RENAL ALLOGRAFT HISTOPATHOLOGY IN DOG LEUKOCYTE ANTIGEN

(DLA) MISMATCHED DOGS FOLLOWING

RENAL TRANSPLANTATION

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VITA

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THESIS ABSTRACT

RENAL ALLOGRAFT HISTOPATHOLOGY IN DOG LEUKOCYTE ANTIGEN (DLA) MISMATCHED DOGS FOLLOWING RENAL TRANSPLANTATION

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Reciprocal renal transplantation and bilateral nephrectomy were performed in 10 healthy, adult, mongrel dogs to evaluate allograft histopathology in dog leukocyte antigen (DLA)-mismatched dogs undergoing renal transplantation with transient immunosuppression. Immune conditioning consisted of nonmyeloablative (200cGy) total body irradiation (TBI), bone marrow transplantation (BMT) (7/10 dogs), cyclosporine (CSA)(15 mg/kg BID), mycophenolate mofetil (MMF) (10mg/kg BID) and intermittent prednisone. Biopsies were collected at transplantation, during full immunosuppression (44 to 90 days), once medications were reduced or discontinued (228 to 580 days) and at necropsy or open surgical biopsy. Biopsies were evaluated for interstitial, tubular, vascular, and glomerular lesions. Blood urea nitrogen (BUN),

creatinine (Cr), and clinical score were determined at each biopsy. Seven of 10 dogs survived > 200 days (average of 600 days). Transient CSA toxicity was suspected in 6 dogs. Lymphocytic, plasmacytic interstitial inflammation and tubulitis progressed when immunosuppressive medications were reduced. All seven dogs had histologic lesions consistent with some degree of allograft rejection at biopsy three. Four dogs were euthanized due to persistent azotemia and histologically end-stage organ failure was confirmed. Two dogs are still alive at 500+ and 1500+ days post-transplantation.

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I. INTRODUCTION

Chronic renal failure (CRF) is the most common disease of the kidney in dogs.[1] It can result from congenital abnormalities, toxic and infectious insults, metabolic alterations, and age related pathology. Replacement of damaged nephrons by fibrotic tissue leads to destruction of neighboring interdependent nephrons, ultimately affecting the entire organ. The exact cause of the initial insult may be difficult to define; however, once seventy-five percent of the total mass of nephrons are irreversibly damaged, end-stage renal disease (ESRD) ultimately develops. [1]

Once renal pathology is deemed progressive and irreversible, supportive medical care becomes costly and unrewarding. The ideal solution for ESRD is renal transplantation. Feline renal transplantation has become a feasible therapeutic option; however, canine renal transplantation has had only limited clinical success. Improvements in immunosuppressive drugs have helped canine renal transplantation become a short-term clinical option, but the side effects associated with long-term immunosuppression are significant. The development of life-threatening opportunistic infections, an increased risk of developing tumors, the high cost of immunosuppressive drugs, drug-related toxicities, and eventual chronic rejection of the kidney have all limited routine application of renal transplantation in the dog.

An additional challenge of renal transplantation is accurate post-transplantation monitoring of potential renal allograft pathology. Hematological analysis of blood urea nitrogen (BUN) and creatinine (Cr) may underestimate the extent of renal

allograft disease.[2] Protocol biopsies, which are taken according to a preplanned time schedule, are an acceptable approach for diagnosing subclinical rejections in human patients.[2-5] In humans, identification and prompt treatment of subclinical rejection results in increased renal allograft survival compared to patients who are treated solely on clinical evidence of rejection.[4]

Recently, it has been shown that a novel nonmyeloablative bone marrow transplantation protocol which induces stable mixed hematopoietic chimerism in DLA-matched dogs can be used to induce donor specific tolerance to skin and renal allografts. [6;7] The current study uses the same immunosuppressive induction protocol which includes 200cGy total body irradiation (TBI), +/- bone marrow transplantation (BMT), and short-term immunosuppression with cyclosporine (CSA), mycophenolate mofetil (MMF) and intermittent prednisone. The purpose of this study was to evaluate the progression of allograft histopathology in relation to renal biochemical parameters (BUN and Cr), and clinical status of DLA-mismatched dogs undergoing renal transplantation.

II. LITERATURE REVIEW

Renal Transplantation:

The solution to end-stage organ failure is organ transplantation. Since 1952 when the first human kidney transplantation was performed and subsequently rejected, researchers have been deciphering the complexities of transplantation immunology. Several years later, after a successful kidney transplantation was performed from one identical twin to another, investigators began to question the role of the immune system in transplantation medicine. Human, non-human primate, and dog models have been useful for creating protocols that promote successful allograft acceptance. Recently both veterinarians and pet owners have pushed to offer renal transplantation for end-stage renal failure in cats and dogs. Cats have been successfully managed with kidney transplants; however, dogs face many of the same challenges seen in people, particularly, chronic renal allograft rejection. Within the past decade, veterinary medicine has offered several immune modulating therapies with the hope of long-term success for transplantation in dogs. Pre-transplantation blood transfusions, antithymocyte serum, donor bone marrow transplantation (BMT), and various combinations of immunosuppressive medications have all yielded useful information for transplantation medicine.[7-12]

Renal Allograft Rejection

Histocompatibility

It is now well known that pre-transplantation major histocompatibility complex (MHC) matching significantly improves the success of organ transplantation. In humans, a sibling who is human leukocyte antigen (HLA) identical to the recipient, is

chosen for the most favorable outcome. Non-related HLA-identical matches still incite a significant immunologic response, as HLA typing is imprecise and the MHC molecule is very complex and polymorphic. Unrelated identical matches still require some level of immune regulation. Similarly, a dog leukocyte antigen (DLA) identical donor-recipient match can be created to minimize allograft rejection. However, it is often difficult to locate a sibling available for organ donation, and only 25% of these are probable DLA-identical. [13]

Rejection at the cellular level

Given that leukocyte antigen identical siblings are rarely available for organ donation, renal allograft rejection is a very real issue in both human and veterinary medicine. Allograft rejection has been studied extensively in order to understand and control the recipient's immune response to the donated organ. This rejection process can be broken down into cell-mediated and humoral components. The cell-mediated immune response requires the binding of antigen to CD4-positive or CD8-positive T cells. CD4-positive cells, sometimes called T-helper cells, help to coordinate the various activities of the immune system. CD4-positive T cells also generate cytokines IL-2, IL-4, and IL-5 which increase vascular permeability and attract lymphocytes and macrophages. CD8-positive T cells are classified as T-suppressor cells or killer T-cells. T-suppressor cells inhibit or suppress immune responses. Killer T cells attack cancerous cells and cells infected with viruses. In order for these cells to initiate rejection they must first receive specific cellular signaling.

Naïve T cells require two independent signals from the same antigen presenting cells (APC) (macrophages, B cells and dendritic cells) for activation. The

MHC binding to the naïve T cell provides the first antigen-dependent signal. The MHC receptor presents antigen to the T cell receptor (TCR) and associated CD3 molecule. The second antigen-independent signal is the result of B7 (CD80/86) molecules on the APC binding to CD28 molecules on the T cell. This binding is required for clonal activation. Once activated, a T cell can express CD40L which binds to CD40, a glycoprotein expressed on APCs and thymic epithelial cells. The binding of CD40-CD40L further sustains the costimulatory response. [14]

Dendritic cells of the donor and recipient are key players in allograft rejection. Dendritic cells are the most effective APCs because of their expression of MHC I, MHC II, and costimulatory molecules. Dendritic cells present antigen to naïve T cells and stimulate a T-cell response to these antigens. Consequently, their removal promotes allograft survival. Recipient dendritic cells generate an immune response by presenting donor alloantigen in the cleft of "self" MHC to T cells in the lymph nodes. Once these dendritic cells bind to recipient T cells with the corresponding receptor, the T cell is then primed, initiating clonal expansion. This provides a large population of antigen specific T cells that are able to bind and kill allograft cells. Donor dendritic cells (passenger leukocytes in allograft) can also stimulate recipient's alloreactive T cells by migrating to a local lymph node and presenting donor antigen. A close MHC match, i.e. similar MHC structure between donor and recipient, should promote a less vigorous immune response against the allograft.

An antibody-mediated immune response is also a component of allograft rejection. A hyperacute rejection episode can occur if preformed antibodies to the donor are present in the recipient at the time of transplantation. Preexisting

alloantibodies within the recipient against donor blood group antigens and donor MHC antigens will cause allograft rejection within minutes of transplantation. The vascular endothelium of the graft is the main target of this humoral attack. Complement and coagulation cascades are rapidly initiated. This is visible immediately as a blood engorged organ with extensive hemorrhage. Soon the allograft is deoxygenated and rejected.

With current blood typing and MHC matching hyperacute rejection in human transplant patients is a rare event whereas chronic rejection is the main factor typically precipitating renal allograft dysfunction and eventual loss. At a histopathologic level, the hallmarks of renal allograft rejection in humans are tubulitis and vasculitis. Most rejection processes begin subclinically as interstitial mononuclear cell infiltration, edema and potentially interstitial hemorrhage. Clinical rejection becomes apparent as renal tubular and vascular changes occur. Renal tubule invasion by lymphocytes and narrowing of the interlobar, interarcuate, and interlobular arteries by intimal thickening occurs. CD4-positive, CD8-positive and IL-2 staining all reveal an increased response. Later an antibody-mediated endothelial response occurs causing necrotizing vasculitis and eventual thrombosis. Eventually a majority of renal transplant patients will suffer from the consequences of chronic rejection which are primarily vascular injury, tubular atrophy, interstitial fibrosis and loss of renal parenchyma. [15;16]

Widespread acceptance of human renal transplantation created a need for a standardized system of renal allograft biopsy evaluation. This ideology evolved into the "Banff 97" scheme which has been accepted as a universal system of renal

allograft analysis among human pathologists. This system of classification has helped the renal transplant community "to guide therapy and establish an objective end point for clinical trials." [17] It also provides a system of communication between clinician, pathologists and researchers. The "Banff 97" scheme is based upon the second, third, and fourth Banff conferences, and the Syntex/Roche mycophenolate mofetil trials [17]. It serves as the universal language for interpreting rejection episodes as well as predicting a prognosis. It aims to provide "international uniformity" in the evaluation of renal allograft biopsies [5]

"Banff 97" classifies renal allograft rejection into three categories (I, II, III) based on the specific location of inflammation. Type I rejection is tubulointerstitial inflammation without arteritis. Type II rejection is vascular rejection with intimal arteritis. Type III rejection is severe rejection with transmural arterial changes. Tubulitis and intimal arteritis in more than one focal region are considered hallmarks for allograft rejection. Interstitial inflammation alone is not a signal of rejection. Classic infiltrates are T lymphocytes, monocytes, and macrophages. The presence of other inflammatory cell types such as eosinophils, neutrophils, or plasma cells should be noted. Vasculitis is defined as lymphocyte infiltration beneath the vascular endothelium and ranges from arteritis characterized by focal inflammation in the vascular media to transmural fibrinoid necrosis of the vessel wall. The total number of arteries in the renal allograft biopsy specimen and the presence of hemorrhage or infarction should be noted. Glomerulitis is defined by mononuclear infiltration and endothelial cell enlargement, and is classified as segmental or global based on the

extent of these changes. Under current Banff 97 guidelines, the significance of glomerulitis is unclear.

Accurate renal allograft grading using the Banff 97 scheme requires a proper biopsy specimen, specifically, the specimen must have adequate cortical tissue with a minimum of seven glomeruli and at least one artery. Seven slides are prepared with the sections being 3 to 4 microns in thickness. Three slides are stained with hematoxylin and eosin (H&E), three with periodic acid-Schiff (PAS) or silver stain, and one with trichrome stain. Routine H & E staining illuminates general allograft architecture such as alterations in tubular, interstitial, glomerular, or vascular structures. Lymphoid aggregates are visualized as well as the presence of hemorrhage, edema, and fibrosis. The PAS and silver stains are useful for identification of glomerulitis, tubulitis, and any destruction of tubular basement membranes. Furthermore, chronic features such as arteriolar hyaline, increased mesangial matrix, double contours in glomerular capillaries, and thickened tubular basement membranes are enhanced by PAS and silver stains. The silver stain highlights basement membranes of tubules and mesangial matrix as well as the basal lamina of smooth myocytes and elastic fibers. Trichrome stains for collagen, illuminating major vessels and the renal pelvis as well as emphasizing the location and the extent of allograft fibrosis. [17]

The "Banff 97" classification system encourages routine allograft biopsies to help identify episodes of subclinical rejection and enable early intervention. It is well established that hematological analysis of urea nitrogen and creatinine often underestimates renal allograft disease, requiring biopsies to document the pathology of

renal allograft. [2]. Based on biopsies, subacute rejection episodes occur in about 30% of human patients within the first three months of transplantation.[4] Subclinical rejection at 6 months has been proven to be an independent predictor of long-term graft dysfunction. [18] Additionally, treatment of subclinical rejections with prednisone at 1 and 3 months resulted in a decreased rate of early and late graft rejection, less tubulointerstitial disease, and lower creatinine values. [4]

There is not an established system of renal allograft analysis for veterinary patients. A few veterinary studies have tried to adapt the "Banff 97" scheme with moderate success.[11;12;19;20] A recent feline study looked at renal biopsies and necropsies of feline allograft recipients on a CSA and prednisone protocol. The average mean survival time of renal allografts was 270 days (1 day to 9 years). Out of 77 cats, 69% of recipients showed signs of chronic allograft nephropathy and 51% had evidence of CSA toxicity. Eighty-percent of biopsies revealed histopathologic evidence of rejection, whereas clinical rejection was evident in only 50% of these cats[21]. Vasculitis, tubulitis, and lymphocytic glomerulitis, all major criteria for rejection in humans, were not seen in the cats. Overall, the allograft rejection in cats appears different from humans and potentially involves a humoral component [21].

Most veterinary accounts are descriptions of necropsy specimens from patients with clinical evidence of rejection or at the timed end of a study. The lack of intermediate stage biopsies specimens; e.g. scheduled needle biopsies, makes the predictive use of Banff 97 difficult to assess. Serial biopsies in dogs and cats would be useful to document the progression of renal pathology before end-stage renal disease occurs.

Immunosuppression and Transplantation

Historically, chronic medical immunosuppression has been the primary modality used to suppress the recipient's immune response in order to avoid allograft rejection. Immunosuppressive drugs such as prednisone, azathioprine, calcineurin inhibitors, mycophenolate mofetil (MMF), and others, have been used alone or in combination to prevent renal allograft rejection in people and dogs.

