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TITLE: Adoptive Immunotherapy Combined with Hematopoietic Cell Transplantation as a Therapeutic Approach to Treatment of Prostate Cancer

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14. ABSTRACT We determined that cell lysates prepared from canine prostate tissue was immunogenic when injected in female dogs. In addition to the known prostate antigen, canine prostate specific esterase (CPSE), we identified by molecular weight several other proteins against which the dog made IgG antibodies. Using a mixed lymphocyte reaction, we found that a cellular response was generated against the prostate cell lysate after the female dog was injected 3 times with antigen. Sensitization of female dogs with prostate antigen suspended in incomplete Freund's adjuvant appeared to be superior to prostate lysate antigen-loaded canine autologous dendritic cells when both were injected subcutaneously near the popliteal lymph node. Peripheral blood lymphocytes and lymph node-derived lymphocytes reacted to prostate lysate in the mixed lymphocyte reaction. An immune response to prostate lysate or CPSE was not adequately detected by a delayed-type hypersensitivity reaction 10 days after the last of three subcutaneous injections of antigen. Two of three male dogs transplanted with dog leukocyte identical female bone marrow engrafted, but additional time is need to ensure engraftment is stable before prostate antigen sensitized donor lymphocytes can be infused into the recipient for an anti-prostate immune response to be evaluated.					
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Introduction

Allogeneic hematopoietic cell transplantation (HCT), or the transplantation of a hematopoietic system from a tissue matched normal donor has developed into a highly effective therapy for the treatment of hematological malignancies. The canine model has played a major role in the development of these HCT therapies, to the extent that regimens for successful allogeneic HCT in the canine model have been directly translated to the clinic (1-3). We and others have shown that the donor cytotoxic lymphocytes mediate an attack against the host hematopoietic system including often successful elimination of the underlying hematopoietic tumor. This has been termed the “graft-versus-tumor effect”.

Oncologists have recently attempted to apply this remarkably efficient form of cancer therapy to solid tumors. Treatment of renal (4), breast (5), and ovarian (6) cancer has met with only limited success in part due to the inability to separate graft-versus-host disease (GvHD) from anti-tumor effects. What is evidently needed is a method wherein the host lymphocytes are sensitized against tumor or tissue antigens specific to the tumor to improve anti-tumor efficacy.

Prostate cancer is an ideal candidate for HCT therapy as co-elimination of this organ with the cancer is not life-threatening to the patient. In addition to tumor antigens, several male androgen sensitive antigens exist which are essentially foreign to a female immune cells, and if expressed on the tumor and presented by the major histocompatibility complex (MHC) could be targeted by the female donor prostate antigen-sensitized lymphocytes after infusion into the male recipient.

The concept of this grant has been to test the hypothesis that immunotherapy can be combined with allo-HCT to treat prostate cancer. In this model, HCT is required as a platform for adoptive immunotherapy. In this capacity, female prostate antigen-sensitized lymphocytes are expected to survive in the chimeric environment which, by definition, is tolerant to them. We hypothesize that the sensitized donor lymphocyte infusion will provide the anti-tumor activity.

Body

The study design was divided into two aims. The first was to identify the better of two methods for immunizing a female HCT donor against prostate antigens. One way was to sensitize female dogs with three injections of a mixture of prostate cell lysate and keyhole limpet hemocyanin (KLH) antigen in Incomplete Freund's Adjuvant (IFA). For the other, we cultured female canine bone marrow derived dendritic cells in the presence of the same antigen mixture, and administered three injections of these cells within two centimeters of the popliteal lymph nodes in two dogs. The second aim of this study was to establish mixed hematopoietic chimerism in a male recipient using marrow from a female dog leukocyte antigen (DLA)-identical littermate, sensitize the female donor using the optimum method identified in Aim 1, and to perform an infusion of sensitized donor lymphocytes into the male recipient. At this point we planned to monitor chimerism, prostate inflammation, serum levels of CPSE, and GvHD. Over the past year we have completed Aim 1 and initiated Aim 2 by successfully transplanting 1 out of 2 male dogs with female marrow grafts. Two additional dog pairs have been assigned to this study. We are behind schedule for the 1.5 years allotted to this grant period due to the intense competition with other studies and investigators in obtaining dogs.

Aim 1. Establish methods to induce immune responses to prostate lysate in female dogs.

