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EFFECTS OF DIFFERENT ORAL DOSES OF CYCLOSPORINE ON T-LYMPHOCYTE BIOMARKERS OF IMMUNOSUPPRESSION IN NORMAL DOGS

By

Todd Marlow Archer

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

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IN NORMAL DOGS

By

Todd Marlow Archer

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Candidate for the Degree of Master of Science

Cyclosporine is a potent immunosuppressive agent used to treat a wide range of canine inflammatory diseases. Unfortunately, optimal dosing protocols for achieving immunosuppression with cyclosporine in dogs remain unclear, and standard methods that objectively monitor effectiveness of immunosuppression have not been established. We evaluated an already established panel of biomarkers of immunosuppression *in vivo* with two oral dosages of cyclosporine in seven normal dogs, a high dosage known to induce immunosuppression and a lower dosage used to treat atopy, with a washout period between the two dosages. The biomarker panel included the flow cytometric evaluation of T-lymphocyte cytokine expression (IL-2, IL-4, & IFN-gamma). High dosage cyclosporine resulted in significant decreases in IL-2 and INF-gamma expression, but not IL-4 expression. Low dosage cyclosporine was associated with a significant decrease in INF-gamma expression, while IL-2 expression was not affected. The results demonstrated suppression of biomarkers in a dose-dependent manner.

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DEDICATION

First and foremost, I want to give all thanks to God, who has directed and blessed my life more than I deserve or could ever ask for. I would like to dedicate this research to my wonderful and amazing wife Meridith Howell Archer and to my four beautiful and fantastic children James Howell Archer, Meridith Laci Archer, Fleming Cross Archer, and Martha Morris Archer, for all of the time, love, and support they have given me during my time coming back to school and throughout my residency and Masters program. I would also like to dedicate this research to my parents, Barb and Jim Archer, for all of the love and support they have given me throughout my lifetime. I would also like to dedicate this research to my mother and father in-law, Martha Jo and Jimmy Howell, for the love and support they have shown me and my family during my journey in veterinary medicine. Lastly, I would like to dedicate this research to our dog, Kloey Annabelle Archer, who has served as a motivation and inspiration to learn all that I could during my endeavors in the veterinary profession.

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I would like to personally thank Dr. Andrew Mackin for allowing me the opportunity to pursue a residency in small animal internal medicine at Mississippi State University College of Veterinary Medicine and pursue a dream in life. I would like to especially thank Dr. Mackin for his mentoring and friendship along the way during veterinary school, internship, residency and now as a junior faculty member. He has had an essential and crucial impact on my development as well as provided a significant contribution towards my training, all through the giving of his time, patience, guidance, expertise, and willingness to be available for the benefit of my learning and understanding in many different aspects of the veterinary profession. I would like to thank Drs. Kari Lunsford and Patty Lathan for their support and mentorship throughout these projects as well as during my residency. I would like to thank Drs. Lesya Pinchuk, Cory Langston, and Stephen Pruett for serving on my Master's committee and for their support, assistance, and mentorship through this research project. Dr. Claire Fellman deserves considerable recognition for her significant contributions to this research and her help in providing insight and valuable assistance to all areas of this research experience. She has put in countless hours and made a considerable sacrifice in making this research a success. I would like to thank John Stokes for his tireless efforts to train and assist in the laboratory and being available when needed for flow cytometry sample analysis. His contributions were significant to the success of this project and this

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research would not have been completed without him. I would like to thank Dr. Robert Wills for his critical help and hard work with the statistical analysis of our research data. The following individuals need to be recognized for their contributions and technical assistance: Leslie Reed, Lisa Chrestman, Joyce Billow, Matthew Raby, Derek Moore, Jamie Allison, Dr. Bridget Willeford, and Jesse Grady. Furthermore, I would like to thank my fellow medicine residents, Drs. Kirstin Johnson, John Thomason, and Christine Bryan for their support during my residency. Dr. Thomason was a significant help, as we spent many hours working together to complete our research, as well as the time he spent measuring cyclosporine blood concentrations in the lab. He has become a great friend, colleague, and research collaborator. Finally, thanks to the dogs who made this research possible, who included Annabelle, Bonnie, Brownie, Cricket, Ella, Freckles, Jasmine, Noodle, and Princess.

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CHAPTER I

INTRODUCTION

Cyclosporine is a potent immunosuppressive agent derived from the fungus *Tolypocladium inflatum*, initially discovered by Borel in the Sandoz laboratory, with the findings of its immunosuppressive effects first described in 1976 at the British Society of Immunology (1). It is used in both human and veterinary medicine to treat a wide range of inflammatory and immune-mediated diseases as well as to prevent organ transplant rejection. In dogs, because of its immunosuppressive, anti-inflammatory, anti-proliferative, and lacrimomimetic properties, cyclosporine is used for the treatment of diseases such as atopy, keratoconjunctivitis sicca, autoimmune skin disorders, perianal fistula, inflammatory bowel disease, granulomatous meningoenchephalitis, and immune-mediated blood disorders such as immune-mediate hemolytic anemia (2-8). Cyclosporine is also used in canine transplantation (9-11).

Mechanism of Action of Cyclosporine

Cyclosporine is a calcineurin inhibitor, which specifically inhibits T-cell function, and potently inhibits cell-mediated immunity with a relatively lesser effect on humoral immunity (12). Cyclosporine initiates its immunosuppressive effects once in T-cells by binding to intracellular proteins called cyclophilins. The most abundant cyclophilin found in T cells is cyclophilin A. The binding of cyclosporine to cyclophilin facilitates the complex's affinity for calcineurin. When this complex binds to calcineurin, inhibition

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of calcineurin occurs through inhibition of phosphatase activity, thus preventing calcineurin's binding and subsequent activation of the nuclear factor of activated T-cells (NFAT). Inhibition of NFAT leads to decreased cytokine gene expression, with the most notable cytokine being IL-2. Other cytokines shown to have decreased expression in response to cyclosporine include IL-4, INF-γ and TNF-α. The role of IL-2 in the inflammatory process is complex and multifactorial, involving both pro-inflammatory as well as regulatory functions. In T-cells, IL-2 recognition by the IL-2 receptor initiates a signaling cascade which ultimately promotes cytokine transcription, cell survival, cell-cycle entry, and proliferation. IL-2 works within T-cells to further induce the expression of IL-2 receptors. IL-2 is the most effective cytokine for T-cell clonal expansion (13). By decreasing IL-2 expression in CD4+Th1 cells, proliferation and activation of both T-helper and T-cytotoxic lymphocytes are inhibited and the immune response is blunted.

Current Use of Cyclosporine in Canine Medicine

Cyclosporine is utilized in the treatment of many inflammatory and immune-mediated diseases in dogs. In dermatologic applications such as canine atopic dermatitis, cyclosporine is often started at a low once daily dosage, and used at that dosage until remission of disease is achieved (14-17). Cyclosporine therapy is then slowly weaned down until the lowest effective dose to maintain disease remission is found. In more critical and life-threatening diseases such as immune-mediated hemolytic anemia and immune-mediated thrombocytopenia, the best way to utilize cyclosporine therapy is not known. It is often started at a much higher dose than that used to treat canine atopic dermatitis, often demonstrating multiple possible side effects as well as being cost prohibitive for medium to large breed dogs. Oral cyclosporine preparations have been

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shown to have an unpredictable bioavailability, and therapeutic drug monitoring is therefore recommended for many disease conditions in dogs in order to achieve a target cyclosporine blood concentration range. Veterinary pharmacology laboratory recommendations have varied widely regarding the type of assay used to determine cyclosporine concentrations, the target cyclosporine concentration range for controlling disease, and whether the target should be based on trough blood concentrations or peak blood concentrations or both. Strictly relying on blood cyclosporine concentrations during treatment, however, may not determine if there is sufficient immunosuppression for disease control.

Cyclosporine target ranges are often empirical and speculative, with the main criteria for adequacy of treatment being response to therapy and disease control. Target blood concentrations do not necessarily correlate with clinical outcome or response to therapy. Many of our canine patients respond to therapy although their blood concentrations are less than the ideal cyclosporine target range, and some do not respond even when their cyclosporine blood concentrations are within the recommended cyclosporine target range. There is currently a lack of a more objective measure for assessing the immune system during immunosuppressive therapy for predicting adequacy of treatment.

