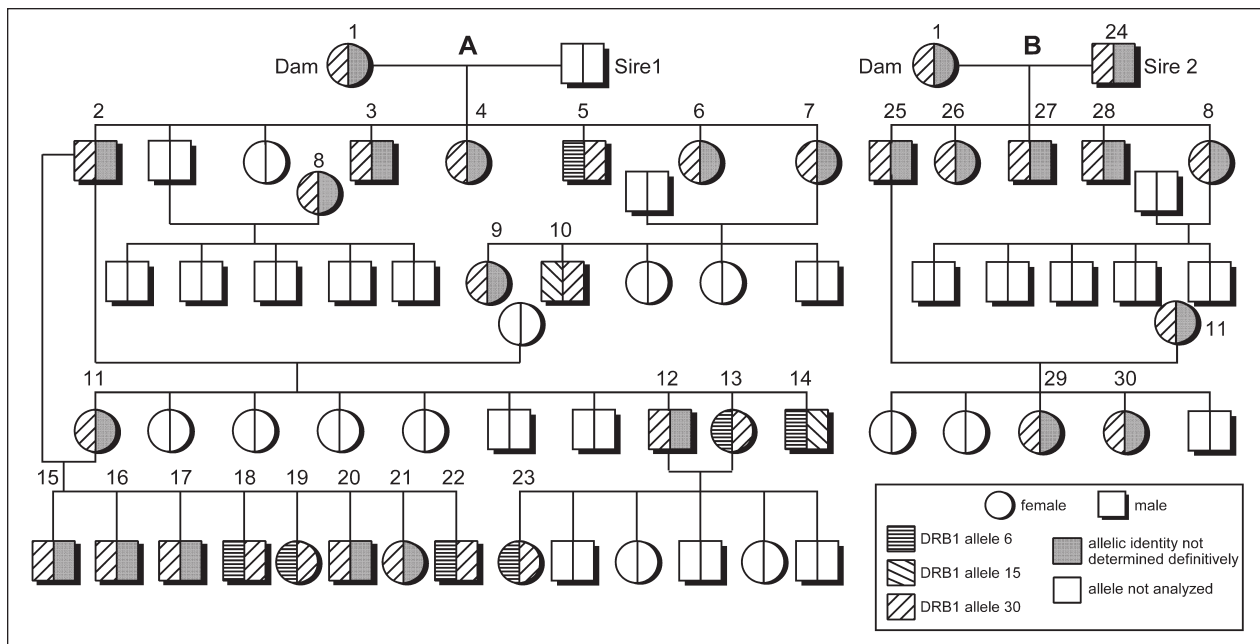


Figure 1—Four-generation genealogy and genotyping profiles of family members of a dog with T-cell lymphoma receiving an HCT from a DLA-matched related donor. A—Affected dog's dam's first litter. B—Affected dog's dam's second litter. 1-30 = Dogs for which blood samples were evaluated for highly polymorphic microsatellite markers within DLA class I and class II regions, and DLA class II DRB1 allele sequencing. A3 = Affected dog. A15, A17, A21, B29, and B30 = Dogs matched with the affected dog on the basis of results of segregation analysis of the polymorphisms and DRB1 allele sequencing. A17 = Dog selected as donor.

DRB1 hypervariable region^{4,9} was amplified by use of an SSCP-PCR method^{10,11} and specific IC and ID primers.^{11c} Results were analyzed by electrophoresis on 0.5% mutation detection enhancement gels⁵ that revealed identical banding patterns for those dogs identified as matched by segregation analysis of microsatellite polymorphisms. The interpretation of DRB1 allele banding patterns was not sufficient for histocompatibility assessment because different DRB1 alleles could have the same size and identical banding patterns. Therefore, DRB1 allele sequencing was performed by use of a genetic analyzer^f to identify potential allelic disparities on the basis of nucleotide alignments. Among the 30 dogs from which sequences were analyzed with the software,⁸ 3 DRB1 alleles were identified: alleles 6, 15, and 30 (Figure 1). The affected dog shared the same DLA-DRB1 allelic sequence profile with the 5 dogs identified to be matched on the basis of the segregation analysis of microsatellite polymorphisms and SSCP gel banding patterns. Among the DLA-matched relatives, a 13-month-old male that weighed 29.5 kg (65 lb) was selected as the HCT donor. The donor's blood group was DEA1.1 negative.