Prednisone was the first immunosuppressive agent to be used in solid organ transplantation. Prednisone stabilizes the cell membranes of endothelial cells and inhibits the chemotaxis of neutrophils, monocytes, and lymphocytes. Monocytes and lymphocytes are sequestered to the lymphatics and bone marrow. T cell activation and macrophage function are impaired. Prednisone inhibits collagenase, elastase, and tissue plasminogen activator. The release of arachidonic acid by cell membrane phospholipids is also inhibited resulting in decreased production of prostaglandins, thromboxanes, and leukotrienes. Although cheap and effective, prednisone alone does not prevent rejection. Furthermore, the long-term complications of prednisone such as gastrointestinal bleeding, iatrogenic hyperadrenocorticism, diabetes mellitus, and increased susceptibility to infection are all significant side effects. [14;22]

Azathioprine replaced prednisone and remained a mainstay of immunosuppressive therapy for approximately 30 years. Azathioprine is a purine antagonist that interferes with DNA synthesis, killing actively dividing lymphocytes. It is metabolized to 6-thioinosinic acid which blocks *de novo* synthesis of adenosine monophosphate and guanosine monophosphate. However, azathioprine affects all

rapidly dividing cells in the recipient, resulting in hepatotoxic effects, bone marrow suppression, anemia, and damage to intestinal epithelium. [23] [22]

The development of newer, more tolerable immunosuppressive agents such as cyclosporine (CSA) and tacrolimus signaled a new age for transplantation medicine. CSA, a cyclic decapaptide derived from the soil fungus, *Tolypocladium inflatum*, was introduced in 1983. It inhibits T-lymphocyte proliferation by inhibiting the phosphatase activity of the calcium activating enzyme calcineurin and prevents select cytokine synthesis (interleukins-2,-3,-4, GM-CSF, TNF-α) [22;23]. The suppression of interleukin-2 further diminishes T cell proliferation. [23] Cyclosporine is a mainstay in transplantation medicine; however, nephrotoxicity due to CSA has been documented in people. A histologic description of isometric tubular vacuolation, arteriolar myocyte vacuolation, arteriolar endothelial cell necrosis, arteriolar subendothelial hyaline deposits, arterial fibrointimal proliferation, and glomerular microthrombi are consistent with CSA toxicity in people [16;17]. Chronic nephrotoxicity is characterized by interstitial fibrosis, tubular atrophy, and non-obliterative arteriolopathy. [16] The widespread use of calcineurin-inhibitors has decreased early acute rejection episodes but paradoxically has induced nephrotoxicity and eventual graft loss. Several authors claim calcineurin-inhibitor-induced nephrotoxicity to be almost universal at ten years post-renal transplantation in people. [24;25]

Tacrolimus, a T-lymphocyte inhibitor, was initially approved for liver transplants and later for kidney transplant patients in 1997. It blocks T-lymphocyte activation genes using a mechanism similar to cyclosporine but is 10 to 100 times

more potent; however tacrolimus has more side effects such as severe vasculitis and intussusception.[22]

In 1995, mycophenolate mofetil (MMF) was FDA approved as an immunosuppressive agent. Similar to azathioprine, MMF inhibits *de novo* purine biosynthesis, affecting rapidly dividing cells.[14] Side effects of MMF include vomiting, diarrhea, gastrointestinal hemorrhage and petechiation, and less frequently pancreatitis (CellCept® [Mycophenolate Mofetil] Labeling, Roche) .[8;26]

The irony of modern immunosuppressive agents is that short-term graft survival has experienced huge strides; however, long-term survival remains largely unimproved. Immunosuppressive agents generally affect the entire immune system impairing the host versus graft response (HVG) as well as lowering the recipient's response to opportunistic infections. The development of life-threatening infections, an increased risk of developing tumors, drug-related toxicities, and eventual chronic rejection of the kidney have all limited the capabilities of long-term immunosuppressive medications.

Immunologic Tolerance

Ideally, an immunotherapeutic agent would be used temporarily to allow the immune system to recognize an allograft as "self" until the recipient's body could maintain this status without exogenous treatment. The immune system would not attack a graft while maintaining all other normal immune functions. This selective alteration of the immune system is called immune tolerance. Donor specific allograft tolerance is defined as the indefinite survival of a normally functioning allograft in the

absence of maintenance immunosuppression.[27] More precisely, 1) the absence of donor-specific alloantibodies in the recipient, 2) lack of destructive lymphocytic infiltration in the allograft biopsies, and 3) verification of donor-specific unresponsiveness and third party responsiveness using functional assays define true tolerance.[28]

Discovery of Immune Tolerance

T cells play a pivotal role in the success or failure of an allograft. In a "nude mouse," which is born without a thymus, and thus without T-cell mediated immunity, skin grafts from various species are successful without any requirement for medical immunosuppression. In one particular experiment, a chicken skin xenograft was successfully performed on a mouse, and the mouse even grew feathers.[29] This emphasizes the important role of T-cell immunity in transplantation rejection; however, a person or dog without T cells will not survive. The key to allograft and patient survival is the lack of a detrimental immune response against the allograft, not the total absence of immune reactivity.[30] This balance is the essence of tolerance.

The goal of modern immune tolerance is to maintain a functional graft without ongoing therapeutic immune suppression.[30] The elimination or inactivation of preexisting mature donor reactive T cells and the lifelong elimination or inactivation of newly developing donor-reactive T-cells are both required for tolerance. [31] The window of opportunity for tolerance induction appears to be during a period of immunodeficiency either naturally or artificially induced. This occurs in young patients whose immune systems are still developing or patients who have undergone full body immune compromise i.e. total body irradiation. Peter Medawar found that

immature mice injected with allogeneic spleen cells could later accept skin grafts from the same donor.[29] This example of tolerance showed the potential manipulation of the developing immune system for acceptance of foreign allografts. Because patients in need of transplantation generally have mature immune systems, temporary immunologic ablation or incompetence is required to induce tolerance in an adult.

Induction of Immune Tolerance

It is important to note that a majority of studies that document successful immune tolerance induction use rodent models. In comparison to laboratory rodents, large animals and people have a much wider exposure to environmental antigens and thus maintain a more highly developed T-cell response. T-cells primed by an antigenic stimulus are more difficult to make tolerant.[31] Additionally, large animals have MHC II molecules expressed on vascular endothelial cells, whereas mice do not.[32] This increases their susceptibility to host surveillance mechanisms.

Deletion, anergy, regulation/immunosuppression, ignorance, and hematopoietic chimerism are all potential mechanisms of immune tolerance induction. The induction of tolerance using clonal deletion can be accomplished at the central and peripheral levels.

Central tolerance to "self" is established at the thymic level. After production in the bone marrow, T lymphocytes travel to the thymus for evaluation. T cells whose TCRs bind too tightly or do not bind at all to "self" MHC molecules are eliminated via apoptosis to prevent future populations of autoreactive and dysfunctional T cells. Researchers have attempted to harness this process of clonal deletion to eliminate alloreactive T cells. Currently, the most robust form of tolerance is thought to be the

result of hematopoietic chimerism, as donor cells are continually migrating to the thymus, promoting clonal deletion.

Chimerism exists when cells from another organism i.e.. the donor, exist in the recipient. A hematopoietic chimera has hematopoietic cells from a donor coexisting within the recipient's hematopoietic population. One of the most plausible theories of tolerance requires there to be mixed chimerism, which is the existence of donor cells within a host, at levels that are greater than 1% but less than 100% of the recipient's total cell population. Mixed chimerism is a subset of macrochimerism which has been well established in the murine model, but is more difficult to attain in a large animal or human model. Several points argue in favor of mixed chimerism. Models for tolerance that use mixed chimerism routinely meet strict experimental standards for tolerance such as the acceptance of a donor skin graft and the maintenance of an immune response to third party antigenic stimulation. Additionally, mixed chimerism can be a quantitative measure for the presence of donor specific tolerance.[31] Hematopoietic stem cell transplantation (from harvested donor bone marrow) provides the recipient's thymus with a continual influx of donor lymphocytes to promote life-long negative selection of donor reactive thymocytes.[28] Ideally, the T cells that express receptors (TCR) that bind allogeneic cells would be negatively selected, thus permitting long-term survival of the graft.[33]

In large animal and human models, macrochimerism requires debilitation of the T cell repertoire in order to eliminate preexisting mature donor-reactive T-cells.[31] This can be accomplished within the thymus, using local or total body irradiation, or through peripheral mechanisms. Initially full body lethal irradiation was promoted as

the best route to immunologic ablation. It was thought that the entirety of the recipient's bone marrow had to be ablated to create a physical space that the new progenitor cells would fill. In reality, this total ablation is only required for hematologic malignancies and does not appear necessary for allograft recipients. Less aggressive sublethal TBI resets the immune system to a lower level of surveillance as seen in immature animals or T-cell depleted adults without necessitating the total elimination of the immune response. This myelotoxic cytoreduction allows for the reception of a donor-derived bone marrow transplant from the donor.[29] Local thymic and nodal irradiation have been shown to serve a similar purpose of weakening the recipient's immune response.

Induction of tolerance in peripheral tissues can be established using repetitive T cell depletion or a costimulatory blockade. Peripheral tolerance is likely most important to "jump start" the induction phase of immunologic tolerance and becomes less important during the maintenance of tolerance.[28] Once T cell populations leave the thymus and undergo clonal expansion (proliferation of a specific and identical T cell line), they can be inactivated or eliminated in the peripheral vasculature. Using monoclonal antibodies, CD4-positive and CD8-positive T cells can be systemically depleted. In rodent models, the combination of monoclonal antibodies against CD8-positive T cells, 3Gy TBI, MHC disparate BMT, and one dose of cyclophosphamide led to multilineage engraftment in a majority of patients.[34] In dogs, when used without additional medical therapy, these antibodies set up an immunosuppressed environment suitable for tolerance induction; however, prolonged use (>13 days) of the antibodies led to recipient antiglobulin production and an eventual anaphylactic

response. When used in conjunction with azathioprine and CSA, the antibody therapy could be prolonged up to 28 days.[35] Anti-CD3 immunotoxin has been documented to induce tolerance in rhesus monkeys by depleting sessile and circulating T cells.[36;37]

As mentioned earlier, costimulation via the binding of T cell surface molecules CD154 to CD40 and CD28 to B7 molecules is required for T cell activation and thus T cell directed allograft cell destruction. If costimulation is absent or blocked with CD154 antibody or CTLA4-Ig, the result is T cell anergy or inactivation, leaving the donor cells viable. The use of CD154 specific monoclonal antibody allowed for the induction and maintenance of tolerance in an outbred group of MHC-mismatched rhesus monkeys for greater then 10 months.[38] Rabbit anti-dog antilymphocyte serum in conjunction with BMT and limited CSA therapy led to long-term canine renal allograft survival (>180 days) with donor specific unresponsiveness.[39] Interestingly, if very high doses of donor bone marrow (12-fold increase) are administered along with antiCD154 (+/- CTLA4Ig), then sublethal irradiation, cytotoxic drugs, and monoclonal antibodies were not required in some models.[40] Bone marrow transplantation has been associated with marginal, nonspecific immune depression documented by the down-regulation of mixed lymphocyte reactions and cytotoxicity assays. This immune depression was directly linked to CD34+ stem cells as well as early progeny lymphoid cells (CD38+, CD2+, CD5+ and CD1+) and early myeloid cells (CD33+).[41] Another study was able to reduce whole body radiation to low levels (3 Gy) or eliminate irradiation entirely if very large quantities of donor bone marrow were utilized.[27] Because such large quantities of bone marrow are not practical in a clinical

setting, a course of conventional immunosuppression, co-stimulatory blockade, low dose irradiation and standard dose BMT are often advocated with similar success.[31]

Veterinary Studies

Many different protocols have been attempted using various pharmacologic combinations in canine models. One 100-day study found that 50% of dogs on microemulsified CSA alone (achieving 500 ng/mL) experienced allograft rejection following mismatched kidney transplantation.[11] Another study administered microemulsified CSA, azathioprine and prednisone combination to four dogs after mismatched renal transplantation with 50% fulfilling the 100 day study without clinical signs of rejection. Two dogs were euthanized, one due to an intussusception (day 8) and one due to an upper respiratory tract infection (day 64). Three dogs had evidence of hepatotoxicity, which resolved when the azathioprine dose was decreased. Of the two survivors, one had no evidence of rejection and the other displayed acute rejection (moderate intimal arteritis).[12] In another veterinary study, a similar protocol of RADTS, prednisone, CSA, and azathioprine was followed and additionally, three of seven dogs also received donor bone marrow (DBM). Immunosuppression was gradually tapered as prednisone, CSA, and azathioprine were withdrawn successively at 200, 450 and 680 days. Four dogs survived to the end of the 2 year study but the other three (no DBM) rejected with total drug withdrawal. One of the surviving dogs that received bone marrow, survived off all drugs.[42]

In another study, fifteen dogs with ESRD received a kidney transplant from a dog erythrocyte antigen (DEA) match with successful cross-matching. Dogs were

treated preoperatively with rabbit anti-dog antithymocyte serum (RADTS) injections, prednisone, and azathioprine and postoperatively with CSA. Rabbit anti-dog antithymocyte serum was given daily for 6 days to achieve less than 10% of normal lymphocyte population but greater than 70,000 platelets. Blood transfusions were started preoperatively and completed intraoperatively, as all dogs were anemic. Prednisone began at 1.0 mg/kg/day and was tapered until 0.25 mg/kg/2 days was achieved. Azathioprine was commenced at 1.0 mg/kg/day and gradually by year two was tapered to an every other day dosing schedule. CSA levels were maintained at 500-600ng/ml initially, reduced to 400ng/ml at 6 months and to 300ng/ml during the second year. The side effects of each of the drugs (CSA, azathioprine and prednisone) were monitored and the drug causing the most serious side effects was eliminated first. Using this protocol, a mean survival time of 8 months was achieved. Three dogs died of surgical technical failures and four dogs died from rejection episodes within the first year. Three dogs were euthanized after 12 months due to non-responsive infection, one was euthanized due to neoplasia, one due to recurrent Lyme's disease, and one due to a fibrocartilagenous embolism. At the end of this study, two dogs had survived longer than 36 months on reduced levels of CSA, azathioprine (EOD) and prednisone (EOD).[10]

The purpose of this study was to evaluate the progression of allograft histopathology in relation to renal biochemical parameters (BUN and Cr), and clinical status of DLA-mismatched dogs undergoing renal transplantation.

III. STATEMENT OF RESEARCH OBJECTIVES

a. Perform and evaluate renal allograft transplantation in mismatched donor-recipient mongrel pairs using a protocol that includes total body irradiation, bone marrow transplantation, and short-term immunosuppression.

b. Analyze serial biopsies for evidence of renal allograft pathology using hematoxylin and eosin and immunohistochemical stains.