We successfully generated prostate antigen using a freeze/thaw method that produced milligram quantities of sterile antigen. Briefly, prostates were removed from cadaverous dogs and frozen at -70°C until processing. Once six prostates were collected, the tissue was minced by scalpel and refrozen in dry ice and ethanol. After repeated freeze/thaw cycles, the tissue was sonicated and then the tissue debris was separated from the solution by centrifugation at $10,000 \times g$. Protein concentration was determined using the BioRad protein DC colorimetric assay.

Five dogs were vaccinated with three injections of prostate lysate-KLH, 10 days apart, within 2 cm of the popliteal lymph node. Ten days after the third injection, a skin test was performed for delayed type hypersensitivity response against 1) prostate lysate, 2) KLH, 3) Freund's incomplete adjuvant (IFA), 4) a lysate of the canine kidney cell line, MDCK (negative control), and 5) PBS.

An example of the skin test is shown in **Figure 1** of dog G768, 48 hours after a subcutaneous injection of the prostate lysate, KLH and the controls of IFA, MDCK lysate and PBS. The results of this assay were not remarkable at the 24- and 48-hour periods, where only very mild erythema was noted for the prostate lysate (0.5 cm in diameter) and KLH (0.5 cm) injection sites as well as the IFA (1.0 cm) injection site. No notable erythema was seen at the MDCK or PBS injection site. Similar findings were observed in the other dogs tested in this manner.

Mixed leukocyte reactions were done to determine whether autologous dendritic cells (DC) could present antigen to sensitized lymphocytes collected either from peripheral blood or from popliteal lymph nodes draining the injection sites. This was an empirical procedure, where we tested different methods of assessment in order to determine effective sensitization. In dog G651 a single stimulation or primary MLR reaction was used. For this study, bone marrow cells were collected by aspiration of the head of the humerus, red blood cells were lysed, and the CD34+ cells labeled with a monoclonal antibody (2E9). Next, rat anti-mouse IgG1 magnetic beads were added and the labeled cells collected by magnetic column chromatography (Milteny Automacs). CD34+ cells were cultured for 7 days in medium containing GM-CSF, Flt3-L, and TNF α according to established procedures (2). The cultured cells were harvested and added to 96-well microtiter plates containing PBL or lymph node-derived lymphocytes and prostate lysate (0.1 to 10 $\mu\text{g/ml}$) KLH (0.1 to 1 $\mu\text{g/ml}$) MDCK (1 $\mu\text{g/ml}$) or canine vaccine (Parvo, rabies distemper at a 1:500 dilution of vaccine). Concanavalin A (Con A) (5 $\mu\text{g/ml}$) was added on day 3 and ^3H -thymidine added on day 6. Plates were harvested and counted on day 7.

Shown in **Figure 2** are the results of a MLR using both PBL and lymph node-derived lymphocytes as responder cells and autologous DC plus antigen as stimulator cells. Although there was no reaction to prostate lysate, there was a strong response to KLH and to a lesser extent the canine vaccine by the PBL. We also observed a response to these antigens by the lymph node lymphocytes. These data indicated that the method of immunization was effective; however, we did not detect at this stage of investigation an anti-prostate antigen immune response.

At this point, we questioned whether a double stimulation in vitro of PBL or lymph node-derived lymphocytes with autologous DC might show better responses to the prostate lysate. Independent studies showed there might be a predominate CD4 T-cell response in a primary MLR but a CD8 T-cell response appeared to dominate a secondary MLR.

Therefore, for dog G768 and G149 we set up both a primary MLR as described above and additional secondary MLR in which lymphocytes were cultured 7 days in the presence of autologous DC plus antigen, harvested and incubated an additional 7 days with antigen and additional DC. Here, the results for the primary MLR were essentially the same as shown in Figure 2. But as shown in **Figure 3**, we were able to observe an immune response against prostate lysate and canine prostate specific esterase, one of the major prostate antigens expected in the prostate lysate. We postulated that the number of prostate antigen-specific lymphocytes either in circulation or in the draining lymph node were few in number and sufficiently expanded only after a second in vitro stimulation detected by the MLR method.

A third dog, G149, was vaccinated using IFA, and 10 days after the third injection evaluated in a primary and secondary MLR. Again, after the second stimulation, we observed an increased response in PBL stimulated with autologous DC pulsed with CPSE at 10 µg/ml relative to the cells stimulated with autologous DC alone (data not shown).