The HCT was performed 18 weeks after diagnosis and 1 month after the second induction chemotherapy, while the dog was still in remission. Two weeks before transplantation, 240 mL of blood was collected from the intended donor and stored for the purpose of priming the apheresis machine. To avoid transmission of any potential blood-borne pathogens to the recipient, the donor was screened for *Dirofilaria immitis*, *Ehrlichia canis*, *Ehrlichia equi*, *Babesia canis*, *Borrelia burgdorferi*, and *Rickettsia rickettsii*.

For mobilization of PBMCs, the donor was given recombinant canine granulocyte colony-stimulating factor^h (5 µg/kg [2.3 µg/lb], SC, q 12 h, for 5 consecutive days). Leukapheresis was performed with a 12-F, 20-cm dual-lumen central venous catheter¹²ⁱ and a continuous flow blood separator,^j with acid citrate dextrose solution as an anticoagulant. The PBMC collection lasted 4 hours with the donor under general anesthesia while body temperature, respiratory rate, and pulse rate were monitored. Electrolytes and the hematologic profile were evaluated every hour by use of a portable clinical analyzer.^k To avoid anticoagulant-induced calcium depletion, 10% calcium gluconate was administered throughout the apheresis procedure via IV pump injection, at 10 mL/h. The donor's body temperature was maintained by use of microwave heating pads. The apheresis product contained 92×10^3 nucleated cells/µL and 0.36×10^3 CD34+ cells/µL. The CD34+ cell count was analyzed via flow cytometry by use of a canine anti-CD34 biotin-conjugated monoclonal antibody.^{13,l} The affected dog was given 2 fractions of 4 Gy total body irradiation with an interfraction interval of 3 hours, delivered at 7 cGy/min from a 4-MV clinical linear accelerator.^m Immediately after irradiation, the dog was given an IV infusion of 440 mL of freshly isolated PBMCs containing 3.6×10^6 CD34+ cells/kg (1.6×10^6 CD34+ cells/lb; Figure 2).

Post-HCT immunosuppression was attained by administration of cyclosporine (5 mg/kg, PO, q 12 h), from the day before HCT until day 35 after HCT. Cyclosporine blood concentration was evaluated twice weekly by use of a monoclonal whole-blood assay.ⁿ To achieve therapeutic concentrations ranging from 400 to 600 ng/mL, as measured by the monoclonal whole-blood assay, the cyclosporine dose was adjusted to 4.5 to 5.5 mg/kg (9.9 to 12.1 mg/lb). The CBC was assessed daily until the WBC concentration exceeded 1,000 cells/µL and platelet concentration exceeded 30,000 platelets/µL, when frequency of CBC was reduced to once per week. Serum biochemical analyses were performed daily to monitor liver and kidney functions.

Supportive treatment after HCT included administration of lactated Ringer's solution (13 mL/kg [5.9 mL/lb], SC, q 12 h) and 20% fat emulsion^o in lactated Ringer's solution (6.5 mL/kg/h [3 mL/lb/h], continuous rate infusion) administered via IV catheter, which was maintained for 4 days after HCT; thereafter, the dog was given nutrition PO. Antimicrobials, consisting of neomycin sulfate (6 mg/kg [2.7 mg/lb], PO, q 8 h), polymyxin B sulfate (25,000 U/kg/d [11,364 U/lb/d], PO, q 8 h), and enrofloxacin (10 mg/kg [4.5 mg/lb], SC, q 24 h), were administered until the WBC concentration exceeded 1,000 cells/µL. *Lactobacillus GG*^p (80 mg, PO, q 12 h) was administered as a balanced probiotic. During the first week after HCT, the dog lost 12% of baseline body weight, all of which was regained during the following month. On day 14 after HCT and hospitalization, the dog was released to its owners, with continuation of administration of the cyclosporine, enrofloxacin, and *Lactobacillus GG*.