Project Significance

Recent studies in human medicine utilizing peak drug concentrations for therapeutic monitoring have had had mixed results, suggesting the optimal pharmacokinetic monitoring strategy for cyclosporine is still undetermined. Assessing the biologically relevant effects of the drug, rather than measuring blood concentration,

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may allow for improved immunosuppressive therapy. Pharmacodynamic monitoring allows for the assessment of the biological effect of a drug on the immune system and the degree of immunosuppression achieved. Several pharmacodynamic biomarkers have been studied in human medicine, including lymphocyte proliferation, enzyme activity (calcineurin), lymphocyte surface antigens, and intracellular cytokine quantification. Clear relationships have been shown between pharmacodynamic parameters and drug concentrations. Furthermore, through pharmacodynamic monitoring, human studies have shown individually distinct degrees of calcineurin inhibitor sensitivity in patients despite comparable drug concentrations. A clinical benefit has been demonstrated when pharmacodynamic monitoring of either calcineurin activity or IL-2 production was utilized, and these methods show great promise for optimizing cyclosporine therapy as well as delivering individualized therapy.

Molecular methods have been used in veterinary medicine to objectively measure cytokines before and after treatment of certain diseases. These methods have not previously been used to correlate immune suppression at differing cyclosporine dosages or to validate target cyclosporine blood concentrations. Offering individualized immunosuppressive therapy for our canine patients based on a better assessment of the status of the immune system would potentially allow for tighter control of cyclosporine therapy, and potentially reduce the side effects seen as well as the costs associated with treatment. Because of the lack of evidence supporting the use of therapeutic drug monitoring to control disease in veterinary medicine, a more objective means to measure the effectiveness of cyclosporine therapy is needed. The objectives of our study were to establish and evaluate a panel of molecular biomarkers of immunosuppression through

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the use of flow cytometry and then to utilize this panel of molecular biomarkers to thoroughly evaluate the pharmacodynamics of cyclosporine in the dog. The end goal of our work was to establish a select panel of biomarkers that could be used to monitor the immunosuppressive effects of drugs such as cyclosporine and to establish the relationship between blood concentrations of cyclosporine and measurable immunosuppression.

Preliminary Studies

Initial work in our laboratory used flow cytometry to investigate a panel of biomarkers published in the human literature which demonstrated suppression of biomarkers in transplant recipients receiving cyclosporine therapy (18). The biomarker panel was also chosen based on the availability of antibodies known to be specific to dogs or those which cross-reacted with canine markers. The biomarker panel included flow cytometric evaluation of activated T-cell intracellular cytokine expression (IL-2, IL-4, & IFN-γ) and T-cell surface antigen expression (CD25 & CD95).

In the initial pilot *in vitro* study, lymphocytes from 3 healthy dogs were incubated for different time periods in varying concentrations of activator and cyclosporine at a concentration of 200 ng/ml. This cyclosporine concentration was chosen because cyclosporine concentrations higher than this resulted in negligible additional suppression of mRNA expression during real time RT-PCR in dogs in an already published study in dogs, and because preliminary studies in our laboratory showed it to be an effective drug concentration. Cells were prepared according to already published methods and incubated with CD3 to identify T-cells, and then double stained with antibodies against the target biomarker. The cells were then assessed by flow cytometry. The CD3 positive T-cells were evaluated for biomarker expression both at baseline and after activation, and

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the results helped to establish optimal sample preparation methodology in subsequent studies. (Figs. 1.1, 1.2, 1.3, and 1.4)

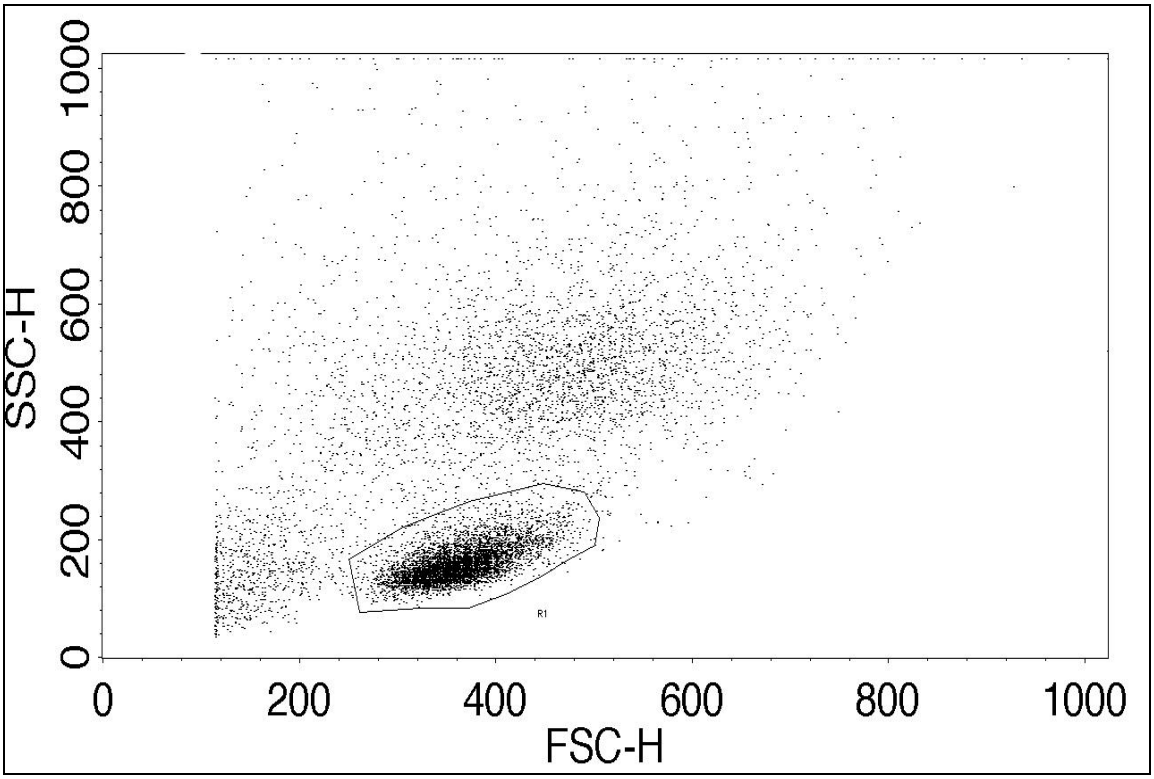

Figure 1

Canine PBMC gated based on their size and granularity, presented as a dot plot

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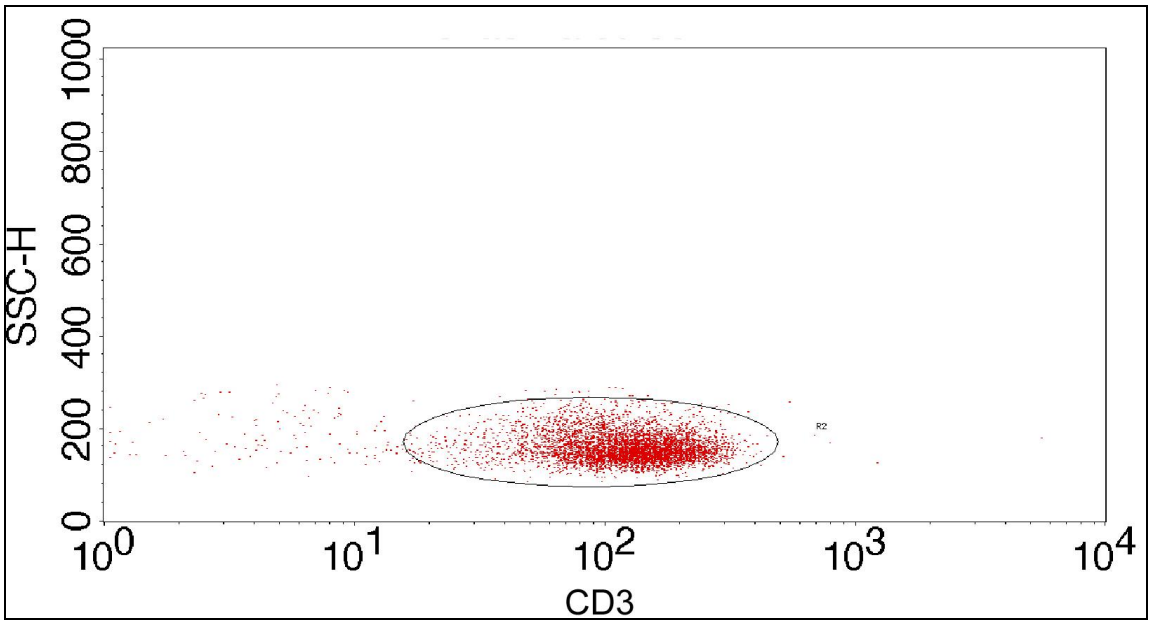