We then entered into the second phase of Aim 1 which was to vaccinate dogs with autologous DC pulsed in vitro with a mix of prostate lysate, CPSE and KLH. For these studies, on three occasions, DC were cultured from female bone marrow CD34+ cells as described above. Two days before injection, DC were pulsed with antigen in vitro. On the day of injection, DC were harvested from culture counted and injected within 1-2 cm of the popliteal lymph node according to the injection schedule in **Table 1**. Due to the limited number of DC collected on the third harvest for dog G703, we elected to collect a fourth bone marrow sample and culture DC for an additional immunization of DC pulsed with antigen.

Using this approach for in vivo sensitization, for dog G703 we failed to observe a significant response to prostate lysate, CPSE or KLH after primary or secondary MLR, perhaps in part owing to the high background response of PBL cultured with DC alone. For dog G809, there was a modest (two-fold over background) response in PBL stimulated with DC pulsed with KLH for the primary and secondary MLR. The second dog, G703 showed only a modest response against the viral vaccine in the primary MLR while any positive response to antigens was masked by an abnormally high background response of the PBL to autologous DC alone (data not shown). Overall, these results were less impressive than those obtained with the IFA vaccine method and resulted in our continued evaluation of two additional dogs using the vaccine technique for further validation.

Dogs H072 and H073 were injected with prostate lysate/KLH in IFA as described above. Ten days after the third vaccination, PBL and popliteal lymph node cells were collected and cultured in 96-well round bottom plates with cultured autologous DC pulsed with antigen. One week later, these cells were collected and stimulated again with fresh DC and antigen for a secondary MLR. Compared to DC stimulators alone, there were modest primary responses to prostate lysate and KLH for dog H073 but not H072 (data not shown). In the secondary immune response, the lymph node cells from H072 responded modestly to prostate lysate and CPSE relative to cells stimulated by DC alone, but in the case of dog H073, no stimulation index was observed owing to the high background of the lymph node cells stimulated by DC alone (**Figure 4**).

These data, together with the studies outlined above, suggest that there was variability among the dogs tested as to their response to prostate antigens and KLH determined either by a primary or secondary MLR. Such variability may require a large number (10 or more) dogs to statistically delineate an anti-prostate immune response in chimeric dogs after injection of sensitized lymphocytes.

In addition to MLR as a read-out for anti-prostate immune responses, we collected sera from H072 and H073 and evaluated prostate lysate CPSE and KLH by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) followed by a Western blot using the dogs immune sera. As shown in **Figure 5**, several bands were indicated for the prostate lysate (lane 2) in addition to strong bands for both CPSE (lane 3) and KLH (lane 4). Antibody responses were also detectable against the antigens of the control virus vaccine the dogs received as pups (lane 5). These results indicated that prostate antigens are immunogenic in female dogs as demonstrated by IgG antibody responses. In addition, CPSE is a major antigen found in the prostate lysate. Future studies will include identifying the proteins of approximately 55 kDa and 62 kDa found in the prostate lysate that induced an antibody responses.

Aim 2. Induction of Prostatitis in female to male chimeric dogs.

The goal of this Aim is to demonstrate that prostate antigen-sensitized DLI from a female marrow donor into a DLA-identical male chimeric recipient can lead to an anti-prostate immune response. To date, we transplanted two dogs each with marrow from a DLA-identical female littermate into male recipients after 2 Gy total body irradiation followed by postgrafting immunosuppression with mycophenolate mofetil and cyclosporine A for 28 and 35 days, respectively. Both dogs engrafted with one remaining a stable chimera (H008), while the other dog (G985) rejected his graft at week 11 and has been taken off study (**Figure 6**).

Two additional dog pairs have been assigned to this study; one was transplanted on 7/8/08, but it is too early to determine chimerism (data not shown). A fourth dog will be transplanted on 8/24/08. In order to ensure engraftment of the donor marrow is stable, a period of 25 weeks should transpire before sensitized PBMC and draining lymph node lymphocytes are injected in the chimeric male recipient.

Key Research Accomplishments

- 1) We have demonstrated that of the two possible methods for inducing an immune response to prostate antigen in female dogs, soluble antigen in IFA gives more reproducible results than antigen-pulsed cultured autologous canine DC. Although DC have been used in other systems to induce immune responses in vivo (for review see 9,10), at this time we have not hit upon an effective method of inducing adequate antigen uptake or maturation of the DC to effectively present prostate antigens to naïve female T cells in vivo.
- 2) A two-step or secondary MLR provided more reproducible stimulation as measured by ³H-Thymidine uptake than did a primary MLR.
- 3) Lymph node cells obtained from the popliteal lymph node responded to the antigen mix and will be added to the donor lymphocyte infusion from the apheresed prostate antigen sensitized donor dogs.
- 4) We were able to detect antibody responses to prostate lysate, CPSE, and KLH.
- 5) Several proteins were detected by Western blot in the prostate lysate, one of which was CPSE. Identification of the higher molecular weight proteins in the prostate lysate by mass spectrum analysis antigens may provide candidate antigens for future studies.
- 6) We have begun establishing DLA-identical mixed chimeras for the adoptive immunotherapy study under Aim 2. Surprisingly, one of the dogs rejected his graft after