IV. MATERIAL AND METHODS

Dogs

Ten mixed breed dogs weighing 8.0 to 27.6 kg were included in the study. The dogs (7 intact females and 3 intact males) were considered normal based on physical examination, prolonged observation (> 6 months), complete blood counts (CBC), serum biochemistry profiles, and urinalyses. Two dogs received kidneys from DLA-mismatched siblings. DNA typing was used to ensure related dogs were mismatched at the class I and II major histocompatibility complex (MHC) using microsatellite analysis through polymerase chain reaction (PCR) techniques according to published protocols.[10] The remaining 8 dogs received a renal allograft from a mismatched, unrelated donor. This project was approved by the Auburn University Institutional Animal Care and Use Committee. Dogs were housed in USDA/AAALAC accredited facilities.

Bone Marrow Transplantation

Seven of 10 dogs received bone marrow transplantations. Bone marrow was harvested from humeri and femora of the donor dogs while under general anesthesia and immediately prior to renal transplantation surgery. Bone marrow was collected

into 12 ml heparinized syringes. Bone marrow mononuclear cells were purified by Ficoll-Hypaque (1.077) density gradient centrifugation (150 x g) for 30 minutes. The low-density cells were washed with Hanks balanced salt solution and infused into the recipient's cephalic vein after the transplantation surgery but within 8 hours of total body irradiation.

Transplantation Surgery

Ten dogs underwent surgery for renal transplantation. The anesthesia protocol consisted of premedication with butorphanol (0.4mg/kg) and midazolam (0.2mg/kg), followed by anesthesia induction with thiopental, propofol, or isoflurane mask induction. General anesthesia was maintained with isoflurane (1-4%), oxygen, and intermittent hydromorphone (Hydromorphone HCL, Baxter, Healthcare Corp., Deerfield, IL) boluses. Epidural anesthesia, using morphine (Astramorph PF®, Astra, Westborough, MA) (0.1 mg/kg) and bupivicaine (Bupivicaine, Abbott Labs, N. Chicago, IL) (1.0 mg/kg) was performed prior to surgery. The dogs were maintained on balanced crystalloid fluid therapy (11ml/kg/hr) and dopamine (3mcg/kg/hr) while under general anesthesia.

Dogs were prepared for aseptic surgery and given intraoperative cefazolin (22 mg/kg IV q2h). A ventral midline incision was made and the small intestines were enteroplicated in gentle loops using simple interrupted sutures of 4/0 polydioxanone (PDS II, Ethicon, Inc., Sommerville, NJ). The native left kidney was removed from the recipient dog. Approximately 4 cm of aorta and caudal vena cava were cleared just cranial to the aorto-ilial bifurcation in the recipient dog. Next, the donor kidney

was harvested and flushed with room temperature heparinized saline. The renal artery was dilated and excess adventitia was removed. The recipient dog received heparin (75 U/kg IV) prior to placement of the aortic clamp, in preparation of the donor kidney anastomosis. The prepared recipient aorta was clamped and the donor kidney positioned on the left side. A 4 mm aortic punch (Allegiance Healthcare Corp., McGaw Park, IL) was used to create the arterial stoma. The aortic anastomosis was completed with 6/0 polypropylene (Prolene, Ethicon, Inc., Sommerville, NJ) using two simple continuous suture lines. The caudal vena cava was occluded with vascular clamps. The recipient caudal vena cava was incised to match the diameter of the donor kidney renal vein. Care was taken to avoid twisting the vein of the allograft and the venous anastomosis was completed using simple interrupted suture of 6/0 polypropylene. Once the artery and vein were anastamosed, the vascular clamps were removed, and allograft reperfusion was established. A gelatin sponge (Gel-foam, Pharmacia-Upjohn, Kalamazoo, MI) was placed around the anastamotic site to aid with hemostasis. Once hemostasis was satisfactory, the urinary bladder was opened and the allograft ureter was inserted through the wall of the bladder using blunt dissection. The ureter was spatulated and sutured with simple interrupted suture of 6/0 polypropylene. One to two additional sutures were placed on the serosal surface between the ureter and bladder. The renal allograft was stabilized with the mesovarium, mesotesticular or mesocolon. The native right kidney was then removed from the recipient dog, leaving the heterotopic allograft as the only kidney.

Post-operative analgesia was provided by the epidural anesthesia, intravenous administration of hydromorphone (0.10 mg/kg IV as needed but no less than q4-6hrs)

and/or butorphanol (0.1mg/kg/hr CRI or 0.2-0.4 mg/kg IV PRN but not less than q4hrs). Acepromazine (0.02-0.05mg/kg) was used for supplemental sedation as needed. Post-operative analgesic administration and fluid therapy (2-4 ml/kg/hour of a crystalloid dextrose solution) were typically continued for 4-7 days. Additional supportive care was provided as needed.

Surgical biopsies

The unused, nephrectomized kidney from each recipient and a needle biopsy of the remaining donor kidney were examined microscopically to determine if sub-clinical pathology was present at the time of the transplant. These specimens were designated as biopsy one. Allograft biopsies were collected at three additional time periods: a) while recipients were on full immunosuppressive therapy (biopsy 2: between 44 and 90 days), b) after maximal drug reduction (biopsy 3: 228-580 days) and c) a final biopsy (biopsy 4: obtained at necropsy [dogs 4, 5, 6, 7, and 9] and as an open surgical biopsy [dog 1]). Allograft biopsies were obtained with a 14 to 18-gauge Tru-cut biopsy needle (Achieve, Allegiance Healthcare Corp., McGaw Park, IL) via a mini-laparatomy. A larger wedge biopsy of the renal allograft was obtained from the initial biopsy (biopsy 1) and from necropsy specimens.

Immunosuppressive Regimen and Monitoring

Immediately before surgery, recipient dogs received TBI using a linear accelerator to deliver a nonmyeloablative total dose of 200 cGy at 28.5 Gy/min. Cyclosporine [CSA] (Sandimmune, Novartis, E.Hannover, NJ) was initiated the day

before surgery (15 mg/kg PO) and continued BID. Mycophenolate mofetil [MMF](Cellcept, Roche Laboratories, Nutley, NJ) therapy was initiated the day of transplantation. Six dogs were initiated at a full dose of MMF (10 mg/kg BID) and 4 dogs were started at a half dose (5 mg/kg PO BID) with all dogs receiving the full dose (10mg/kg BID) by week 2. CSA and MMF were given at these doses until the first allograft biopsy was obtained. All dogs were on antibiotics to minimize infections during the immunosuppressive period.

Immunosuppressive therapy was slowly tapered beginning 24 to 48 hours after biopsy 2. Cyclosporine was tapered first with reductions of 25-30% of the full dose (15 mg/kg). Once the cyclosporine dose was reduced by greater than 50% of the starting dose, oral prednisone was added to the immunosuppressive protocol (1 mg/kg/day). If the BUN and serum creatinine concentrations (Cr) were within normal limits 30 days after starting prednisone, then MMF was reduced by 25-30%. Every 30 days the dose of CSA or MMF was again reduced by 25-30% until prednisone was the only medication being given. After 30 days of prednisone alone, all immunosuppressive drugs were discontinued. If Cr rose to greater than 3.0 mg/dl, in the absence of other causative factors, immunosuppressive therapy was reinitiated. Depending on the severity of the azotemia, concurrent biopsy findings and clinical signs, CSA and MMF dosages were restarted at 50 to 100% of the full dosage. All drug dose calculations were based on current weights.

Complete blood count and serum chemistry profiles were monitored every 24 to 48 hours for the first 7 to 10 days. CBC, BUN, and Cr were then evaluated twice weekly until neutrophil and platelet counts normalized, and weekly thereafter.

Indirect blood pressure (Doppler) was monitored weekly beginning at the time of drug reduction.

Serum Cyclosporine Concentrations

Trough CSA levels were measured from stored serum samples taken prior to each surgical biopsy and submitted to the Auburn University Clinical Pharmacology Laboratory. The CSA assay was performed using a monoclonal fluorescence polarization immunoassay.

Clinical Status Monitoring

Each dog was monitored twice daily for attitude, appetite, and GI signs. Weight was monitored weekly. Eating the designated amount of food, bright attitude, lack of vomiting/ diarrhea and lack of significant weight loss (>3kg) earned a score of 4. Each abnormality would subtract one point from a total score of 4. For example, bright attitude (+1), vomiting (-1), eating meals (+1) and constant weight (+1) would equal a score of 3. If there was a discrepancy between the morning and evening clinical status, the least favorable score was recorded. The clinical score recorded (table 1) was based on the average of the daily scores the week preceding surgical biopsy.

Histopathologic evaluation of biopsy specimens

All renal allograft sections were examined by the author (KDB) and a board certified veterinary pathologist (SDL). Needle biopsy specimens were immersion fixed in 10% neutral buffered formalin for a minimum of 2 hours. Fixed specimens were routinely processed to paraffin and sectioned at 3-4μm. Duplicate sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff's/hematoxylin stain (PASH). Additional duplicate sections were reacted with antibodies to CD3 (catalog #: N1580, Dako Corp., Carpinteria, CA) and CD79a (catalog #: M7051, Dako Corp., Carpinteria, CA), using a standardized ABC immunohistochemical procedure.

Histopathology slides were evaluated for changes of the interstitium, tubules, glomeruli, and vasculature. Renal cortical inflammation was characterized based on the predominant cell type (lymphocytic, lymphoplasmacytic, or neutrophilic), location (interstitial, perivascular or peritubular), and overall severity [1+ (minimal): <10% of parenchyma; 2+ (slight): 10-25% of parenchyma; 3+ (moderate): 25-50% of parenchyma; 4+ (marked): >50% of parenchyma). Tubulitis, interstitial fibrosis, and/or vascular lesions were identified and subjectively characterized as minimal, slight, moderate, marked, or severe.

Immunophenotyping of Infiltrating Lymphocytes

Histopathologic sections reacted with either CD3 antibodies or CD79a antibodies were subjected to morphometric analysis. For each needle biopsy, the number of CD3-positive and CD79a-positive cells were counted and recorded. The area of each section was measured using image analysis software (Image J, National Institute of Health, Bethesda, MD). For wedge biopsies, a systematic sampling

technique using a random start point was employed. Beginning with a randomly selected field (cortex), CD3-positive and CD79a-positive lymphocytes were counted in every other 20X field, for a total of five fields. Counts were summed and the results expressed as the number of each lymphocyte phenotype per unit area (mm^2).

V. RESULTS

Clinical Results

All ten dogs had a pre-transplantation clinical score of four. Seven of ten dogs survived more than 200 days and were available for long-term follow-up. Two dogs had a series of three biopsies and five dogs had four biopsies obtained. Three dogs were euthanized or died between 5 and 20 days after renal transplantation (dogs 5, 8, and 10). Dog 5 was on a full immunosuppressive dose of CSA (15 mg/kg PO BID) and MMF (10mg/kg PO BID). After the transplantation surgery, dog 5 had azotemia, persistent mucoid stool, periodic melena and vomiting. This dog did not undergo donor BMT. After 4 days of immunosuppressive therapy, MMF was discontinued and a blood transfusion was given for anemia. On day 5, dog 5 had hemoptysis and dyspnea. Laboratory findings showed azotemia (BUN: 110, Cr: 7.4), severe thrombocytopenia (5,000 cells), and anemia (Hct: 21%). Dog 5 was euthanized and a gross necropsy revealed 500-600 ml of serosanguinous fluid abdominal fluid, a hemorrhagic and edematous pancreas, and diffusely, petechiated, compromised bowel. The renal allograft appeared grossly normal. Histologically, the allograft showed T-lymphocytic multifocal perivascular, interstitial nephritis which was most severe at the corticomedullary junction. Approximately half of the lymphoid-like cells did not react

with CD3 or CD79a antibodies. In the corticomedullary region, tubule lumens and glomeruli are filled with a protein-like fluid. Several arteries and veins appear occluded with fibrin and there is local interstitial hemorrhage. Diffuse gastrointestinal inflammation and hemorrhagic pancreatitis were noted. Histopathologic findings could not differentiate between allograft rejection and progressive renal inflammation secondary to sepsis.

Dog 8 was on full immunosuppressive therapy of CSA (15 mg/kg PO BID) and MMF (10mg/kg PO BID) since transplantation. Dog 8 displayed vomiting, diarrhea, melena and lethargy. Four days after transplantation, dyspnea was noted and thoracic films documented a generalized, severe, pulmonary interstitial pattern with moderate pleural effusion and cardiomegaly. Furosemide (1mg/kg) was administered and MMF was discontinued. On day 5, a whole blood transfusion was administered for anemia (16%) and 500ml of fluid evacuated from the chest via thoracocentesis. On day 7, dog 8 was euthanized due thrombocytopenia (15,000 platelets with gingival petechiation), persistent azotemia (BUN: 49-126; Cr: 3.2-4.5), protein:creatinine ratio of 6.5, anemia (18%) and lethargy, intermittent neurologic deficits and inability to stand. Gross necropsy revealed a hemorrhagic pancreas, grey to black petechiated intestinal tract, and fluid in the abdomen and chest. The renal allograft appeared normal. Histopathology was consistent with an acute hypoxic event. Renal histopathologic analysis revealed multiple, prominent intravascular fibrin formation and local coagulative necrosis. Scant neutrophilic and T-lymphocytic interstitial nephritis, tubular atrophy and regions of tubule dilation with proteinaceous material were noted. Arteries and veins were congested. There was minimal inflammation.

Histopathologic lesions were not considered to be consistent with allograft rejection. Based on histopathologic findings of allograft thrombosis and gastrointestinal disease, gastrointestinal toxicity was suspected as the cause of death.

Dog 10 began therapy on subtherapeutic levels of MMF (5mg/kg PO BID) and therapeutic levels of CSA. After one week, MMF was increased to a full dose (10mg/kg PO BID). Dog 10 had persistent vomiting and diarrhea and developed an infected mammary gland. Initial prophylactic trimethoprim sulfa (*Tribrissen*®, Schering-Plough, Union, NJ) was changed to enrofloxacin (*Baytril*®; Bayer Healthcare Corp, Shawnee Mission, KS). The dog was severely thrombocytopenic (5,000 platelets) and anemic (12.7%). A whole blood transfusion was administered. Eighteen days after transplantation, the dog became hyperthermic (105°F) and anuric and later died. Same day blood work showed only a mildly elevated BUN (39 mg/dl) and Cr (1.4mg/dl). Initial gross necropsy showed mild hemorrhagic changes of the serosal surface of the kidney with blood clots in the bladder. Histopathologic lesions were multifocal lymphoid aggregates at the corticomedullary junction, mild hemorrhage, acutely degenerative tubules, and distention of Bowman's capsule and venules with protein. The lymphoid aggregates had a small CD3-positive and CD79a-positive population but the majority did not stain. The death of dog 10 was attributed to bacterial sepsis resulting from the infected mammary gland.

Biopsy One (normal specimens)

At the time of transplantation a kidney biopsy, either wedge or needle biopsy, was taken from all donor and recipient dogs. These biopsies were analyzed for any signs of pathology. A wedge biopsy was analyzed in 10 dogs and a needle biopsy in 4 dogs. All slides were stained with H&E, and reacted with CD3, and CD79a antibodies. No abnormalities were noted on any slide with the exception of nine CD3-positive cells seen within the interstitium of one biopsy (dog 3)(Table 2A).