Figure 2

CD3 expression analyzed using dot plots and single gate statistics

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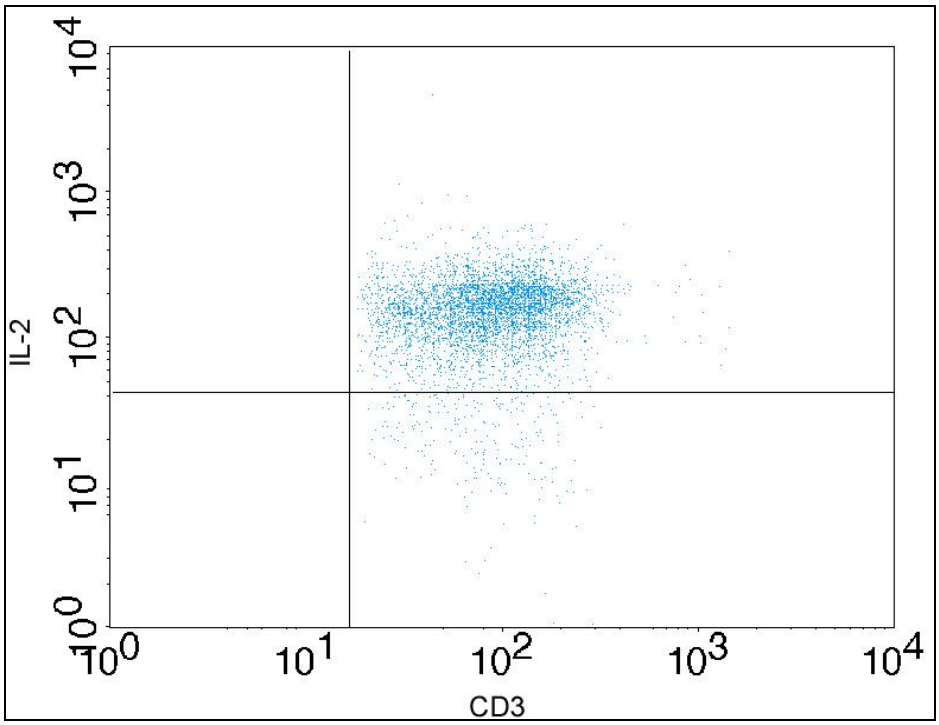

Figure 3

Two-color analysis for CD3 and IL-2 staining performed using dot plots with quadrant statistics

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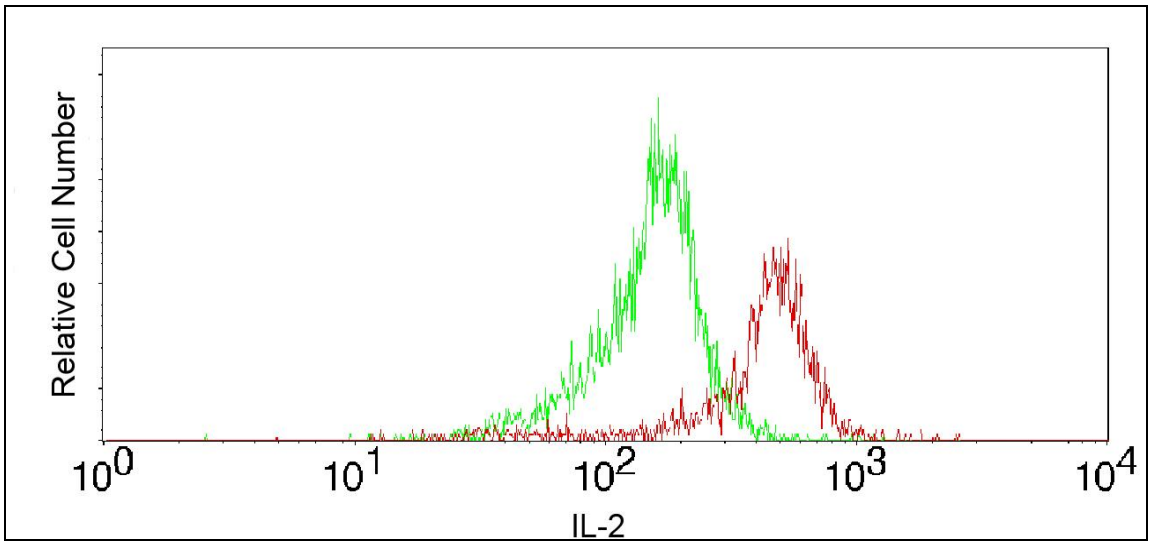

Figure 4

Phorbol 12-myristate 13-acetate (PMA) and ionomycin activated CD3+ T cells increase expression of IL-2 (red line) compared to their non-activated counterparts (green line)

This pilot study demonstrated significant cyclosporine-associated reduction in biomarker expression at 12 hours after exposure to the activators phorbol 12-myristate 13-acetate (PMA)/Ionomycin for intracellular cytokines and 36 hours after exposure to the activator concanavalin A. IL-2 expression was reduced by 54%, IL-4 by 34%, and IFN-γ by 81%, and CD25 was reduced by 25% and CD95 by 18%. This confirmed consistent cyclosporine-induced suppression of the selected biomarkers.

A subsequent larger scale *in vitro* study was then performed utilizing our already established sample preparation protocols. Blood was drawn from six healthy dogs, and peripheral blood mononuclear cells (PBMC) were isolated and activated. Half of the cells were incubated with a cyclosporine concentration of 200 ng/mL prior to activation, while the other half was not exposed to cyclosporine. All samples were analyzed using flow cytometry, and the expression of intracellular cytokines IL-2, IL-4, and IFN-γ was

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evaluated after 6, 12, and 24 hours of cyclosporine exposure. Each cytokine exhibited a time-dependent suppression profile, with activated samples exposed to cyclosporine demonstrating a lower cytokine expression than untreated controls. We also evaluated the expression of the surface T-cell activation molecules CD25 and CD95 by flow cytometry after 36 hours of drug exposure. Expression of these surface molecules decreased significantly when activated in the presence of cyclosporine as compared to untreated controls. The results from this larger scale *in vitro* study suggested that suppressed expression of the markers related to T-cell activation could potentially be utilized as an indicator of the efficacy of cyclosporine therapy in dogs. (Figs 1.5 and 1.6).