11 weeks after transplantation. The method used to establish chimerism rarely results in graft rejection. Nonetheless, we intend to continue with the engrafted chimeric dog and will look for stable engraftment in two additional dogs on this portion of the protocol as well.

Reportable Outcomes

At present we can only report on studies conducted under Aim 1, as the limited supply of dogs has slowed the expected progress for Aim 2. We expect to report in a publication once the study is completed that canine prostate tissue lysate contains antigens that are immunogenic to female dogs when administered in Freund's incomplete adjuvant. These antigens can be presented by autologous marrow derived DC and a response determined by a MLR. In addition, we expect to be able to report on the immunogenic proteins in prostate lysate that induce an antibody response once they have been identified by liquid chromatography/ mass spectrum analysis.

Conclusions

We conclude at this stage that a female dog can be sensitized against a mixture of prostate antigens. However, there is variability in this response that is presumably MHC class I or class II antigen dependent. We intend to continue the efforts outlined under Aim 2 in which lymphocytes from prostate antigen-sensitized females dogs will be adoptively transferred to stable mixed chimeric male littermates and induce prostatitis via recognition of antigens specific to prostate tissue. For this, we will request an extension of 9 months for this grant period.

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Appendix

Abbreviations

HCT	hematopoietic cell transplantation
MHC	major histocompatibility complex
IFA	incomplete Freund's adjuvant
KLH	keyhole limpet hemocyanin
DLA	dog leukocyte antigen
CPSE	canine prostate specific esterase
GvHD	graft versus host disease
MDCK	Madin-Darby canine kidney
PBS	phosphate buffered saline
MLR	mixed lymphocyte reaction
DC	dendritic cells
SDS-PAGE	sodium dodecyl polyacrylamide gel electrophoresis
kDa	kilodaltons
VNTR-PCR	variable number tandem repeat-polymerase chain reaction

Supporting Data



Figure 1. Skin test of dog G768 48 hours after injection of antigen. Ten days after the third injection of prostate lysate in adjuvant, dogs received 100 μ l injection of antigen intradermally. In the left photograph, the circled areas indicate injection sites for prostate lysate (left), KLH (middle), IFA (right). In the right photograph, the circled areas indicate injection sites for MDCK lysate (left) and PBS (right). Only mild erythema was noted for prostate lysate, KLH, and IFA.