Biopsy Two (full immunosuppression)

At the time of the second biopsy, the BUN for the seven dogs ranged from 5.8-20.9 mg/dl and Cr ranged from 1.0-2.7 mg/dl. Six of seven biopsy samples had some degree of lymphocytic, plasmacytic interstitial inflammation (Figure 1b)[minimal (2 dogs) and slight (4 dogs)]. While on full immunosuppression, no renal allograft biopsy sections had more than slight (25%) interstitial inflammation. Vacuolar degeneration of the tubular epithelium was observed in 6 dogs (Figure 2). Slight to moderate tubular atrophy (4 dogs) was also noted. One dog had focal intimal arteritis (Table 2B). Minimal tubulitis was seen in 2 allograft biopsies (dogs 2 and 3). Dog 3, which had slight tubulitis at the time of transplantation, had the most tubule invasion (35 cells). Monocytic arteritis was seen in one allograft biopsy (dog 6).

Inflammatory infiltrates were composed primarily of mononuclear cells with CD3-positive lymphocytes being the major cell type. The median CD3-positive cells/mm^2 was 157 (range: 63-428 cells/mm^2) and the median CD79a –positive cells/mm^2 was 40.1 (range: 3-132 cells/mm^2).

Biopsy Three (modified immunosuppression)

Timing of biopsy three ranged from 228 days to 580 days after surgery. Renal allograft biopsies were obtained within 48 hours of immunosuppressive therapy reduction or elimination, or at necropsy (dog 3). The BUN for the seven dogs ranged from 6.1 mg/dl to 43.4 mg/dl and creatinine ranged from 1.1 mg/dl to 5.5 mg/dl in 6 dogs (Table 2C). Dog 3 was the most severely azotemic. Five of seven dogs were off of MMF and CSA; 3 were still receiving low dose prednisone. Diagnostic biopsies were obtained from two dogs (dogs 3, 7) when the serum creatinine concentration increased to greater than 4.5mg/dl despite aggressive medical management. At the time of biopsy, dog 3 had been returned to a full dose of MMF and a reduced CSA dose (50%). Dog 7 had been reduced to 10% of the initial CSA dose and 60% of the initial MMF dose but the drug doses were returned to full immunosuppression when the BUN and Cr dramatically (140 mg/dl; 6.5 mg/dl, respectively) increased one week after the final drug reduction.

Biopsies from six of the seven renal allografts showed a histological progression of interstitial inflammation of at least one level of severity when compared to biopsy 2. One renal allograft biopsy specimen (dog 7), with no inflammation on biopsy 2, had advanced to minimal inflammation at biopsy 3. Another allograft biopsy specimen (dog 4) with minimal inflammation at biopsy 2 progressed to slight inflammation at biopsy 3. The allografts previously graded as slight inflammation progressed to moderate (dogs 1 and 2), marked (dog 9), and severe (dog 3) inflammation (Figure 1c). One renal allograft biopsy specimen (dog 6) had been rated as minimal inflammation at biopsy 2, but then progressed to marked at

biopsy 3. All biopsy specimens showed a peritubular and perivascular distribution of lymphocytic, plasmacytic interstitial inflammation. Membranoproliferative glomerulonephritis (dogs 4 and 9) and glomerulosclerosis (4 dog) were also observed. Five dogs had developed minimal (1 dog), slight (2 dogs), moderate (1 dog), or marked (1 dog) interstitial fibrosis since the second biopsy. In three biopsy specimens neutrophilic infiltration had increased from slight to moderate from biopsy two to three. Tubulitis developed in two dogs and progressed in 3 dogs (Figure 1d). All allograft biopsies showed progressive in degree/severity of tubular atrophy between biopsy 2 and 3. All dogs with evidence of vacuolar degeneration of tubules on biopsy 2 had less severe pathology on biopsy 3, going from multifocal to absent in one dog, diffuse to multifocal in two dogs, and diffuse to absent in one dog. The remaining dog still had multifocal tubular degeneration but the severity had decreased from slight to minimal. Slight arteriosclerosis was seen in one dog.

Based on morphometric analysis, the number of CD3-positive cells (range: 38-TNTC cells/mm^2) typically exceeded the number of CD79a-positive cells (range: 8-TNTC cells/mm^2) in the interstitium of the biopsy. Morphometric analysis was not possible in 4 specimens (dogs 2, 3, 6, and 9) because the lymphocytes were too numerous to quantify.

Dog 3 was on a reduced dose of CSA (5mg/kg/day) and MMF (10mg/kg q24h), with the addition of prednisone (2mg/kg/day) and azathioprine (2mg/kg/day) as rescue agents at the time of euthanasia. This dog was mildly but persistently azotemic from the time of transplantation (average Cr: 3.16 mg/dl; range of 2.0-6.6mg/dl). Once Cr reached 5.5 mg/dl on day 228, the dog was euthanized. Samples from the

renal allograft obtained at necropsy showed several temporally distinct morphologies of insult: acute, subacute and chronic. Hemorrhage and edema, lymphocytic, plasmacytic nephritis with occasional neutrophilic infiltrates, moderate tubulitis, fibrosis and sclerosis were all seen in separate regions of the allograft histopathologic samples.

Fourth Biopsy

Dog 1 was the only dog to have a fourth surgical biopsy. The biopsy was taken at day 1466. The dog had been off all immunosuppressive therapy for over 1300 days and had a creatinine of 1.5 mg/dl. The biopsy of this dog had minimal lymphocytic, plasmacytic interstitial inflammation with mild interstitial fibrosis. The remaining biopsy 4 specimens were obtained at necropsy. Dogs 4, 6, 7, and 9 were euthanized with suspected allograft rejections at day 550, 348, 471, and 658 days after transplantation. (Table 2D) Dog 2 was 500 days post-transplantation at this time a fourth biopsy has not been obtained.

Dog 4 had been off of CSA for about 5 months and off of MMF for one month while maintaining low dose prednisone (0.5mg/kg/day), when creatinine rose to 5.8 mg/dl and BUN reached 96.5 mg/dl. MMF was resumed at full immunosuppressive dose and prednisone was increased (1mg/kg/day). Based on the persistent azotemia refractory to treatment and the presence of moderate glomerulosclerosis at biopsy three, as well as hypertension, dog 4 was euthanatized 545 days after transplantation.

The necropsy allograft specimen had moderate primarily T-lymphocytic interstitial inflammation, severe tubulitis with tubular atrophy, severe striped fibrosis and severe glomerulosclerosis. The capsule had a very thickened granular coating. Arteries had severe fibrointimal proliferation and more than 50% narrowing of the lumens. Several artery lumens were completely obliterated. The histopathologic diagnosis was chronic allograft rejection.

Dog 6 was euthanized at day 348 after transplantation with a BUN of 101.8 mg/dl and a Cr of 7 mg/dl. This dog had been off of CSA and MMF for 130 days and was only on prednisone (1 mg/kg/day). Once off of CSA and MMF, BUN and Cr rose steadily from 17 mg/dl and 2.1 mg/dl to an average of 43 mg/dl and 4.1 mg/dl over several months. The wedge biopsy of allograft tissue had severe lymphocytic nephritis and moderate interstitial fibrosis. The regions of fibrosis were often accompanied with lymphocytic inflammation that were most pronounced at the corticomedullary junction (perivascular). Moderate tubulitis (5-10 cells/tubular cross section), moderate to severe tubular atrophy and tubular degeneration were also noted. Mild glomerulosclerosis was present. Several large arteries had severe fibrointimal thickening which resulted in a greatly reduced lumen size. A few arteries were no longer patent. The intima of one large artery was infiltrated with CD3-positive cells, signaling arteritis. A diagnosis of allograft chronic rejection was made.

Dog 7 was euthanatized 471 days after transplantation with a BUN of 93.7 mg/dl and a Cr of 8.0 mg/dl. This dog had come off of immunosuppressive therapy intermittently but was persistently azotemic and had a chronic urinary tract infection that was refractory to routine antibiotic therapy. The wedge biopsy of allograft tissue

had severe lymphocytic nephritis and moderate interstitial fibrosis. The regions of fibrosis were often accompanied with lymphocytic inflammation, most pronounced at the corticomedullary junction (perivascular). Moderate tubulitis (5-10 cells/tubular cross section), moderate to severe tubular atrophy and tubular degeneration were noted. Mild glomerulosclerosis was present. A diagnosis of chronic allograft rejection was made.

Dog 9 was euthanized 658 days after transplantation with a BUN of 79.5 mg/dl and a Cr of 4.2 mg/dl. Immunosuppressive therapy had consisted of only low-dose prednisone for 30 days. One month prior to euthanasia a bacterial urinary tract infection was detected and treated. The wedge biopsy of the renal allograft revealed chronic degenerative changes consistent chronic rejection and end-stage kidney failure. The capsule had a thick covering of granulation tissue. The interstitium was infiltrated with severe fibrosis and moderate lymphocytic, plasmacytic inflammation. The fibrosis was diffuse and somewhat striped, emanating from the medulla to the cortex. The inflammation was perivascular and periglomerular with additional lymphoid aggregates associated with the regions of fibrosis. Tubulitis was present with severe tubular atrophy and degeneration. A large region of edema was present. Severe glomerulosclerosis was present throughout the wedge biopsy specimen. No vascular changes were evident. Chronic allograft rejection was the final diagnosis.

Cyclosporine levels

At the time of the second biopsy, all seven dogs were on full dose CSA (15 mg/kg BID). Since histopathology was consistent with reports of allograft CSA toxicity, a retrospective analysis was performed in six of the seven dogs. Two dogs had trough levels just below the serum therapeutic range of 200-300 ng/ml, two had moderately elevated concentrations, and two had extremely elevated CSA concentrations. At the third biopsy, four of six dogs had undetectable serum CSA concentrations. Of the two dogs with detectable CSA levels (dog 3 and 7) both had resumed immunosuppressive therapy when allograft rejection was suspected based on BUN and Cr levels. (Table 1)

Immunohistochemistry

At biopsy 1, only dog 3 had evidence of positive reactivity (positive uptake of immunohistochemical stain), defined as rare CD3--positive cells (< 10) in the interstitium. Infiltrates composed of CD3-positive lymphocytes predominated over CD79a-positive lymphocytes at the time of the second biopsy in all seven dogs' biopsy specimens. The average number of CD3-positive cells/mm^2 was 157 (range: 63-428 cells/mm^2) and the average number of CD79a-positive cells/mm^2 was 40.1 (range: 3-132 cells/mm^2). In the third biopsy specimens, CD3-positive cells (range: 38 cells/mm^2-TNTC) appeared to grossly outnumber CD79a-positive cells (range: 8-155 cells/mm^2-TNTC). Morphometric analysis was not possible in 3 biopsy samples (dogs 2, 6, and 9) because CD3-positive and CD79a-positive cells were too numerous to quantify. At biopsy 4, dogs 4, 6, 7 and 9 allograft specimens had B-lymphocyte and

T-lymphocyte populations that were TNTC. Dog 1 had 103 CD3-positive cells/mm^2 and 8 CD79a-positive cells/mm^2 at the fourth biopsy.

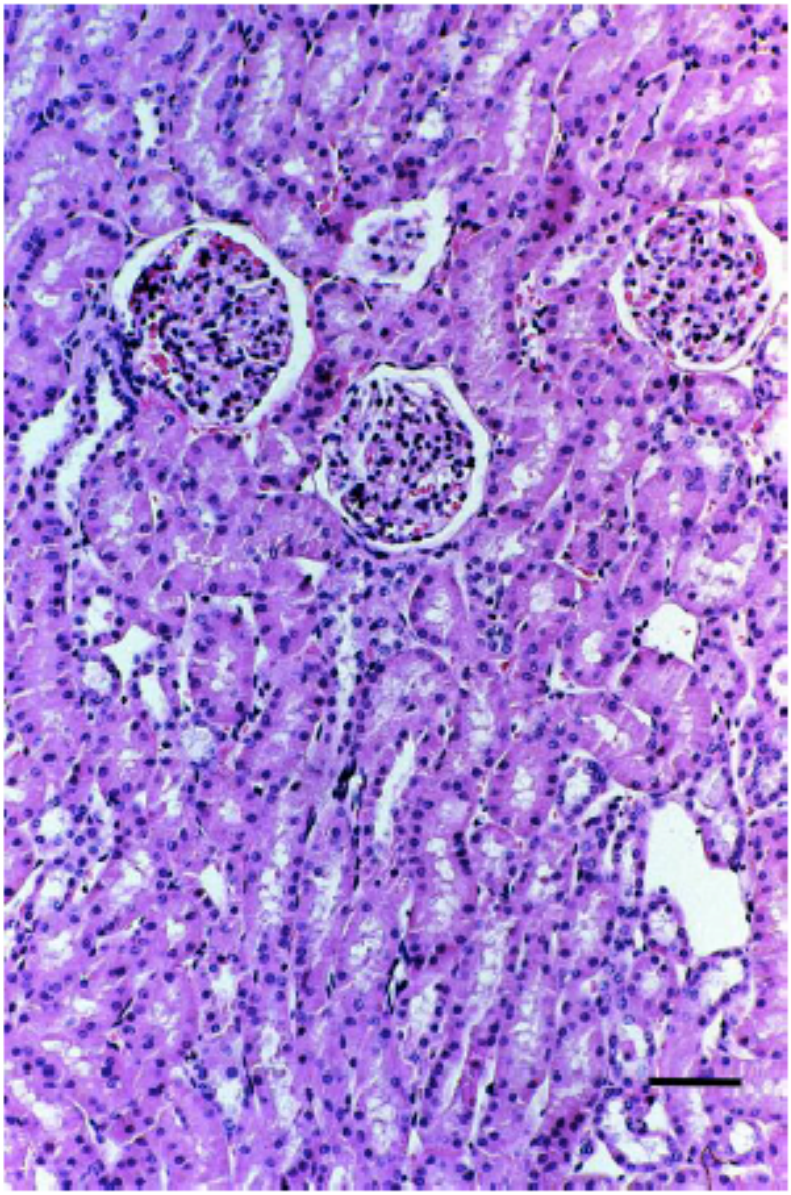

Figure 1A. Dog 6, renal cortex, biopsy 1. Microscopically normal. (Stain: HE. 330x, Bar = 60μm).