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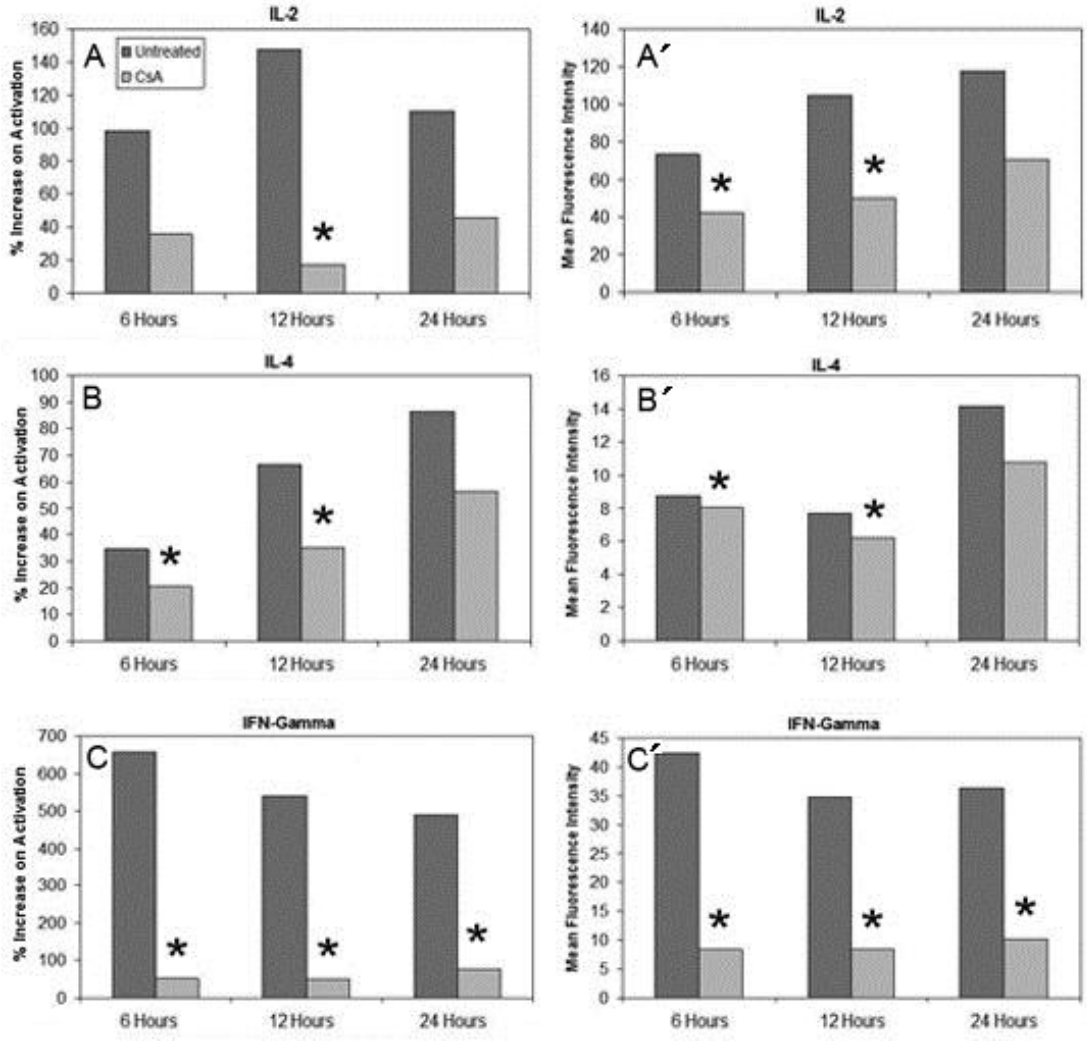

Figure 5

Increase on activation of MFI of IL-2, IL-4, and IFN-γ for cyclosporine (CsA) treated activated T-cells relative to untreated controls

Data are analyzed by calculating the percent increase on activation and by comparing the activated sample MFI values. * indicates a statistically significant difference due to the effects of cyclosporine ($P < 0.05$).

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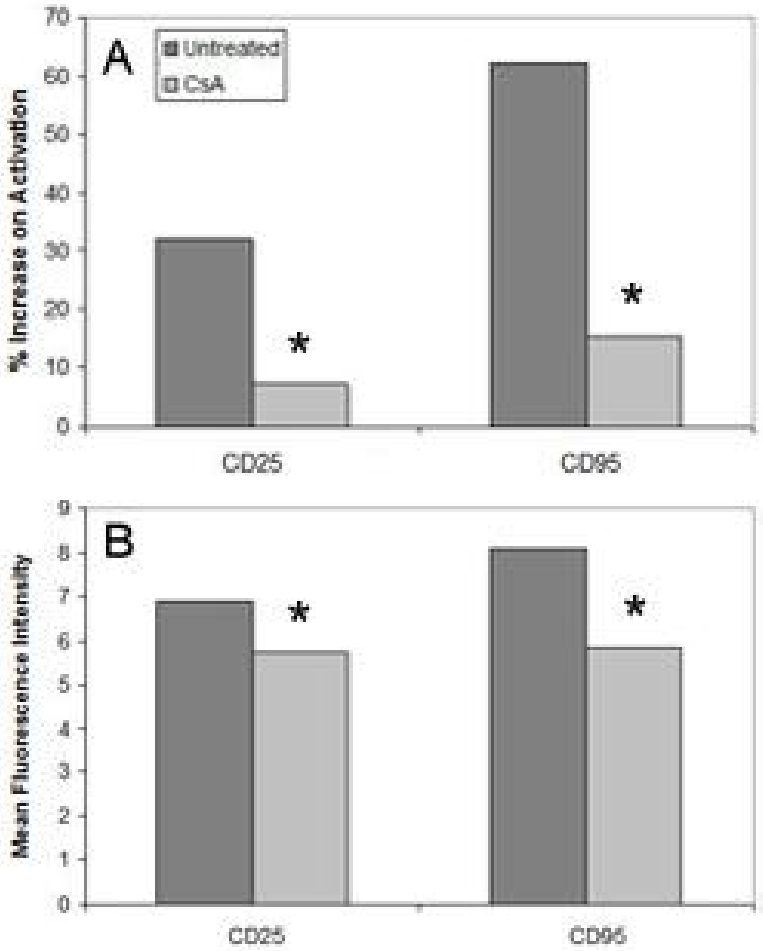

Figure 6

Percent increase in expression on activation (A) and MFI (B) for T-cell surface molecules CD25 and CD95 after 36 hours of incubation

Data are analyzed by calculating the percent increase on activation and by comparing the activated sample MFI values. * Indicates a statistically significant difference due to the effects of cyclosporine ($P<0.05$).

This provided the evidence needed to move forward with *in vivo* studies in normal dogs to further evaluate the pharmacodymanics of cyclosporine in dogs.

In a small pilot *in-vivo* study, oral cyclosporine was administered to two normal dogs at standard twice daily immunosuppressive doses for one week, with doses adjusted based on high performance liquid chromatography (HPLC) measurement of blood

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cyclosporine concentrations to achieve currently recommended target trough cyclosporine concentrations for immunosuppression (500 – 600 ng/ml trough concentrations). Treated animals had a decreased expression of IL-2 (reduced by 51% and 42%), IL-4 (33% and 26%), and INF-γ (51% and 38%). There were variable results for the surface expression markers CD25 and CD95, with neither dog showing a decreased expression of CD25 and only one dog showing decreased expression of CD95. Due to the variable results and lack of suppression in the pilot *in-vivo* study, the surface expression markers CD25 and CD95 were not further analyzed in subsequent studies. (Figs 1.7 and 1.8).

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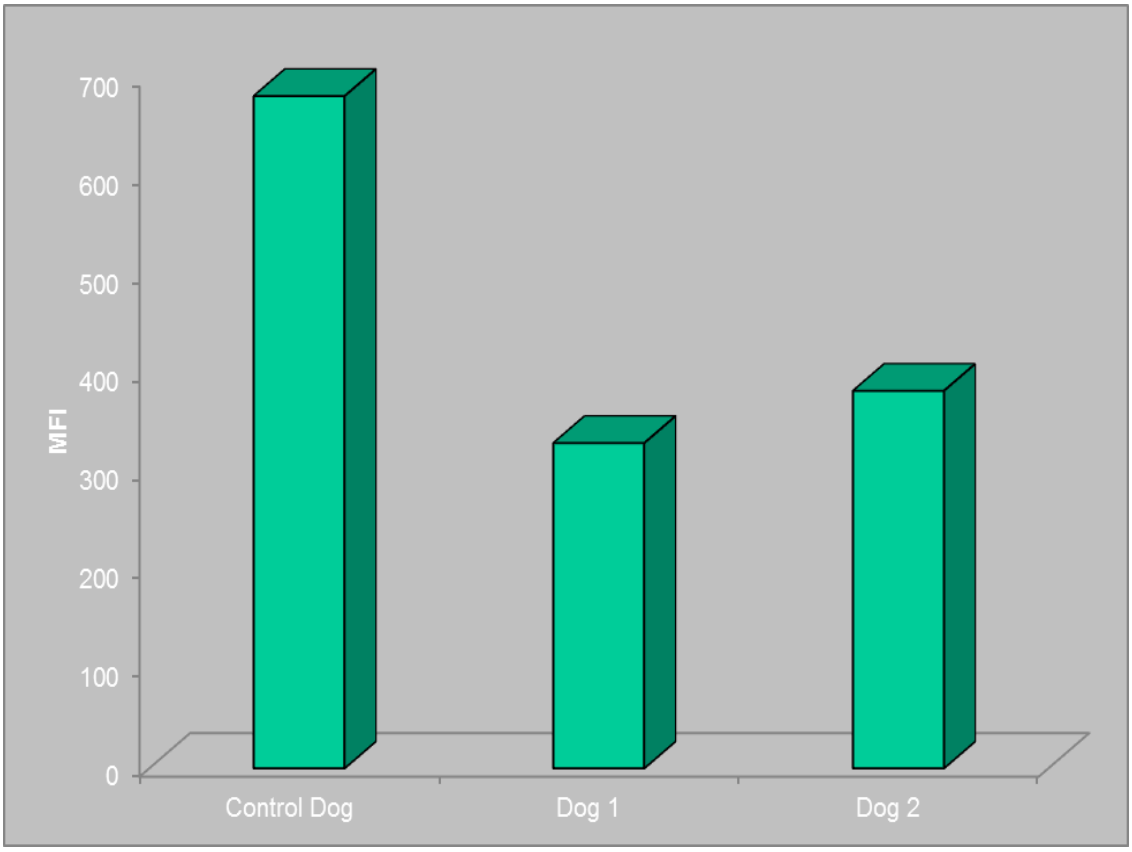