Hematopoietic engraftment was evident as manifested by sustained increases in granulocyte and platelet counts after the postradiation nadir and detection of donor cells in the peripheral blood by use of microsatellite marker polymorphism analyses.^{8,14} Granulocyte count was within reference range by day 10 and platelet count by day 22 (Figure 3), and the dog did not require transfusion. For analysis of donor engraftment, granulocyte and lymphocyte fractions were separated from the recipient's blood by use of a gradient method, and genomic DNA was extracted via standard protocols.⁴ A PCR-based assay was performed with a 2001 primer pair.^{8,14c} The donor contribution to recipient hematopoiesis was quantified by estimating the proportion of donor-specific DNA among host DNA after autoradiography. This revealed mixed hematopoietic

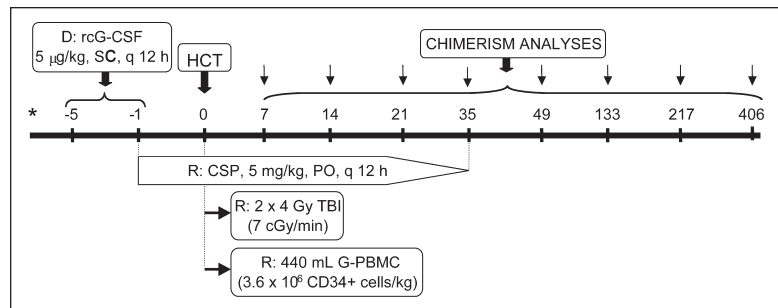


Figure 2—Myeloablative HCT schema of a dog with T-cell lymphoma. *Days before or after HCT. CSP = Cyclosporine. D = Donor. rcG-CSF = Recombinant canine granulocyte colony-stimulating factor. G-PBMC = rcG-CSF mobilized PBMCs. R = Recipient. TBI = Total body irradiation.

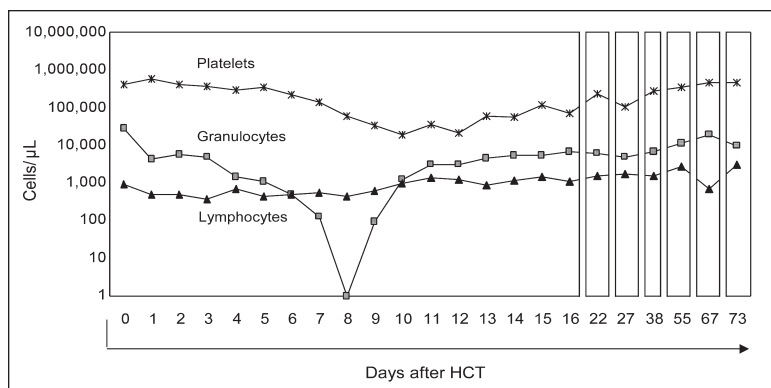


Figure 3—Blood concentrations of WBCs and platelets after HCT in a dog with T-cell lymphoma.

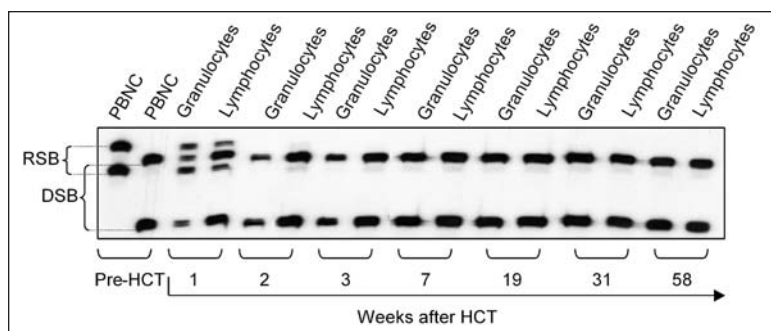


Figure 4—Chimerism analyses of blood from a dog with T-cell lymphoma receiving an HCT from a DLA-matched related donor. DSB = Donor specific bands. RSB = Recipient-specific bands.

chimerism by week 2 after transplantation and, subsequently, progression to full donor chimerism in the granulocyte and lymphocyte fractions, which has been maintained for at least 58 weeks (Figure 4).