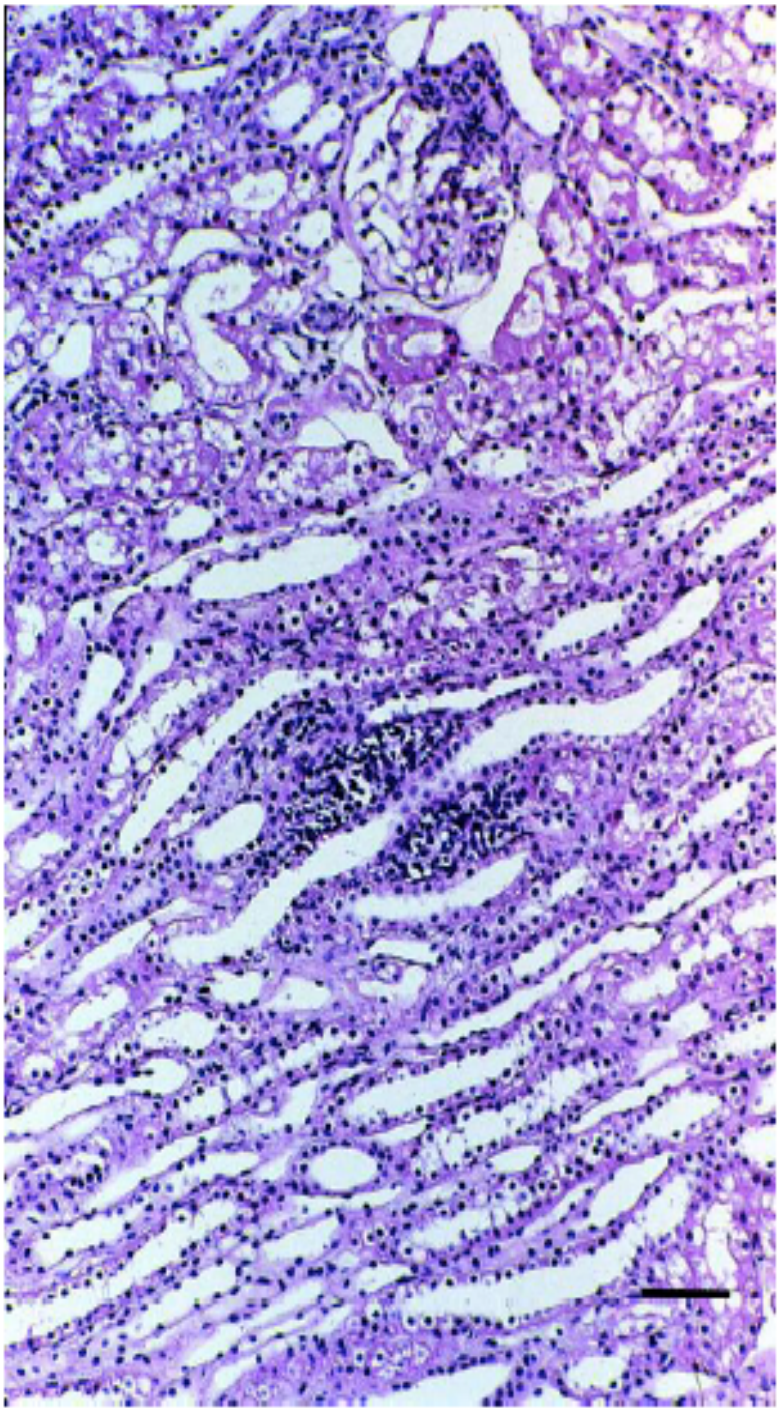

Figure 1B. Dog 6, renal cortex and outer medulla, biopsy 2. Note 2 small foci of interstitial, peritubular, lymphocytic, Plasmacytic inflammation. (Stain: HE. 330x, Bar = 60µm).

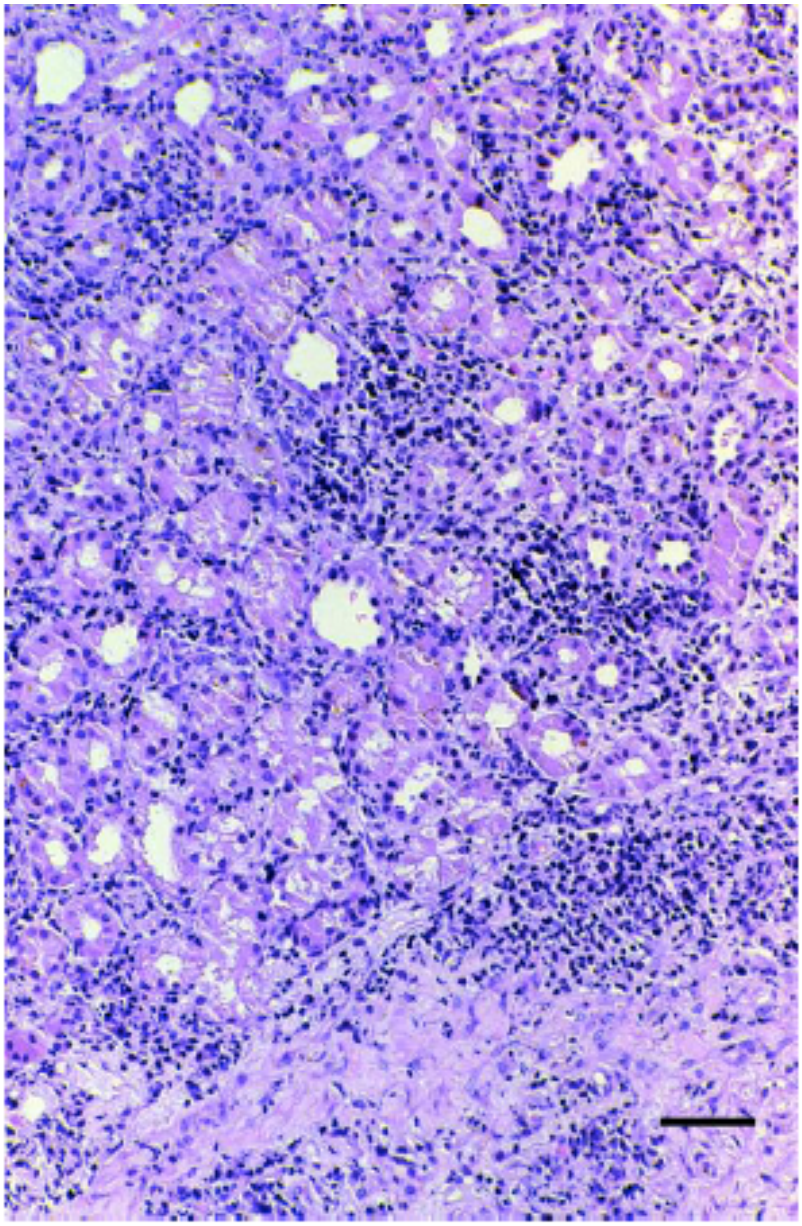

Figure 1C. Dog 6, renal cortex, biopsy 3. Note multiple foci of Interstitial, peritubular, lymphocytic, plasmacytic inflammation. (Stain: HE. 330x, Bar = 60μm).

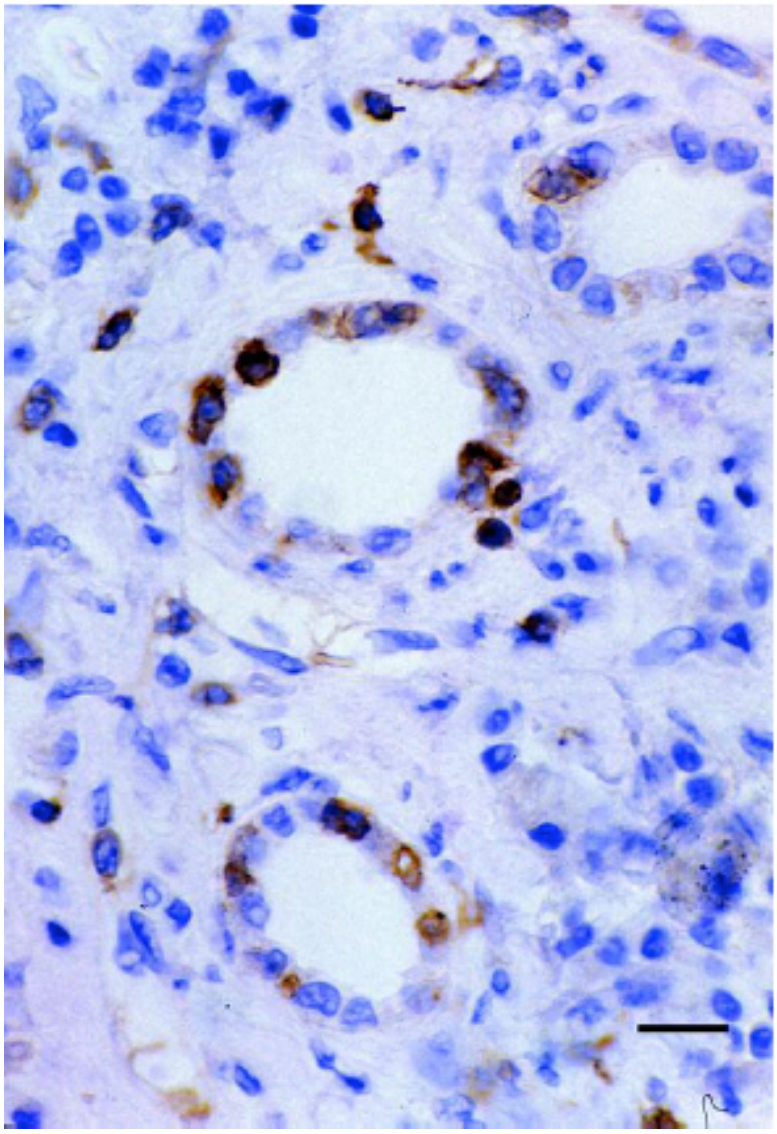

Figure 1D. Dog 6, renal cortex biopsy 3. Note the CD3-positive Lymphocytes (brown) infiltrating tubule epithelium.
(Stain: ABC-DAB as chromagen. 1320x, Bar = 15µm).

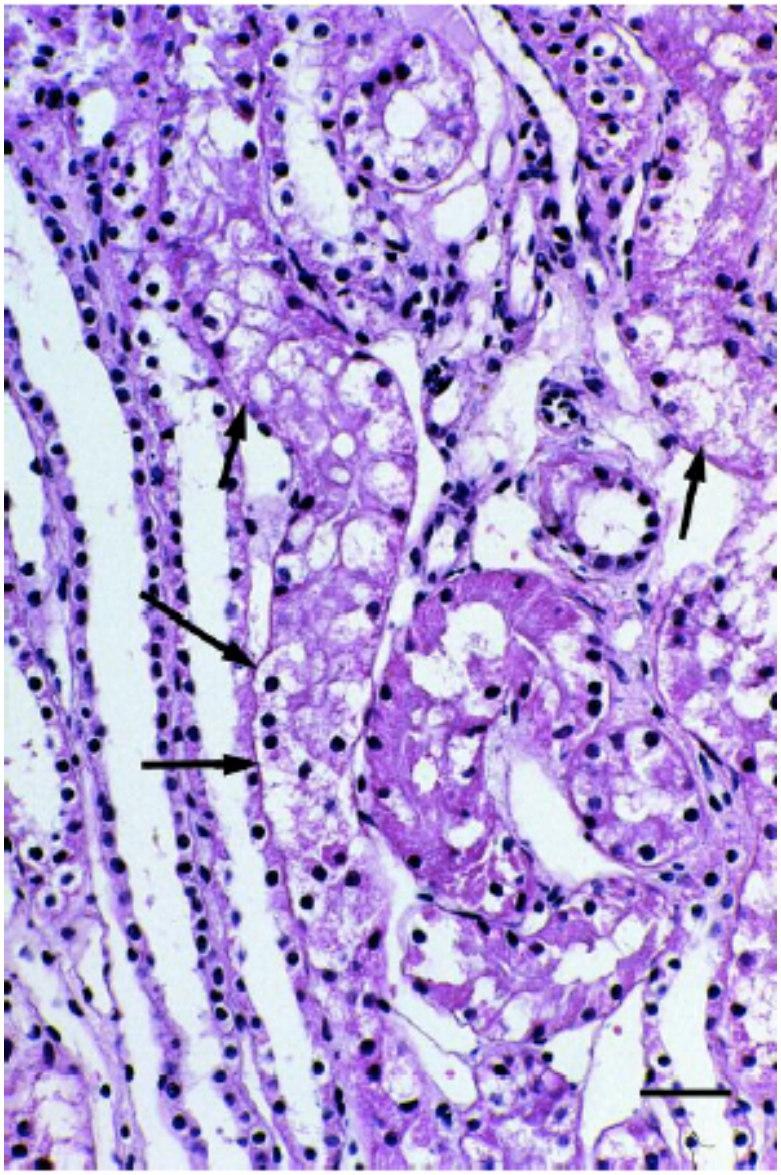

Figure 2. Dog 3, renal cortex, biopsy 2. Suspected cyclosporine Toxicity. Note markedly vacuolated tubule epithelial cells, pars recta (arrowheads) (Stain: HE. 660x; Bar = 30µm).

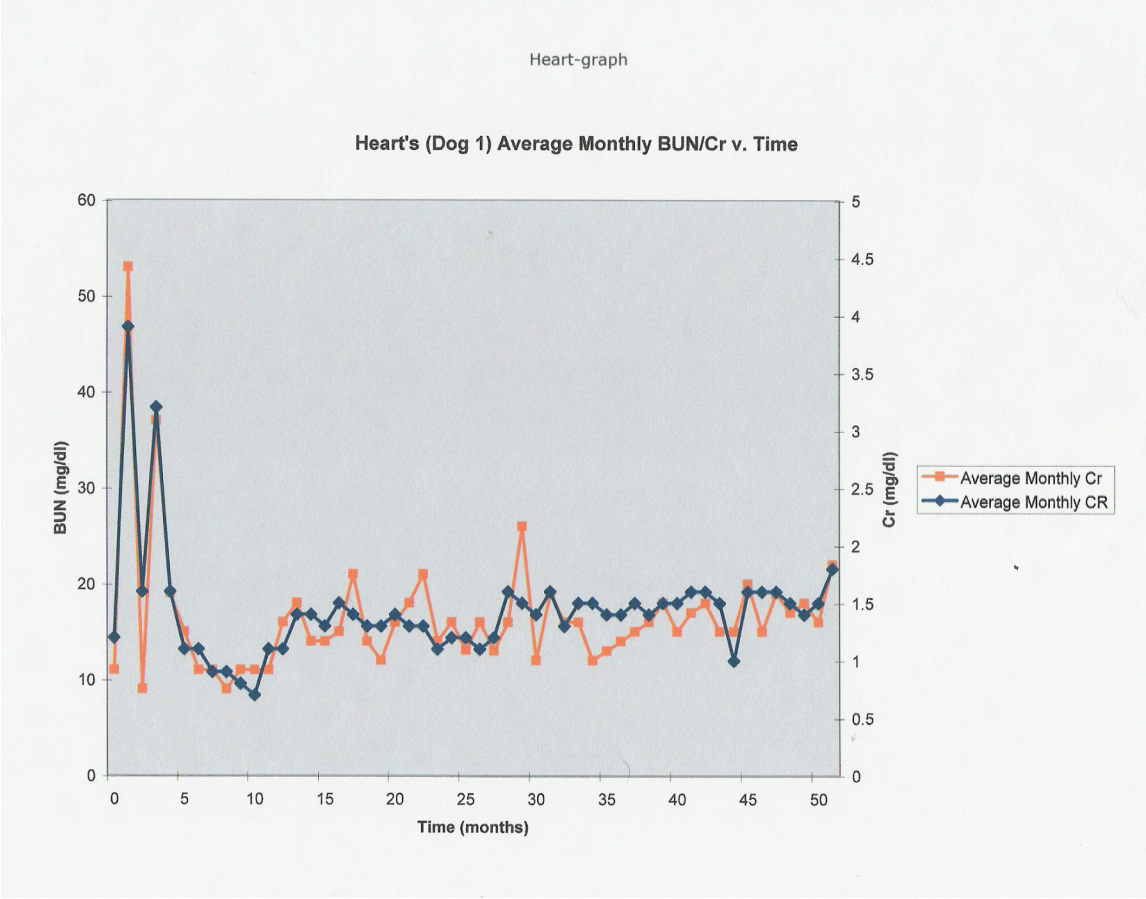

Figure 3. Graph of dog 1: BUN/Creatinine versus Time.