Figure 7

IL-2 expression in activated T-cells from 3 dogs, with one dog serving as a control dog and the other two dogs being dosed at standard twice daily immunosuppressive doses of cyclosporine

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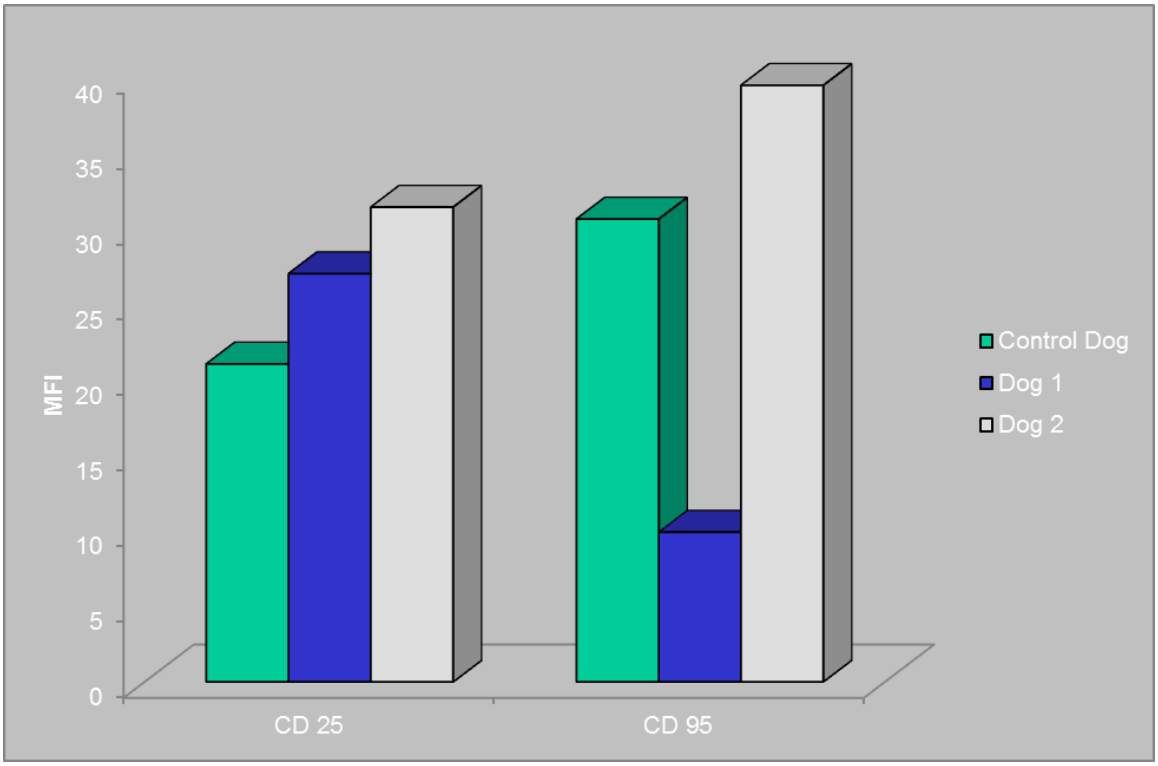

Figure 8

CD25 and CD95 expression in activated T-cells from 3 dogs, with one dog serving as a control dog and the other two dogs being dosed at standard twice daily immunosuppressive dosages of cyclosporine

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CHAPTER II

LITERATURE REVIEW

Cyclosporine is a potent immunosuppressive drug with treatment applications in both human and veterinary medicine for autoimmune diseases and organ transplantation. It is currently being applied to a spectrum of inflammatory and immune-mediated diseases in dogs. It is a cyclic polypeptide derived from the soil fungus *Tolypocladium inflatum*. Cyclosporine was discovered in the Sandoz laboratory by Borel in Switzerland in the 1970s, demonstrating *in vitro* inhibition of human immune reactions as well as prolonging survival of skin grafts (1-3). This data was presented in 1976 at the British Society of Immunology, paving the pathway towards its use as an immunosuppressive agent for organ transplantation (1,2). Experiments then pursued initially in rats which showed prolonged survival of heart allografts and then later in dogs which showed prolonged survival of renal allografts (1). Cyclosporine A was first described in human medicine by Calne in 1978 to prevent organ rejection in patients receiving renal allografts (2,3). Once these results were shown to be repeatable, cyclosporine became and still remains a cornerstone of immunosuppression in human organ transplant medicine (4,5). Cyclosporine was approved by the U.S. Food and Drug Administration in 1983 for the treatment and prevention of transplant rejection in human medicine (4). Novartis Animal Health received FDA approval in 2003 for oral cyclosporine capsules marketed as Atopica® for the treatment of atopic dermatitis in dogs.

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Oral Cyclosporine Formulations

Two oral formulations of cyclosporine exist. The initial formulation of cyclosporine was a vegetable-oil based preparation called Sandimmune®. Sandimmune® demonstrates significant variation in absorption from patient to patient, having wide intra-individual and inter-individual differences in pharmacokinetics. Because of this, a newer formulation was introduced into the human market by Novartis in 1996. The newer formulation is an ultramicronised formulation which forms a microemulsion upon contact with aqueous fluids and has a much more consistent and predictable absorption (6). The ultramicronised formulation is estimated to have as much as 50% better absorption than Sandimmune, with lower intra-individual variability. Novartis manufactures the approved human ultramicronised formulation product (Neoral®) as well as the veterinary approved ultramicronised formulation product (Atopica®). There are also generic formulations available, both as the ultramicronised formulation and the non-ultramicronised formulation. The generic ultramicronised formulations demonstrate equivalent product bioavailability as the FDA approved product, but only the FDA approved veterinary product (Atopica®) has undergone extensive pharmacokinetic testing in dogs to demonstrate its clinical utility and therefore the recommended product. Because of the poor absorption and extreme variability of the non-ultramicronised Sandimmune® preparation, it is not recommended in dogs.

Mechanism of Action

With antigenic stimulation (either endogenous or exogenous) comes antigen presentation and initial cell surface signaling to T-lymphocytes. This signaling begins a cascade of cellular events, starting with antigen binding to CD3 receptors on the surface

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of T-lymphocytes. This interaction causes an increase in intracellular calcium and the activation of calcineurin. Activated calcineurin then dephosphorylates inactive nuclear factors of activation of T-cells (NFAT), leading to NFAT translocation into the nucleus and the intra-nuclear transcriptional gene upregulation of several important cytokines including IL-2, IL-4, TNF-α, and INF-γ (4,7,8). IL-2 production plays a key role in the activation and proliferation of T-lymphocytes. IL-2 receptors on T lymphocytes are composed of three trans-membrane proteins consisting of α (CD-35), β (CD122), and γ (CD132). Once the IL-2 receptors are stimulated, they initiate a feed forward mechanism accelerating the immune response (9). Both Th1 and Th2 cells demonstrate increased proliferation in response to IL-2.

Cyclosporine is a calcineurin inhibitor, thereby modulating the adaptive immune system and specifically inhibiting T-cell function, potently inhibiting cell-mediated immunity with a relatively less effect on humoral immunity (10,11). Calcineurin inhibitors such as cyclosporine begin their biological effects by binding intracellular proteins called cyclophilins. The most abundant cyclophilin found in T lymphocytes is cyclophilin A. The binding of cyclosporine to cyclophilin A facilitates the complex's affinity for calcineurin. When this complex binds to calcineurin, inhibition of calcineurin occurs by inhibiting its phosphatase activity, thus preventing its binding and the subsequent activation of NFAT (4). This leads to decreased cytokine gene expression, with the most notable cytokine being IL-2. Other cytokines shown to have decreased expression in response to exposure to cyclosporine include IL-4, INF-γ, and TNF-α. By decreasing IL-2 expression in CD4+ Th1 cells, proliferation and activation of both T helper and T-cytotoxic lymphocytes are inhibited and the immune response is blunted.