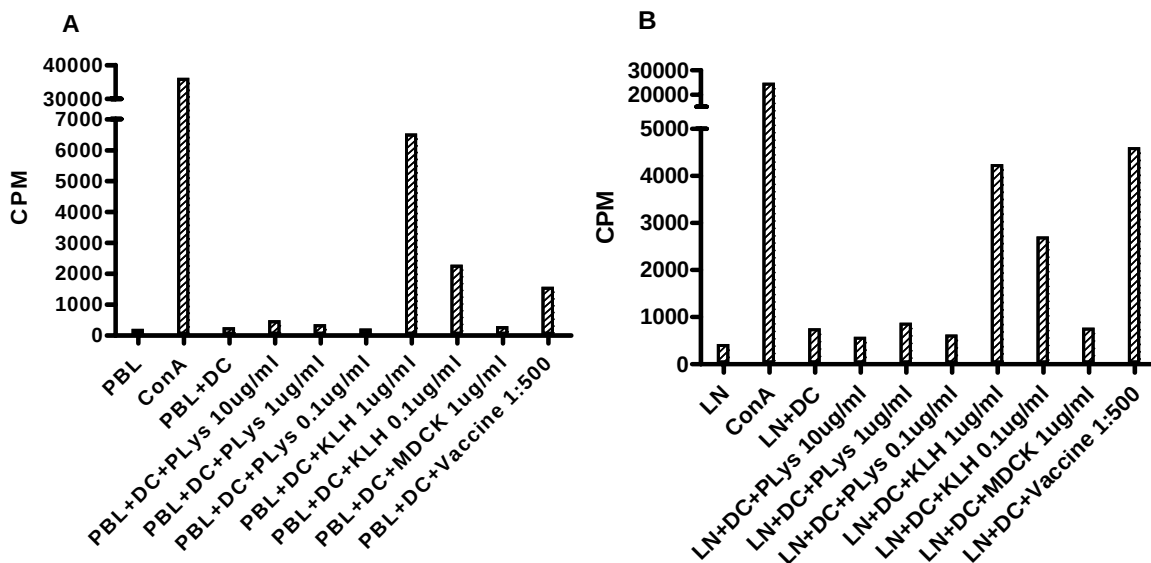


Figure 2. Mixed lymphocyte reaction of antigen pulsed autologous dendritic cells (DC) and PBL (A) or lymph node lymphocytes (LN) (B) for dog G651. Cells were pulsed with antigen prostate lysate (PLys), keyhole limpet hemocyanin (KLH), Madin-Darby canine kidney cells (MDCK), vaccine (Parvo virus, Coronavirus at 1:500 dilution) at the concentrations indicated for 7 days. Concanavalin A (ConA) was added on day 3 at 5 μ g /ml as a positive control for the reaction.

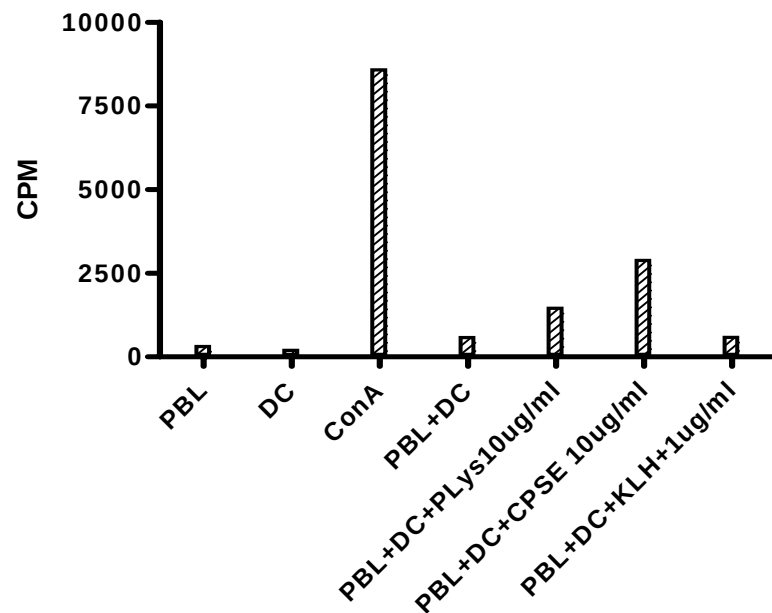


Figure 3. Secondary MLR of dog PBL cultured with autologous DC and pulsed with antigen. PBL from dog G768 were collected 10 days after three subcutaneous injections of prostate lysate, CPSE, and KLH in Freund's incomplete adjuvant. PBL were cultured with prostate lysate (PLys), canine prostate specific esterase (CPSE), keyhole limpet hemocyanin (KLH) or dendritic cells (DC) alone for 7 days. The cells were collected and cultured for an additional 7 days with DC and the antigens indicated, medium alone or concanavalin A. On day 6 ³H-Thymidine was added and the cells harvested on day 7. Shown are mean counts per minute (CPM) of triplicate samples.

Table 1. Number of Prostate Lysate/CPSE/KLH Pulsed Dendritic Cells Injected ($\times 10^7$)