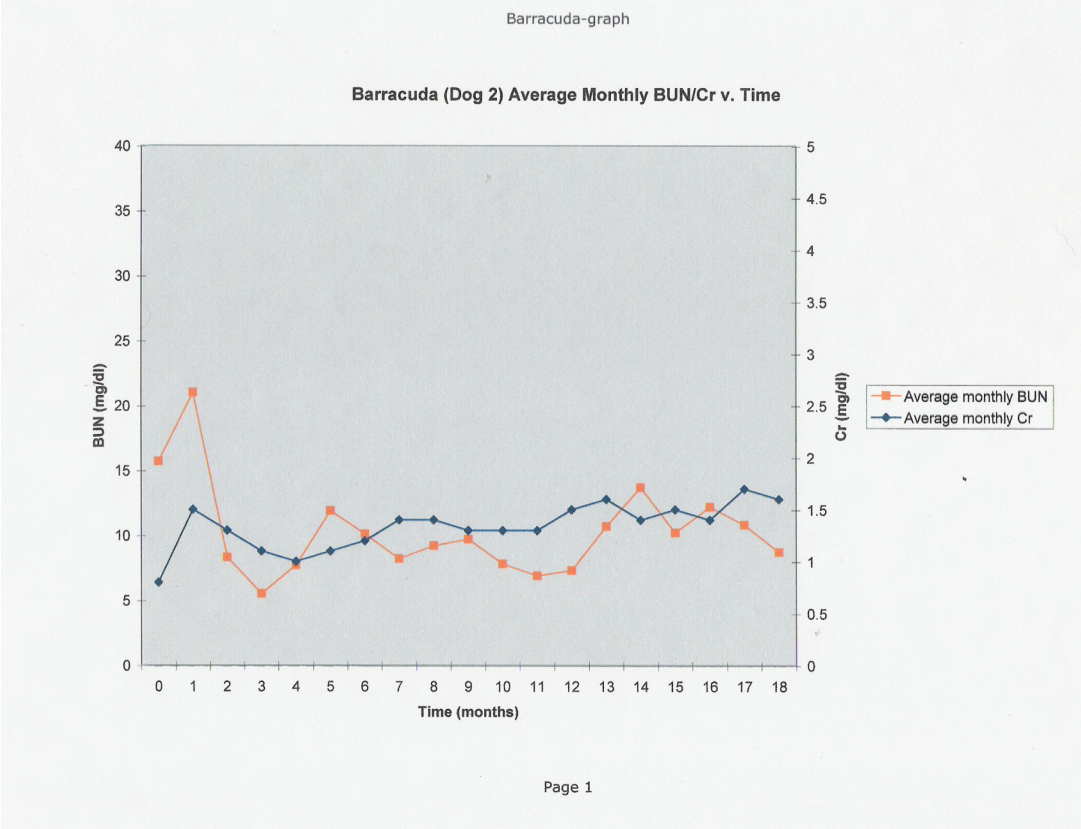

Figure 4. Graph of dog 2: BUN/Creatinine versus Time.

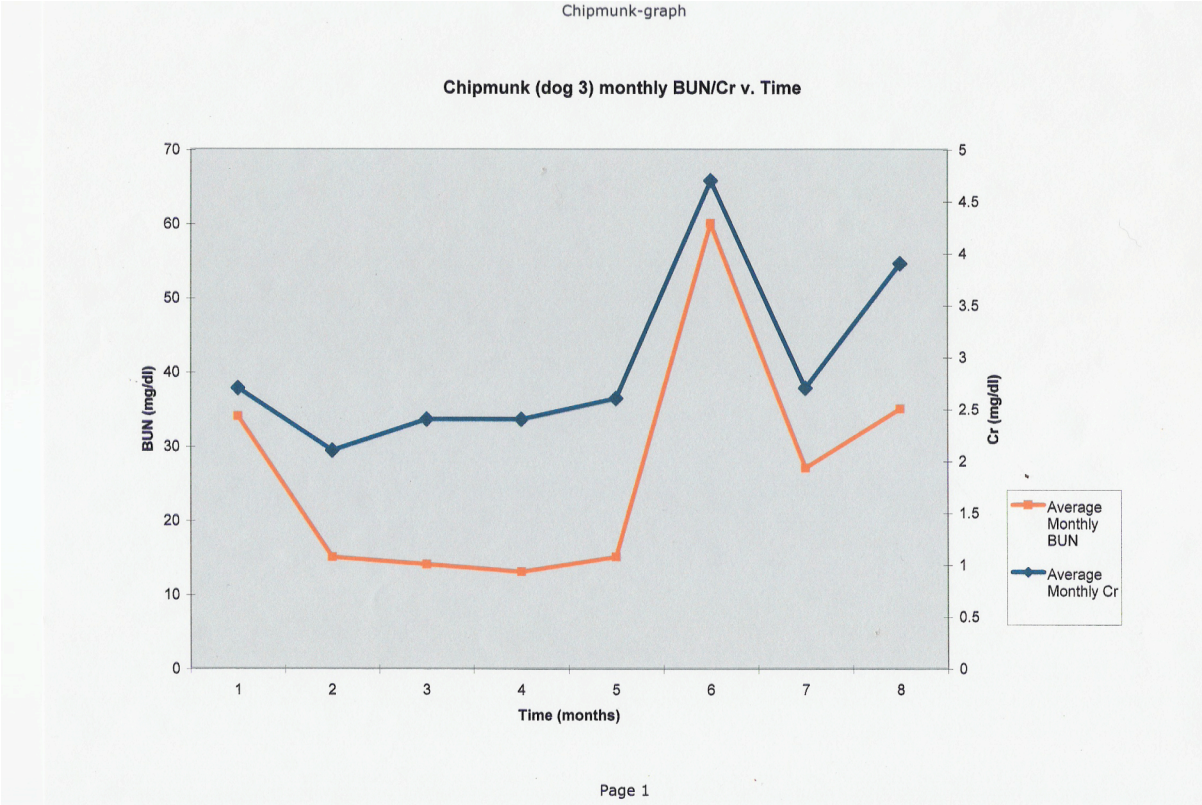

Figure 5. Graph of dog 3: BUN/Creatinine versus Time.

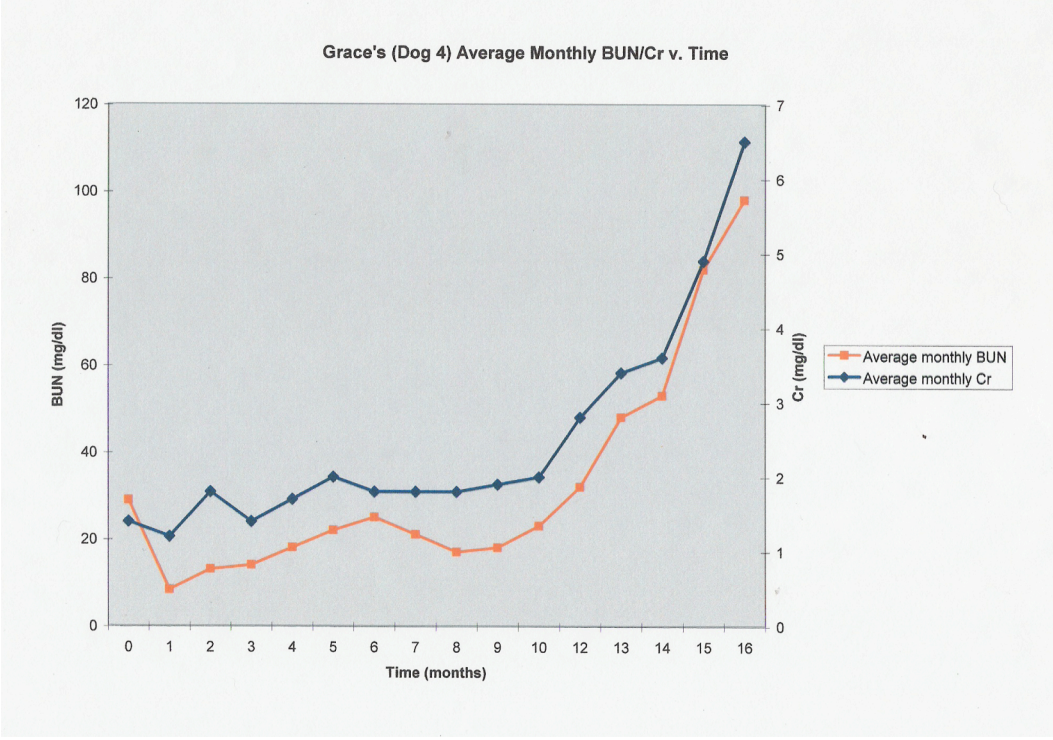

Figure 6. Graph of dog 4: BUN/Creatinine versus Time.

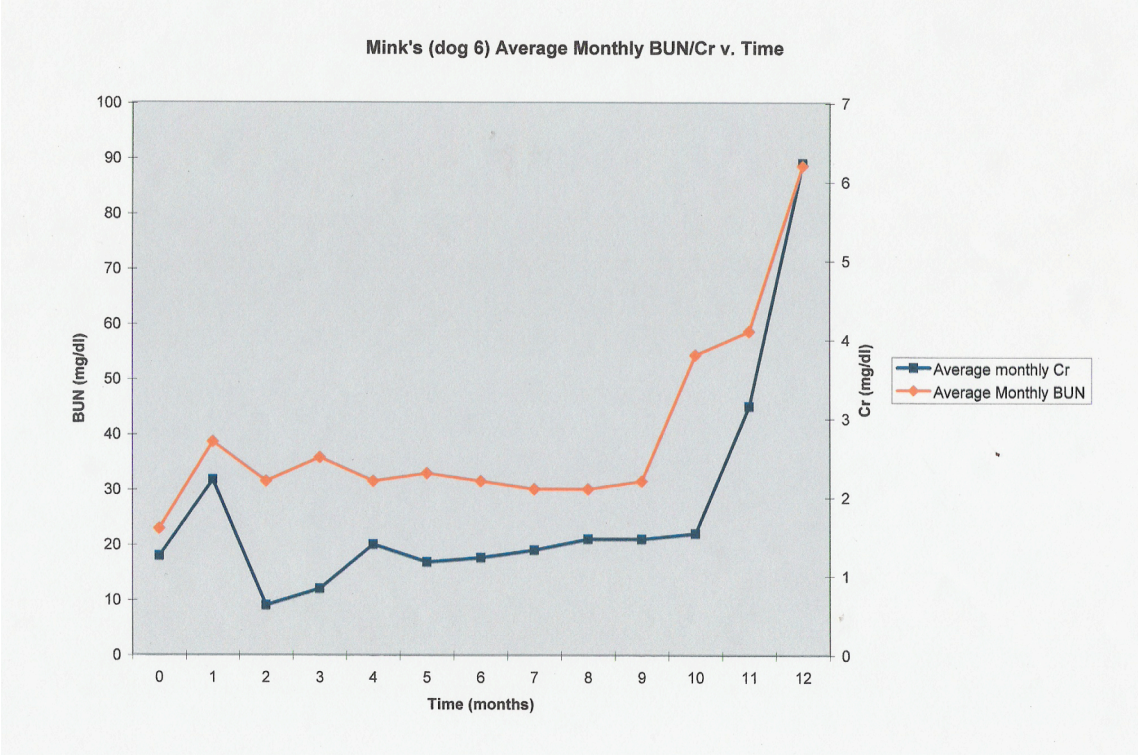

Figure 7. Graph of dog 6 : BUN/Creatinine versus Time

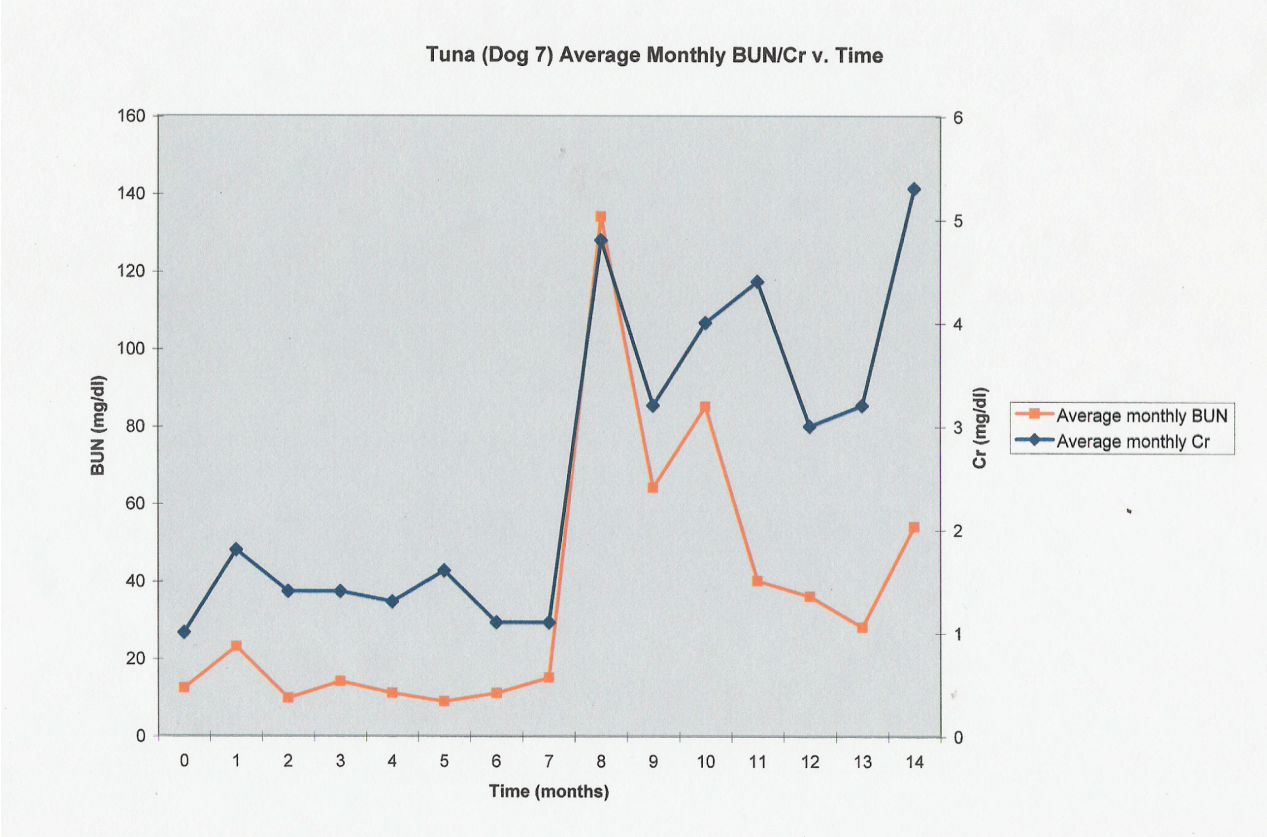

Figure 8. Graph of dog 7: BUN/Creatinine versus Time.