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Pharmacokinetics

Absorption

With oral dosing of cyclosporine, absorption occurs through the gut epithelium in the small intestine. The Sandimmune® formulation has an extremely variable absorption profile, being dependent on factors such as bile flow and the presence or absence of food and gastrointestinal motility (12). The newer ultramicronised formulation does not depend on biliary action nor enzymes or small intestinal secretions for absorption, leading to a much more consistent and predictable absorption (12). The ultramicronised formulation is estimated to have up to as much as 50% better absorption than Sandimmune, with lower intra-individual variability. In dogs dosed with Atopica®, bioavailability is, however, still variable and can range from 23 to 45 percent (13).

One study demonstrated a 22 percent decrease in bioavailability as well as an increase in variability of individual blood concentrations when oral Atopica® was given to dogs with food, and recommendations were made to administer Atopica® two hours before or after feeding (14). A subsequent study evaluated the clinical response in dogs with canine atopic dermatitis treated with Atopica®, to determine whether dosing with food or not made a difference. In this study, there was not a significant difference in clinical efficacy between dogs receiving cyclosporine with food compared to those that did not receive cyclosporine with food (15).

P-glycoprotein is a transporter protein located within the brush border of the enterocytes within the gut epithelium which is able to pump a wide variety of xenobiotics out of the cell and back in to the gut lumen. Cyclosporine is one drug that is transported by P-glycoprotein. A recent study of six dogs (three normal dogs and three dogs with P-

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glycoprotein deficiency) did not show a difference in cyclosporine pharmacokinetics when administered orally as well as intravenously, suggesting that intestinal P-glycoprotein levels do not significantly affect intestinal absorption of cyclosporine in dogs (16). This differs from studies in human medicine, where intestinal P-glycoprotein levels were shown to play a significant role in first-pass metabolism, presumably being a rate-limiting step in absorption (17).

In one study, cimetidine, an H_2-receptor antagonist, given concurrently with oral cyclosporine (Neoral®), caused a significantly longer time until maximal blood cyclosporine concentrations in healthy dogs as compared to those dogs not receiving cimetidine (18). In dogs concurrently treated with cimetidine, there was not however a concurrent increase in the overall maximum concentration of cyclosporine. This suggested a cimetidine-associated altered and prolonged absorption of cyclosporine from the gastrointestinal tract.

Distribution

Cyclosporine has an increased binding affinity for red blood cells as well as plasma lipoproteins. Once in systemic circulation, cyclosporine distributes widely, accumulating in the skin, liver, kidneys, and fat of dogs (12). Tissue levels exceed levels in serum by a factor of 3 to 14 (12). Peak blood concentration times are variable, but usually occur around 2 hours after oral administration of Atopica® (14,19). Blood levels then fade away over the dosing interval.

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Metabolism

Cyclosporine is extensively metabolized by hydroxylation and/or N-demethylation, with many different metabolites being produced (20). Metabolism occurs in the liver, small intestine, and kidneys, with the liver being the major site of metabolism. In dogs, hepatic metabolism is extensive and occurs quickly, with 70 to 100% of the drug being metabolized within 30 minutes in one study (12,20). In the liver, the P-450 3A microsomal enzymes serve as the key pathway of metabolism. The microsomal enzymes in the small intestine are much more variable and slower in their metabolism of cyclosporine. In one study, the small intestine metabolized approximately 34% of what was metabolized by the liver (20). The major metabolites produced by the liver in dogs include the 9 γ-hydroxylated cyclosporine metabolite, 4 N-desmethylated metabolite, 1-β-(8´) hydroxylated metabolite, and 1-β-1-ε-cyclized metabolite (20).

Drugs known to decrease the metabolism of cyclosporine in humans by inhibiting the hepatic P-450 enzyme system, leading to increased cyclosporine blood concentrations, include allopurinol, amiodarone, colchicine, bromocriptine, calcium channel blockers (such as diltiazem), cimetidine, cisapride, danazol, digoxin, doxycycline, erythromycin, fluconazole, flavonoids in grapefruit juice, ketoconazole, itraconazole, metoclopramide, omeprazole, sertraline, verapamil, and high dose methylprednisolone (12, 13, 21). Drugs known to decrease blood concentrations of cyclosporine in humans through either induction of enzymes involved in the metabolism or increased excretion of cyclosporine include ciprofloxacin, nafcillin, rifampin, phenobarbital, phenytoin, terbinafine, and trimethoprim-sulphadimidine (13).

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In dogs, drugs given concurrently with cyclosporine to decrease cyclosporine dosages include ketoconazole and fluconazole. Ketoconazole decreases oral cyclosporine requirements by as much as 75 percent, depending on the dose of ketoconazole administered (22). Fluconazole has been shown to significantly reduce the oral cyclosporine dosage needed to maintain target trough concentrations by between 30 to 50% (23,24). With concurrent fluconazole and cyclosporine, significant increases in cyclosporine blood concentrations are seen, suggesting that adjusted doses may be necessary when dogs are treated with both drugs (24). High dose powdered whole grapefruit has been shown to increase the cyclosporine blood concentration in dogs and has the potential to reduce orally administered cyclosporine requirements, through the proposed mechanism of furanocoumarins in grapefruit juice inhibiting P-450 3A microsomal intestinal enzymes. In one study with experimentally induced diabetes in dogs, there was an accelerated metabolic clearance of cyclosporine under hyperglycemic conditions.

Excretion

Most cyclosporine metabolites are excreted through the biliary system, with minimal renal excretion (13).

Adverse Effects

Many adverse effects are associated with cyclosporine therapy in dogs. In a multisite, placebo-controlled field study safety analysis, 265 dogs were administered either placebo or Atopica® at a dosage of 5 mg/kg/day, with treated dogs receiving Atopica® for a total of 4 months. The following were the most common side effects seen

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in dogs receiving Atopica®: vomiting (30.9%), diarrhea (20.0%), persistent otitis externa (6.8%), urinary tract infections (3.8%), anorexia (3.0%), lethargy (2.3%), gingival hyperplasia (2.3%), and lymphadenopathy (2.3%). Other less common clinical signs noticed included constipation, flatulence, Clostridial organisms in the feces, nausea, regurgitation, polyuria/ploydipsia, strong urine odor, proteinuria, pruritus, erythema/flushed appearance, pyoderma, sebaceous adenitis, crusty dermatitis, excessive shedding, coarse coat, alopecia, papillomas, histiocytoma, granulomatous masses or lesions, cutaneous cysts, epulis, benign epithelial tumors, multiple hemangiomas, raised nodules on pinnae, seizures, shaking/trembling, hind limb twitching, panting, depression, irritability, hyperactivity, becoming quieter, increased light sensitivity, reluctance to go outside, weight loss, and hepatitis. The most common clinicopathology changes noticed during Atopica® therapy included an elevated creatinine (7.8%), hyperglobulinemia (6.4%), hyperphosphatemia (5.3%), hyperproteinemia (3.4%), hypercholesterolemia (2.6%), hypoalbuminemia (2.3%), hypocalcemia (2.3%), and an elevated BUN (2.3%). Other clinicopathological changes were less common, and included hypernatremia, hyperkalemia, elevated ALT, elevated ALP, hypercalcemia, and hyperchloremia.

In other studies published throughout the literature, the most notable adverse effects of cyclosporine are gastrointestinal in nature, including diarrhea, vomiting/nausea, and anorexia (25). These side effects are seen across a range of dosages, with an increased incidence seen at higher dosages (25). Dermatological side effects include hirsutism, coat shedding, gingival hyperplasia, gingival eruption cysts in neonatal dogs, cutaneous papillomatosis hyperplasia, hyperkeratosis of footpads, psoriasiform-lichenoid-like dermatitis, and lymphoplasmatoid dermatitis (26-30). Clinicopathological

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abnormalities seen in conjunction with cyclosporine therapy which were not reported in the field study safety analysis include lymphopenia, eosinopenia, transient thrombosis, anemia, and leukocytosis (25). Concurrent infections have been documented to occur during cyclosporine therapy, including acute toxoplasmosis, bacterial respiratory infection, bacterial urinary infection, purulent pericarditis, *Neospora caninum* central nervous system infection, demodicosis, pyoderma, pyelonephritis, pyometra, and septic arthritis (28, 31, 32). Other adverse cyclosporine reactions reported in the literature include hepatotoxicity, defective hepatic protein synthesis, inhibition of insulin release, lameness, lethargy, nephropathy, increase in peripheral insulin resistance, transient hypoalbuminemia, anaphylactic reaction, tremors, angioedema, emergence of neoplasia, and cyctic nodules of unknown etiology located in the pericardium and diaphragm (6,33). Recent findings have also suggested cyclosporine may increase platelet procoagulant activity in dogs (34). In a single experimental study in dogs evaluating the effects of cyclosporine on blood pressure, results indicated that cyclosporine increased blood pressure through activation of the renin-angiotensin-aldosterone system (35).