Injection	Dogs	
	G703	G809
1st	3.5	1.9
2nd	0.19	2.2
3rd	0.025	0.5
4th	1.9	

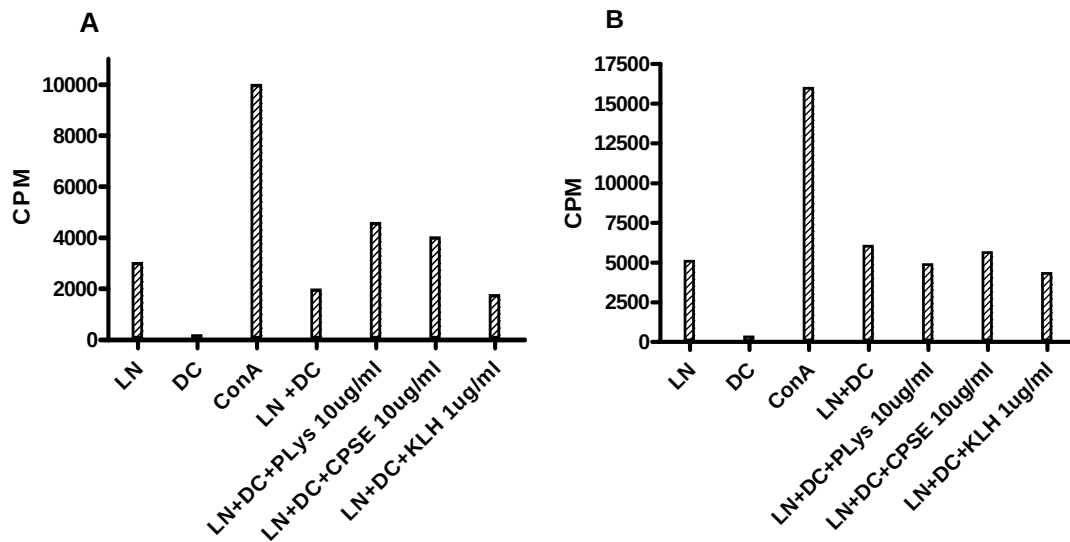


Figure 4. Secondary MLR of lymph node lymphocytes stimulated with autologous DC and antigen. Ten days after a third injection of prostate lysate/KLH antigen, popliteal lymph nodes were collected from two dogs, H072 (A) and H073 (B). The nodes were macerated in PBS, washed and resuspended in culture medium + 10% pooled canine serum and incubated for 7 days in the presence of autologous DC with or without prostate lysate, KLH or CPSE. Cells were harvested and incubated again in the same manner and pulsed with Con A on day 3 and ^3H -Thymidine on day 6. Proliferation measured by CPM on Y-axis.

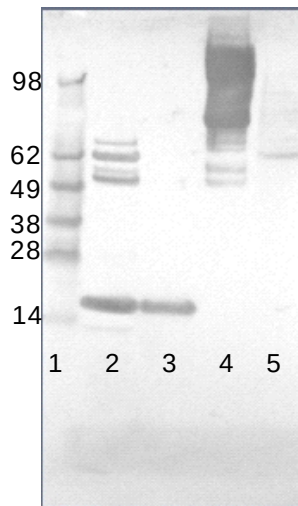


Figure 5. Antibody responses to prostate lysate, CPSE, and KLH in vaccinated dogs. Dogs were vaccinated with prostate lysate/KLH in IFA by three injections. Ten days after the third injection, blood was collected for serum. A SDS-PAGE was run with molecular weight markers indicated in kDa (lane 1), prostate lysate (lane 2), CPSE (lane 3), KLH (lane 4), and canine virus vaccine (lane 5). A Western blot was made of the gel using serum from dog H073 and developed with mouse anti-dog IgG-horse radish peroxidase.

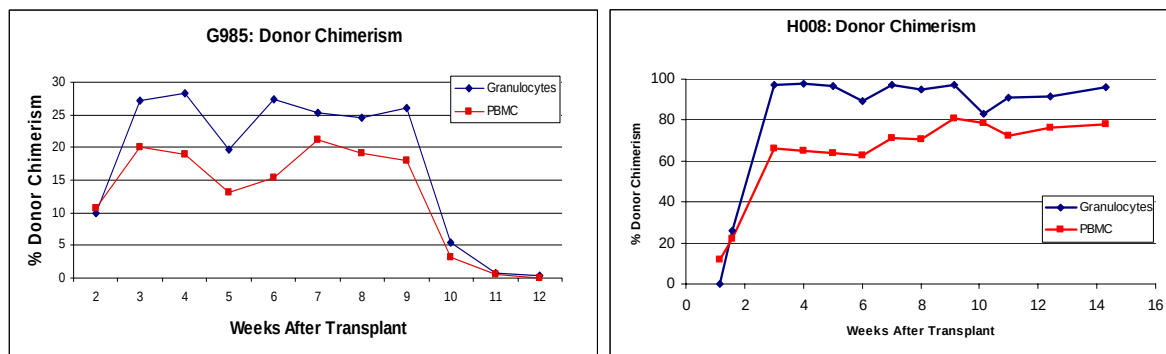


Figure 6. Female-to-male marrow transplantation for mixed hematopoietic chimerism. Dogs G985 and H008 were given 2 Gy TBI followed by 3.5 and 3.7×10^8 marrow cells/kg, respectively, on day 0. Cyclosporine A was given on days -1 through 35 and mycophenolate mofetil given on days 0-28. Chimerism was determined by variable number tandem repeat-polymerase chain reaction (VNTR-PCR) analysis (7,8).