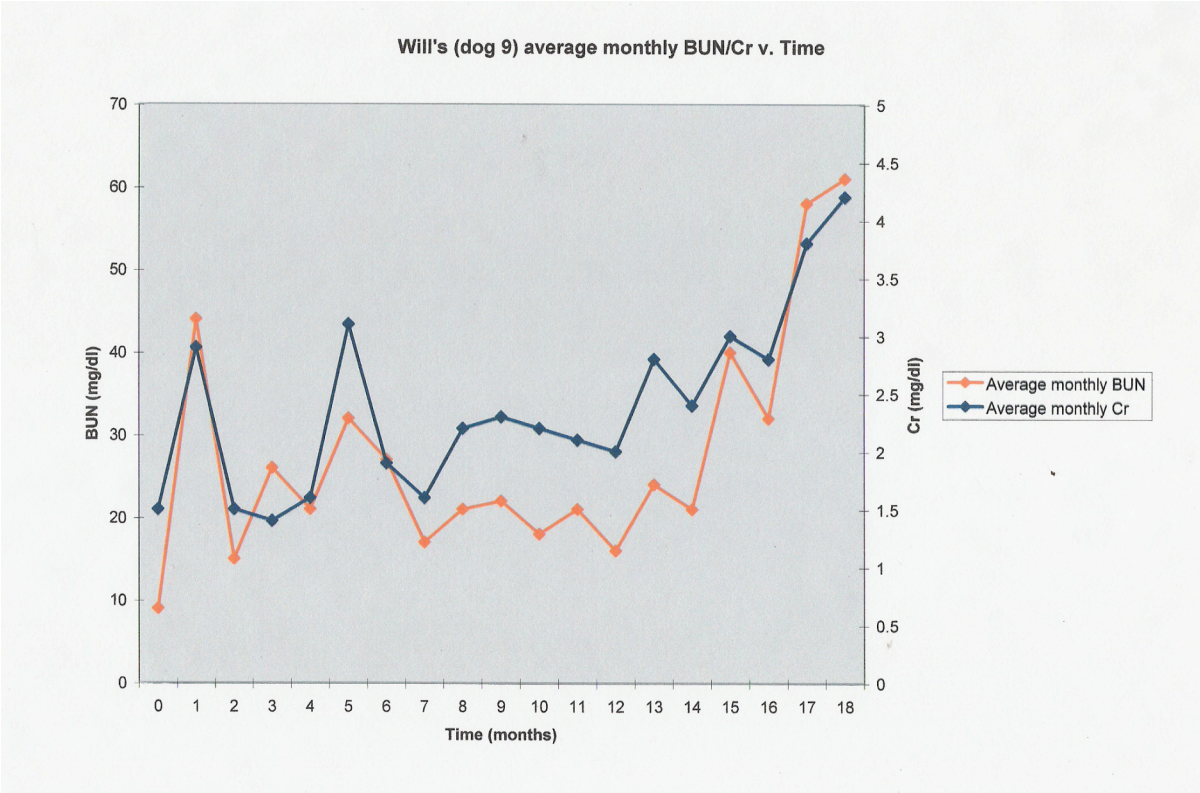

Figure 9. Graph of dog 9: BUN/Creatinine versus Time.

Time (days)	Dog	Drug levels	CSA levels*	Clinical Score
Biopsy 1				
0	1	Pre	NA	4
0	2	Pre	NA	4
0	3	Pre	NA	4
0	4	Pre	NA	4
0	5	Pre	NA	4
0	6	Pre	NA	4
0	7	Pre	NA	4
0	8	Pre	NA	4
0	9	Pre	NA	4
0	10	Pre	NA	4
Biopsy 2				
44	1	Full	ND	
90	2	Full	331 ng/ml	4
78	3	Full	1446 ng/ml	4
77	4	Full	199.52 ng/ml	4
85	6	Full	176 ng/ml	4
75	7	Full	365 ng/ml	4
75	9	Full	>2000 ng/ml	2
Biopsy 3				
580	1	Off	ND	4
281	2	Off	<25 ng/ml	4
228	3	Reduced	60 ng/ml	1
484	4	Off	<25ng/ml	4
268	6	Off	<25ng/ml	4
260	7	Full	276 ng/ml	2
560	9	Off	<25ng/ml	4
Biopsy 4				
580	1	Off	ND	4
550	4	pred	ND	2
348	6	pred	ND	3
471	7	Off	ND	2
658	9	Off	ND	3

CSA: cyclosporine Pred: prednisone ND: not determined
NA: not applicable

Table 1. Clinical score, cyclosporine levels, and renal biopsy schedule in renal transplant dogs.

Day	Dog	BUN/CR	Histopathology
Biopsy 1			
0	1	11/1.2	normal cortical sample
0	2	15.7/0.8	normal cortical sample
0	3	14.8/1.2	rare (<10) CD3+cells, rare hemosiderin in tubules
0	4	18.6/1.4	normal cortical sample
0	6	17.9/1.6	normal cortical sample
0	7	12.3/1.0	normal cortical sample
0	9	12.8/1.2	normal cortical sample

BUN: Blood urea nitrogen CR: creatinine

Table 2A. Biochemical and histopathological data in renal allograft dogs, Biopsy 1.

Biopsy 2	Dog	BUN/Cr	Histopathology
44	1	12/1.7	slight lymphocytic plasmacytic interstitial inflammation
90	2	5.8/1.0	slight, multifocal, lymphocytic plasmacytic, perivascular inflammation, minimal tubulitis, slight tubular degeneration, Atrophy
78	3	14/2.7	slight multifocal, peritubular lymphocytic plasmacytic inflammation, moderate tubular degeneration, minimal tubulitis
77	4	15/1.5	minimal, multifocal, perivascular, lymphocytic plasmacytic inflammation, moderate tubular Degeneration
85	6	12/2.5	minimal, multifocal, perivascular, lymphocytic,plasmacytic inflammation, moderate tubular degeneration, tubular atrophy and focal moderate arteritis
75	7	15.7/1.6	no significant inflammation, moderate tubular degeneration
75	9	20.9/1.3	slight multifocal, peritubular, lymphocytic plasmacytic, inflammation, slight tubular degeneration, minimal tubular atrophy, minimal tubulitis

BUN: Blood urea nitrogen CR: creatinine

Table 2B. Biochemical and histopathological data in renal allograft dogs, Biopsy 2.

Biopsy 3	Dog	BUN/Cr	Histopathology
580	1	14.8/1.4	moderate, diffuse, perivascular, lymphocytic, plasmacytic inflammation,minimal fibrosis, tubular atrophy
281	2	6.1/1.1	moderate, multifocal, perivascular, peritubular lymphocytic, plasmacytic inflammation, slight tubulitis, slight tubular atrophy
228	3	43.4/5.5	severe, diffuse, lymphocytic, plasmacytic, neutrophiilic inflammation with marked tubulitis, moderate fibrosis, tubular atrophy, marked glomerulosclerosis
484	4	61.6/4.1	slight, multifocal, perivascular/tubular plasmacytic, lymphocytic, neutrophilic inflammation with slight fibrosis, glomerulosclerosis/iitis, slight tubulitis, tubular degeneration, atrophy
268	6	16/2.8	marked multifocal,perivascular, peritubular plasmacytic lymphocytic, neutrophilic, inflammation, moderate tubulitis, slight tubular atrophy
260	7	204/4.6	Minimal perivascular, plasmacytic, lymphocytic inflammation, marked fibrosis, focal arteriosclerosis
560	9	26.2/4.2	marked multifocal, peritubular, perivascular, periglomerular, lymphocytic, plasmacytic, neutrophilic, inflammation, moderate tubulitis, slight fibrosis, moderate tubular atrophy, tubular loss

BUN: Blood urea nitrogen CR: creatinine

Table 2C. Biochemical and histopathological data in renal allograft dogs, Biopsy 3.

Biopsy 4	Dog	BUN/Cr	Histopathology
1466	1		slight, diffuse, perivascular, lymphocytic, plasmacytic inflammation,minimal fibrosis, tubular atrophy
550	4	169/7.6	severe, multifocal, perivascular/tubular plasmacytic, lymphocytic, neutrophilic inflammation with severe fibrosis, glomerulosclerosis/iitis, moderate tubulitis, severe tubular degeneration, atrophy
348	6	101.8/7.0	marked multifocal,perivascular, peritubular plasmacytic lymphocytic, inflammation, moderate tubulitis, moderate tubular atrophy, tubular degeneration
471	7	93.7/8.3	severe perivascular, lymphocytic, plasmacytic inflammation marked fibrosis, focal arteriosclerosis
658	9		marked multifocal, peritubular, perivascular, periglomerular, lymphocytic, plasmacytic, neutrophilic, inflammation, moderate tubulitis, slight fibrosis, moderate tubular atrophy, tubular loss

BUN: Blood urea nitrogen CR: creatinine

Table 2D. Biochemical and histopathological data in renal allograft dogs, Biopsy 4.

VI. DISCUSSION

Seven of 10 renal transplant dogs conditioned with 200cGy TBI and limited immunosuppression with CSA, MMF, and intermittent prednisone achieved long-term survival (average of 600 days). Tubule epithelial vacuolar degeneration consistent with CSA toxicity was observed in six of seven biopsy specimens when the dogs were on full dose immunosuppression. These lesions disappeared or diminished after the discontinuation of CSA. Later biopsy specimens (biopsy 3) from these seven dogs had an inflammatory component consistent with early rejection; however, the classic vascular lesions of allograft rejection were not observed. The severity of azotemia did not correlate well with the severity of the histopathologic lesions at biopsy three. Five dogs had a fourth histopathologic evaluation (4 necropsy, one surgical). Biopsy 4 allograft specimens obtained at necropsy were consistent with chronic allograft rejection in all 4 dogs. Two dogs (dogs 1 and 2) are still alive with functional renal allografts at 500 and 1500 days, respectively. In contrast to recent reports, this study provides an extended observation time in which canine renal allografts were monitored both histologically and biochemically.

All ten dogs began the study with normal BUN and Cr values and histologically normal kidneys. Histologic lesions of greater severity than slight

interstitial inflammation were not observed in allograft biopsies while dogs were on full immunosuppressive therapy; however, diffuse vacuolar tubular degeneration was documented in six of seven dogs at this time. In renal allograft biopsies from humans, this is considered a sign of renal allograft cyclosporine toxicity.[16;17] Vacuolar tubular degeneration was most prominent in the proximal tubules and pars recta. These tubular lesions were most severe when dogs were on full dose CSA for 75 days or greater and lesions decreased in severity or were absent on later biopsies specimens, when CSA dosages had been reduced or eliminated (Table 1). Because of the relatively high dose and lengthy duration of CSA administration as well as the concurrent characteristic histopathologic lesions that were reversible once CSA was reduced, CSA toxicity seems to be a likely cause for these changes.

Cyclosporine allograft nephropathy in human beings is characterized by the degree of isometric vacuolization of proximal tubules.[16;17] A concurrent arteriolopathy and striped, focal, interstitial fibrosis is seen in chronic cases.[16] Cyclosporine-induced nephropathy has also been documented in cats.[20] The literature suggests CSA toxicity does not occur in dogs at comparable doses. [43;44] There was no reported increase in Cr, BUN, alkaline phosphatase, or total bilirubin in two dogs that received 5, 20 or 40 mg/kg/day of CSA for one month in one study; however, these were healthy dogs that had not undergone renal transplantation.[44] The authors of another study modeling acute allograft rejection did not report histological evidence of CSA toxicity, but these dogs were only on CSA for 20 days.[43] In contrast to these reports in which dogs were on CSA for a maximum of 30 days, the dogs in the present study had lesions consistent with CSA toxicity, as described in the human literature, and were on CSA

(30 mg/kg/day) for a minimum of 75 days. Thus, CSA toxicity in the dog may be time-related as well as dose-related. In support of this conclusion, dog 1, which was on full dose CSA for the shortest period of all dogs (44 days) did not have vacuolar degeneration at biopsy 2. Other sources cite that CSA is not nephrotoxic in dogs unless whole blood levels are greater than >3000 ng/ml (>1500 ng/ml for serum).[22] Four of the six dogs with vacuolar tubular degeneration in the current study had supratherapeutic levels (>300 ng/ml) of CSA, but all six dogs were on full dose CSA for at least 75 days (average of 80 days). It is important that all diffuse tubular lesions diminished in severity and distribution once the CSA dose was lowered or eliminated. It is also interesting that these dogs had more fibrosis in biopsy 3 specimens. This suggests that CSA toxicity in dogs may result in chronic allograft changes associated with fibrosis. CSA toxicity has been documented in 50 to 100 percent of human allograft patients 7 to 10 years after transplantation on the basis of fibrosis and hyalinosis.[2] Calcineurin-inhibitor induced nephrotoxicity has been suggested as the reason for late histologic injury and the ongoing decline in renal allograft function in human beings[25] and CSA toxicity may have contributed to the renal allograft failure in some of the dogs in the current study. This problem deserves further investigation.

CSA levels in this study were retrospectively determined from serum samples obtained at the time of biopsy two and three. It may have been advantageous to monitor cyclosporine levels prospectively especially when the dogs were on high dosages (15 mg/kg) of CSA. This would allowed modification of CSA dosing to reach and maintain therapeutic levels before tapering the dosage. However, the ultimate goal of this study was to initiate a standard dose of immunosuppression and then taper

and eliminate the medication rather than maintain therapeutic levels of CSA. Furthermore, modifying the initial dosage could have further confounded the results as the significance of the CSA levels during the dosage reduction would have been difficult to determine.