Precautions

The safety and efficacy of cyclosporine has not been evaluated in dogs less than 6 months of age or in dogs less than 4 pounds in weight, and the drug should therefore not be used or be used cautiously in these patients. Because of the dampened immune system associated with cyclosporine therapy, vaccine efficacy may be impacted. Modified live vaccines are not recommended with concurrent cyclosporine therapy because of the potential for reactivation of the pathogen (13, 36). Cyclosporine is not for use in breeding, pregnant, or lactating dogs. Malignancies, including lymphoma, have occurred

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in conjunction with concurrent use of cyclosporine, and it is therefore contraindicated in dogs with a history of neoplasia. Nephrotoxicity and renal dysfunction have rarely been reported in the veterinary literature, although renal disease is a common adverse effect in people during cyclosporine therapy. Nephrotoxicity has not been reported in dogs receiving therapeutic dosages, but has occurred with prolonged significantly increased serum blood drug concentrations (> 3000 ng/ml) (6).

Measurement of Cyclosporine Blood Levels

The assay used to measure cyclosporine blood levels as well as the sample type must be considered when interpreting results. Plasma values will be lower than whole-blood values due to the concentration of cyclosporine in erythrocytes and leukocytes. While either whole blood or plasma cyclosporine concentrations can be measured, most laboratories recommend measuring whole blood concentrations. Cyclosporine concentrations can be measured by high-pressure liquid chromatography (HPLC), fluorescence polarization immunoassay (TDx method), and specific monoclonal antibody radioimmunoassay (RIA) methods. HPLC measures only the parent drug, and does not measure cyclosporine metabolites. Both TDx and RIA methods measure parent drug as well as metabolites, and blood cyclosporine concentrations will therefore be higher compared to the same sample analyzed using HPLC. Although HPLC is labor intensive and not routinely offered for patient monitoring, it is considered the gold standard for measuring cyclosporine blood concentrations. TDx and RIA are most often clinically employed, with the laboratory providing the assay typically providing recommendations regarding ideal target blood drug levels.

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Therapeutic Drug Monitoring

Because gastrointestinal absorption is variable in patients receiving cyclosporine, monitoring blood concentrations to ensure therapeutic dosing is often recommended. However, there are only a limited number of clinical studies in veterinary medicine which address target therapeutic cyclosporine blood concentrations. Pharmacokinetic studies demonstrate a wide variability in cyclosporine pharmacokinetics, with differing blood levels in dogs administered the same oral dosage (14, 37-39). Monitoring blood levels is a way to attempt to optimize the dose for individual patients. Current recommendations evolved from initial work with organ transplantation in dogs (40-41). Initial recommendations for immunosuppression centered on achieving a minimum target trough blood drug concentration of 500 ng/ml, with blood collected just before the next oral dose (6).

While human medicine initially utilized measurement of trough concentrations, laboratories have since changed to using peak concentrations as well as pharmacodynamic monitoring. Trough concentrations did not adequately predict sufficient immunosuppression, and clinical outcomes were not acceptable (3). Drug concentrations at which nephrotoxicity and hepatotoxicity occurred were identified using trough levels, but not the ideal levels to induce and maintain immunosuppression. Further work identified that the concentration measurement of the area under the curve (AUC) for hours 0-12 in the dosing interval provided a much more reliable and successful indicator of clinical immunosuppression, with patient outcome significantly improving. To calculate the AUC for hours 0 to 12, multiple blood samples had to be collected. Based on the idea that absorption of the Neoral® preparation is more rapid and

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complete during the first four hours after dosing, the AUC for hours 0 to 4 were investigated as a simpler alternative to the AUC 0 to 12 hours. There was a close correlation between the two methods, with fewer samples needed for the AUC for 0 to 4 hours. Further work in human medicine identified that the peak drug concentration, or the concentration in plasma 2 hours post-dosing, approximated the patient outcome and success as that seen when using the concentration measurement of the area under the curve for hours 0-4, while trough levels correlated poorly with the area under the curve for hours 0-4. These findings apply to the Neoral® formulation, as the peak blood concentration reliably occurs between 1 and 2 hours, and the 2 hour peak captures virtually all patients. In contrast, these findings cannot be applied to the Sandimmune® formulation, as the peak measurement varies between 2 and 6 hours, nor for generic versions of Neoral®, as testing has not validated the peak measurement and studies have shown decreased efficacy with generic formulations compared to the Neoral® formulation. With the peak measurement only requiring a single blood collection, it has now become the single best blood concentration measurement for use during organ transplantation when monitoring cyclosporine pharmacokinetics using the Neoral® formulation.

In veterinary medicine, measurement of trough cyclosporine concentrations prevailed for many years based on initial work done in renal transplant studies. Current recommendations from laboratories offering therapeutic drug monitoring in dogs receiving cyclosporine therapy typically involve the evaluation of both peak and trough levels. Exact recommendations depend on the laboratory reference ranges as well as the assay used to measure cyclosporine concentrations.

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Pharmacodynamics

Pharmacokinetic studies provide evidence that cyclosporine blood concentrations in patients are within the estimated target therapeutic range recommended by referral laboratories. For some patients, this estimation may accurately predict immunosuppression, while for others, there may not be clinical efficacy despite achieving a target therapeutic range of blood cyclosporine concentrations, or there may be adverse side effects. This variability in response to comparable blood concentrations may be attributed to individual pharmacological responses to cyclosporine between patients. A wide variability in the relationship between clinical efficacy and 'therapeutic' drug concentrations is seen in clinical practice (42). For this reason, several pharmacodynamic assays have now been developed in human medicine in an attempt to better estimate the amount of cyclosporine needed for the treatment of disease and prevention of organ rejection in transplant patients as well as to minimize side effects.

Pharmacodynamic assays investigate a drug's effect on target cells. Several pharmacodynamic biomarkers of immunosuppression have been studied in human medicine, including lymphocyte proliferation, enzyme activity (calcineurin), lymphocyte surface antigens, and intracellular cytokine quantification. Through pharmacodynamic monitoring, human studies have shown individually distinct degrees of calcineurin inhibitor sensitivity in patients. Pharmacodynamic monitoring shows great promise for optimizing cyclosporine therapy as well as delivering individualized therapy.

Few such pharmacodynamic studies are found in the veterinary literature. One study demonstrated suppression of lymphocyte proliferation via flow cytometry after the use of topical cyclosporine for the treatment of keratoconjunctivitis sicca in dogs. In

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dogs treated with 2 percent topical ocular cyclosporine, there was decreased lymphocyte proliferation by one month and a statistically significant reduction at three months (43). In more recent veterinary studies utilizing cytokine analysis, quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) assays are being used to measure cellular messenger ribonucleic acid (mRNA) expression. One recent study investigated the effects of cyclosporine on activated canine mononuclear cells *in vitro,* and demonstrated a concentration-dependent reduction in IL-2, IL-4, and IFN-γ mRNA expression via QRT-PCR (44). Another recent study evaluated cellular IL-2 and IFN-γ mRNA expression within lesional biopsies from German shepherd dogs with anal furunculosis before and after therapy with oral Atopica® for 4 weeks duration, and showed an initial significant increase in cytokine mRNA expression in diseased tissue as compared to control tissues. Diseased tissues which were evaluated post-treatment with cyclosporine demonstrated a significant reduction in IL-2 expression, and a lesser decrease in IFN-γ mRNA expression. A correlation was not found between post-treatment T-cell cytokine mRNA expression and the severity of disease in dogs with residual disease (45). There is clearly a need to develop and investigate pharmacodynamic assays that have the potential to more accurately predict the immunosuppressive effects of cyclosporine in the individual patient, and that allow dose adjustments that improve clinical outcomes.