On day 55 after HCT, serum activities of alanine aminotransferase (233 U/L) and aspartate aminotransferase (106 U/L) increased. At the same time, an erythematous rash developed, affecting the plantar region of the paws, periocular and perioral regions, dorsal and lateral nasal skin, pinnae of the ears, abdomen, and dorsal region of both forelimbs. Histologic examination of multiple skin biopsy specimens revealed diffuse lymphocytic infiltrates and apoptotic cells in the follicular epithelium and occasionally in the glandular epithelium, confirming graft versus host disease. Treatment was initiated with cyclosporine (7.5 mg/kg [3.4 mg/lb], PO, q 12 h) and enrofloxacin (10 mg/kg, SC, q 24 h). The skin lesions improved after 20 days of cyclosporine administration, which was continued at the same dose for 5 months and then gradually tapered by 5%/wk until month 13 after HCT, when it was discontinued. While receiving cyclosporine, the dog was without clinical signs with the exception of sporadic vomiting and diarrhea without weight loss. Clonal rearrangement studies of blood T-cell antigen receptor genes¹ and serum thymidine kinase assays¹⁵ performed after HCT have revealed no evidence of residual lymphoma.

Discussion

Systemic chemotherapy is presently the treatment of choice for canine lymphoma, with remission rates

approaching 58% to 96%.² However, most dogs succumb to recurrence of their disease, typically by 12 months after diagnosis¹⁶ because of development of multidrug resistance. When the COAP protocol alone is used for the treatment of dogs with lymphoma, a median survival time of 25 weeks has been reported.³

The canine species has served as an excellent random-bred preclinical model for human HCT,¹⁷⁻¹⁹ providing a valuable basis for many of the principles and techniques of transplantation. Increasing knowledge of the molecular immunogenetics of the canine major histocompatibility complex has provided unique tools for rapid identification of suitable DLA-matched family members, which could serve as hematopoietic donors.^{4-6,8,10,11,20-23} Treatment of spontaneous lymphoma in dogs with a high dose of total body irradiation and chemotherapy followed by autologous²⁴⁻³⁰ or allogeneic³⁰⁻³² marrow transplantation has been reported. Among dogs in chemotherapy-induced remission that were given autologous marrow grafts, approximately 25% became disease-free long-term survivors.^{26,29,30} However, allogeneic grafts were complicated by high rates of fatal graft versus host disease³¹ and other complications, although there was evidence of beneficial graft versus lymphoma effects.³³

Since those early studies, outcomes of allogeneic HCT have improved, in part because of improved donor-recipient selection by use of molecular DLA typing^{5,11} and in part because of better prevention of graft versus host disease,^{34,35} better supportive care, and substitution of PBMCs for bone marrow.³⁶ The dog described here has remained in remission for more than 15 months after HCT and 19 months since the initial diagnosis and has been thriving, with complete donor chimerism.

- Northwest Radiologists, Bellingham, Wash.
- Neupogen, Amgen, Thousand Oaks, Calif.
- Invitrogen Corp, Carlsbad, Calif.
- Ultra Pure Sequagel Sequencing System Kit, National Diagnostics, Atlanta, Ga.
- MDE Gel Solution, Cambrex Bio Science Rockland Inc, Rockland, ME.
- ABI PRISM 3100, Applied Biosystems, Foster City, Calif.
- SeqEd, Applied Biosystems, San Jose, Calif.
- Supplied by Amgen Corp, Thousand Oaks, Calif.
- Arrow International, Reading, Pa.
- COBE Spectra apheresis system, Gambro BCT, Lakewood, Colo.
- I-STAT CG8+, I-STAT Corp, East Windsor, NJ.
- 1H6 canine anti-CD34 antibody, Biologics Facility, Fred Hutchinson Cancer Research Center, Seattle, Wash.
- Clinac 4/80, Varian Associates, Palo Alto, Calif.
- Abbot Laboratories, Abbot Park, Ill.
- Liposyn, Intralipid 20%, Baxter Healthcare Corp, Clintec Nutrition Division, Deerfield, Ill.
- Culturelle, ConAgra Foods, Omaha, Neb.

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