Although vacuolar tubular degeneration was diminished on the third biopsy specimens, 6 of 7 dogs had diffuse perivascular and peritubular interstitial inflammation and tubulitis, all consistent with a progressive immunologic response. This inflammation was characterized as lymphocytic, plasmacytic interstitial infiltration (with occasional neutrophilic infiltration) of varying severity. In biopsies 2 and 3, T-lymphocytes outnumbered B-lymphocytes when inflammation was present. The lymphocytic, plasmacytic interstitial inflammation and tubulitis seen in the current study is consistent with allograft rejection pathology documented in previous veterinary studies.[9;43]

Five dogs had a fourth histopathologic examination. Four specimens were obtained at necropsy (dogs 4, 6, 7, 9) ranging from 348 to 550 days after transplantation and one was surgically obtained (dog 1) at day 1466. In the necropsy allograft wedge specimens, lymphocytic, plasmacytic interstitial inflammation had progressed in all dogs. Dogs developed lymphoid aggregates populated with both B and T lymphocytes, with inflammation being the most severe at the corticomedullary junction as well as in periglomerular and perivascular regions. Inflammation was typically accompanied with interstitial fibrosis, often "striped" in nature i.e. rays emanating from the medullary region to the cortex. Amongst these four dogs, there was a definite temporal distinction in allograft pathology. The two dogs that were

euthanized under 500 days had more diffuse interstitial inflammation and moderate fibrosis. These allografts reflected more active immunologic rejection. The two dogs that were out the longest, at days 528 and 550, had the most interstitial fibrosis, and edema, tubular atrophy and less diffuse interstitial inflammation. These allografts appeared to be end-stage, displaying the effects of a diminished blood supply and dysfunctional nephrons. Only one dog (dog 1) had a regression of interstitial nephritis, down-graded from moderate to slight, focal interstitial nephritis. One other surviving dog (dog 2) has not had a fourth biopsy.

In human transplantation medicine, the importance of tubulointerstitial inflammation is deemphasized compared to vasculitis.[16;17] On human renal allograft biopsies, vasculitis is indicative of a poorer response to therapy. Vasculitis has been reported in canine renal allograft specimens during episodes of rejection.[9;12;43] Fibrinoid necrosis of large vessels has also been reported in dogs that had acutely rejected a renal allograft.[9;43] Another study documented intimal arteritis in biopsies from renal allografts in mongrel dogs receiving microemulsified CSA and azathioprine.[12] The dogs in the current study had only rare vascular lesions on biopsy 2 and 3. At biopsy 2, one dog had focal moderate intimal arteritis with medial inflammation. At biopsy 3, another dog had slight arteriosclerosis. Vascular lesions were more diffuse at biopsy 4. The most prominent vascular changes at biopsy 4 reflected a diffuse arteriopathy. Two dogs (dogs 4 and 6) had severe fibrointimal thickening of arterial walls ranging from fifty-percent diminished caliber to advanced obliterative arteriopathy. Both CSA toxicity and chronic rejection cause fibrointimal thickening of arteries and so discerning between the two etiologies is difficult.

Fibrointimal thickening of arteries is typically a focal change when associated with CSA toxicity whereas chronic rejection results in diffuse vascular changes. Additionally, a truly obliterative arteriopathy is more consistent with chronic rejection.[16] The vascular changes of these two dogs would be more consistent with chronic allograft rejection following human classification; however, both of these biopsies specimens have end-stage changes making specific correlations difficult. The vascular changes noted in these two renal allograft specimens could reflect a combination of insults from both CSA and chronic rejection. It should also be noted that CSA toxicity could lead to a fibrotic and compromised allograft, predisposing it to a chronic allograft nephropathy. The two dogs with arterial changes did have tubular pathology consistent with CSA toxicity at earlier biopsies as well as the characteristic focal, striped fibrosis and tubular atrophy. These renal allograft specimens are end-stage which may make specific correlations difficult.

Throughout the study, there were discrepancies between the histopathologic lesions and the biochemical evaluation of renal status. Although the severity of azotemia and histopathologic lesions progressed overall, individual correlations between biopsies and BUN and Cr levels were unreliable. Two dogs (dogs 2 and 6) had significant progression of tubulointerstitial inflammation without concurrent change in BUN or Cr. Two dogs (dogs 4 and 7) had marked azotemia with minimal renal allograft histopathology. Only two dogs (dog 3 and 9 at biopsy 3) had consistent agreement between biochemical and histopathologic evaluations. The underestimation of pathology in dogs 2 and 6 is not unexpected given that greater than 75% of function must be lost to have biochemical evidence of compromise.[1] Additionally the

persistent azotemia without evidence of rejection at biopsy 3 in dogs 4 and 7 may be explained by clinical factors combined with subclinical rejection. Dog 4 had documented hypertension at the time of biopsy 3 which may have increased the severity of azotemia while dog 7 had a resistant urinary tract infection that was eventually documented on urine culture. Ultimately, at the time of the fourth histopathologic examination of renal allograft specimens, biochemical parameters and histopathologic findings were in agreement. At necropsy, all four dogs had significant azotemia and had histopathologic changes consistent with chronic renal allograft rejection. The surgical renal allograft biopsy from dog 1 had minimal interstitial inflammation and its BUN and Cr are within normal limits.

This inconsistency of biochemical parameters and kidney biopsy results has been reported in both feline and human allograft recipients. In a study examining 77 feline renal transplant biopsies, 36% of biopsies showed some level of rejection despite stable renal allograft function.[20] Protocol biopsies, based on a predetermined time schedule regardless of apparent allograft function, collected from asymptomatic human transplant patients demonstrated histopathologic changes consistent with subclinical rejection in up to 30% of biopsy specimens.[3] Because tubulitis and subclinical rejection are localized processes, the unaffected portions of the allograft are able to maintain normal kidney function.[25] It is also possible that a small needle biopsy may not sample areas demonstrating allograft pathology.

Early detection and treatment of subclinical rejection has been shown to prolong renal allograft survival.[3-5] Surgical biopsy, in conjunction with histopathologic examination, is the "gold standard" for documenting acute and

subacute rejection; however, due to the invasiveness of surgical biopsies and need for a stable surgical candidate, alternative non-invasive and accurate ways to evaluate the kidney function are greatly needed. In human beings, urinary perforin and granzyme mRNA levels have been correlated with acute renal allograft rejection with 83% sensitivity and 77% specificity.[45] Evaluation of the renal segmental arterial resistance index at 3 months after transplantation was shown to be a powerful prognostic indicator of long-term graft survival in human renal transplant patients.[46] Additionally, the usefulness of glomerular filtration rate prediction equations for renal allograft function is being evaluated in human renal allograft recipients.[47] A recent human study linked a 25 % reduction in GFR at three months as the strongest risk factor for subsequent graft failure.[48] The introduction of these diagnostic tests into veterinary medicine may permit more accurate and more frequent monitoring of renal allografts without the invasiveness of surgery. Such monitoring tests could fill in the gaps between biopsies, permitting a more complete evaluation of renal allograft function. Laparoscopic biopsy procedures may be also be helpful as a minimally invasive technique.

The initial mortality rate for this study was 30%. Two of the three dogs that died were transplanted early in the study when MMF was initiated at the full dosage (10 mg/kg BID). These two dogs died within 7 days of surgery with gross evidence of gastrointestinal toxicity (severely petechiated bowel) and hemorrhagic pancreatitis. Side effects of MMF include vomiting, diarrhea, gastrointestinal hemorrhage and petechiation, and less frequently pancreatitis.[8](MMF [Cellcept] label) It is speculated that intestinal and pancreatic toxicity from MMF is the result of reduced pancreatic

perfusion.[49] The use of MMF at high dosages combined with TBI may potentiate gastrointestinal toxicity. It is also possible that MMF potentiates bacterial translocation and subsequent sepsis. Sepsis-associated enteritis was reported in almost 10% of patients receiving immunosuppression with MMF, CSA, TBI and fludarabine.[50] Additionally MMF is known to decrease intestinal cell turnover.[51] Gastrointestinal toxicity was less severe once dogs in the current study were initiated at a half dose of the full immunosuppressive dose of MMF (5 mg/kg BID), starting one to two days after transplantation and gradually increased to full dose (10 mg/kg BID). One dog died from unrelated sepsis, a known complication of medical immunosuppression. The average survival in the 7 remaining was over 600 days. Considering that ESRD is 100% fatal, this protocol offers hope that renal transplantation may become a realistic treatment option for dogs with ESRD.

There were two variables, BMT (7/10) and mismatched related donors (2/10) that prevent direct comparisons among all 10 dogs. Two of the dogs that did not receive BMT died in the first 5 days of therapy of gastrointestinal toxicity. The other dog (dog 9) that did not receive BMT survived more than 658 days. The two surviving dogs which are out 1500 and 528 days did receive donor BMT. Given this broad range of survival in the three dogs that did not undergo BMT, it is not clear if the simultaneous BMT improves survival. It might be significant that two of the three dogs that died in the first twenty days did not receive a BMT. It is possible that donor BMT helps to minimize the number of days of severe neutropenia after surgery.

Two dogs in this study (dogs 1 and 6) received renal transplants from DLA-mismatched siblings. The degree of renal allograft pathology in the DLA-mismatched siblings was similar to that seen with the five unrelated DLA-mismatched recipient dogs until biopsy 4. While there was minimal interstitial inflammation at biopsy two in the two DLA-mismatched sibling dogs, the histologic lesions progressed to marked lymphocytic, plasmacytic inflammation on biopsy three. At biopsy 2, two dogs with non-related renal allografts (dogs 4 & 7) had less inflammatory response than the two mismatched, related dogs. At 348 days, dog 6 was euthanized due to persistent, non-responsive azotemia consistent with rejection. Dog 1, however, decreased from moderate to slight inflammation on the fourth biopsy which was 1466 days after surgery. Clearly, these two similar recipients had two very different immune responses to their renal allografts. With only two dogs that received kidneys from mismatched siblings, it is difficult to make accurate generalizations.

Unlike human transplant medicine, there is not a well-established system of histopathologic analysis of renal allografts for veterinary transplant recipients. A few veterinary studies have adapted the "Banff 97" scheme.[11;12;19;20] A recent feline study found the collection of biopsies for Banff 97 to be useful for the detection of subclinical rejections; however, the severity of lesions was not accurately reflected using this system. Vasculitis, tubulitis, and lymphocytic glomerulitis, all major criteria for rejection in humans, were not seen in the cats. Overall, authors suggest that the rejection of renal allografts in cats is histologically different from humans and potentially involves a humoral component.[20] In the current study, allograft rejection

was indicated as moderate to severe T and B lymphocyte infiltration of the interstitium with mild to moderate tubulitis. A few cases had fibrointimal proliferation of arteries was present. Extensive interstitial fibrosis was found in 4 dogs at biopsy 4. Glomerulitis and true arteritis, classical signs of rejection according to Banff 97,[17] were rarely noted in these canine renal allografts.

The outcome of this study points out the complexity and unpredictability of transplantation immunology despite the various attempts at manipulating the recipient immune system. Of the seven dogs that survived greater than 200 days, 5 eventually died with profound azotemia and histopathologic evidence of allograft rejection +/- concurrent CSA toxicity. With each of these dogs the longer the survival period, the more chronic the renal allograft injury appeared. Two dogs remain survivors, but dog 1 is out significantly longer than all others at greater than 1500 days. This dog received a kidney from DLA-mismatched sibling, similar to dog 6 which was euthanized at day 348 due chronic azotemia and suspect rejection. At biopsy 2 and 3, dog 1 had minimal and moderate inflammation, respectively. At biopsy 4, when dog 1 was completely off of immunosuppressive therapy for greater than 1300 days. The minimal pathology consisted of a minimal inflammatory response present as several small interstitial lymphoid pockets (10-20 cells). Due to this presence of T and B-lymphocytes, true immunological tolerance appears unlikely but not impossible. This focal inflammation may not a signal of rejection. Perhaps accommodation is a better label for this recipient-allograft relationship. Accommodation can be defined as a condition where a vascularized allograft functions despite the development of a humoral immune response by the recipient.[52] Unlike tolerance, where an allograft is

not recognized as foreign, accommodation appears to dampen a persistent immune response for a limited period of time. Diminished antibody binding, generation of endogenous protective substances e.g. heparan sulfate, and desensitized/down-regulated response to complement are all suggested mechanisms of graft accommodation.[52] The host can accommodate by shifting from a Th1 to a Th2 response, lack of T helper response and/or via a form of peripheral tolerance. A recent review of accommodation cited several small animal models where accommodated grafts expressed "survival genes" (Bcl-2 and Bcl-xl) within the endothelium of the allograft vessels. These genes provided anti-inflammatory signs preventing apoptosis by allograft cells.[53] Dog 1 could be in a state of accommodation which provides indefinite recipient survival via a functioning graft; however, this period of graft acceptance is less well defined than life-long tolerance. The negative side of accommodation is an overall decreased surveillance by the immune system resulting in increased susceptibility to environmental pathogens and neoplasia. Any infection could trigger an immune reaction, overcoming the state of accommodation leading to renal allograft rejection.

In comparison to other studies in dogs which terminated at a predetermined time frame of 100 days, there was a prolonged time frame for observation of biochemical and histopathological analysis of dogs with functional renal allografts in the current study.[11;12] Crowell et al reported a 40% mortality at 6 weeks and 100% mortality by one year.[9] A median survival time of eight months was achieved in a study with naturally occurring ESRD.[10] The current study has an average survival time greater than 600 days.

Overall the rejection pattern established in the current study was lymphocytic, plasmacytic interstitial inflammation with severe fibrosis, moderate tubulitis, tubular atrophy, and glomerulosclerosis. Biopsies provided the most accurate assessment of renal pathology in comparison to BUN, Cr and clinical status. Factors other than primary immune rejection, such as CSA toxicity, hypertension, and urinary tract infections may also contribute to eventual allograft failure. Although dogs in the current study appeared to recover from the acute effects of CSA toxicity, long-term effects such as fibrosis and vascular compromise may have contributed to eventual end-stage failure. Starting dogs out at a lower dose or abbreviating the course of the high dose CSA may prove to have significant long-term effects on allograft survival. The dog that has survived the longest in this study was on high dose CSA the shortest period of time in comparison to the other dogs. Complete elimination of immunosuppressive medication may not be possible in unrelated DLA-mismatched mongrel dogs undergoing renal transplantation with the current conditioning protocol. However, if a significant reduction in the immunosuppressive drugs could be achieved, many of the long-term complications of immunosuppression could be minimized and canine renal transplantation may become a widely accepted, clinical treatment for dogs with end-stage renal disease.

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