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CHAPTER III

PHARMACODYNAMIC MONITORING OF CANINE T-CELL CYTOKINE RESPONSES TO ORAL CYCLOSPORINE

Introduction

Cyclosporine is a potent immunosuppressive drug with treatment applications in both human and veterinary medicine. Cyclosporine specifically targets T-cell function, ultimately inhibiting calcineurin within the cell (1-4).

Inhibition of calcineurin prevents activation of nuclear factor of activated T-cells, which regulates the production of several important cytokines including interleukin-2 (IL-2), interleukin-4 (IL-4), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) (5,6). Decreased production of IL-2 is thought to be the main cause of the cyclosporine's immunosuppressive effects (7-9).

Cyclosporine has been used to treat many inflammatory and immune-mediated diseases in the dog (4,10-17). The lipophilic nature of cyclosporine affects drug bioavailability, which has made the use of the drug challenging to clinicians. The oral bioavailability of cyclosporine is highly unpredictable, with wide ranges of blood concentrations seen in dogs receiving similar dosages (13,14,18-20). Because of this variability, therapeutic drug monitoring of cyclosporine blood concentrations is usually

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recommended if a favorable response is not initially achieved (3,20,21). Interpretation of cyclosporine blood concentrations in the individual dog, however, is difficult. There is limited evidence in the veterinary literature correlating cyclosporine blood concentrations with clinical response for many inflammatory and immune-mediated diseases. Target therapeutic ranges are often anecdotal, and recommendations vary considerably among references with regards to desired blood cyclosporine concentrations (20,22,23). Clinical response is often the most reliable means of assessing immunosuppression in treated animals. The relationship between clinical response and drug blood concentrations also appears to be highly variable, with a given cyclosporine concentration being effective in some animals but not others (13,24). Additionally, for some conditions, positive clinical response is often apparent at drug concentrations well below those typically recommended for immunosuppression (19). For this reason, while cyclosporine is marketed for the treatment of canine atopic dermatitis, therapeutic drug monitoring is not routinely recommended for this condition (10,25,26). Based on the unpredictable relationship between blood drug concentrations and clinical response, there is clearly a need to find a better method for monitoring the immunosuppressive effects of cyclosporine, particularly in animals with life-threatening conditions.

Pharmacodynamic assays evaluating selected biomarkers within the immune system are advocated in human medicine to help determine drug efficacy and make dosing recommendations (27). For cyclosporine, these assays include quantification of drug target enzymes (calcineurin), cytokines (IL-2 and IFN-γ), and markers of lymphocyte proliferation or activation (CD25 and CD95) (28-32). These biomarkers are involved in normal and pathological immune responses, so their inhibition serves as a

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quantitative, objective surrogate for inhibition of immune function. Pharmacodynamic monitoring of biomarkers of immunosuppression offers a more individualized approach to immunosuppressive therapy when blood concentrations do not correlate well with clinical response in dogs.

Pharmacodynamic monitoring of biomarkers such as cytokines that are indicative of immunosuppression has yet to be thoroughly explored in the dog. Our study uses flow cytometry to assess the effects of cyclosporine on canine T-cell production of three cytokines, IL-2, IL-4, and IFN-γ, that have potential utility as biomarkers of immunosuppression. These cytokines were evaluated at two drug dosages: a high dosage adjusted to attain trough blood concentrations that are expected to be reliably immunosuppressive, and the much lower dosage used to treat atopic dermatitis.

Materials and Methods

Dogs

Seven healthy intact female Walker hounds were used for the project. Health screening was performed prior to the study and included physical examination, complete blood count, serum biochemistry profile, urinalysis, fecal flotation, and heartworm testing. All animals were cared for according to guidelines approved by the Mississippi State University Institutional Animal Care and Use Committee. The animal facilities and program at Mississippi State University are accredited by the American Association for Accreditation of Laboratory Animal Care.

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Study Design

Blood was drawn for baseline flow cytometric measurement of T-cell expression of the cytokines IL-2, IL-4, and IFN-γ in all dogs. Baseline samples were assessed in duplicate and averaged. Dogs were then given two different dosages of cyclosporine, a high dosage and then, 14 days after completion of the high dosage study, a much lower dosage. For the high dosage study, microemulsified cyclosporine[a] was administered at a starting oral dosage of 10 mg/kg every 12 hours. Dosages were adjusted to achieve a trough blood cyclosporine concentration (measured 12 hours after dosing) of at least 600 ng/mL, a trough concentration that has previously been established to attain sufficient immunosuppression to prevent organ rejection in canine transplant recipients (23, 24, 33). One dog attained this trough concentration at the initial starting dosage, while the other six required dosage increases. On day 8 of cyclosporine administration, after trough drug concentrations of at least 600 ng/mL had been confirmed in all dogs, blood was drawn eight hours after dosing and processed for repeat cytokine measurement. This time point was optimized in an earlier unpublished pilot study. Any cytokine not shown to be suppressed by high dosage cyclosporine was not subsequently analyzed in the low dosage cyclosporine phase of testing. Cyclosporine was then discontinued and, after a two week washout period, cytokine expression was again measured to ensure return to baseline levels before commencing the subsequent low dosage study. For the low dosage study, cyclosporine was administered at the labeled dosage for atopic dermatitis (5 mg/kg orally every 24 hours), and cytokine levels were again measured on day 8 of drug administration. Trough cyclosporine blood concentrations (measured at hour 24, immediately prior to the next dosage) were also measured by HPLC on day 8.

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Cytokine Analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples collected from each dog utilizing density gradient centrifugation with Histopaque®-1077[b], similar to a previously described method (34). The isolated PBMC were reconstituted in complete media with an equal volume to that of the original blood sample in order to approximate the cell density present for each dog. RPMI 1640 medium[c] supplemented with 10% heat-inactivated fetal bovine serum[d], GlutaMAX[e], 1 mM sodium pyruvate[f], 55 µM 2-mercaptoethanol[g], 75 µg/mL gentamicin[h], 2 mM HEPES, and 1 µL/mL MEM Amino Acids Solution without L-glutamine[i] was used for cell culture.

Cytokine expression levels in peripheral blood T-cells were analyzed as described previously (35). Briefly, half of the isolated PBMC were activated with 12.5 ng/mL phorbol-12-myristate-13-acetate[j] and 0.8 µM ionomycin[k], and the other half remained untreated. All cells were then incubated for 12 hours at 37°C in a 5% CO_2 incubator. Brefeldin A at a concentration of 1 µg/mL was added to each well with two hours remaining in the incubation to stop cytokine secretion from T-cells. Cells were then collected and washed with phosphate buffered saline. All samples were incubated with a fluorescein isothiocyanate-conjugated anti-CD3 monoclonal antibody[l] for 30 minutes at room temperature in the dark, and fixed and permeabilized using the BD Cytofix/Cytoperm Plus Kit[m] following the manufacturer's instructions. Cells were then incubated with appropriate antibodies for labeling of the cytokines of interest, either R-phycoerythrin-conjugated anti-bovine IL-4[n] (cross-reactive with canine IL-4 (36)), R-phycoerythrin-conjugated anti-bovine IFN-γ[o] (cross-reactive with canine IFN-γ (36)), or

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biotinylated anti-canine IL-2[p], for 30 minutes at room temperature in the dark. The anti-canine IL-2 antibody has been shown to be canine specific by in-house testing performed by the manufacturer. IL-2 samples had an additional 20 minute incubation with R-phycoerythrin-conjugated streptavidin[q] at room temperature in the dark. A final wash was applied to these samples, and all cells were then resuspended in phosphate buffered saline with 0.2% bovine serum albumin.

Cell staining was evaluated using a BD FACSCalibur Flow Cytometer and analyzed with CellQuest Pro software[r]. Forward scatter and side scatter were used to identify and gate lymphocytes based on their size and granularity. A second gate selected CD3-positive cells. Cytokine levels were measured from cells that were located in both gates, and 10,000 total events per sample were collected. Mean fluorescence intensity (MFI) values with single histogram statistics were used for assessment of cell staining. Negative controls included unactivated samples and isotype controls.

Cyclosporine Blood Concentrations

Blood cyclosporine concentrations were measured via HPLC, with the trough concentration taken 12 hours (high dose phase) or 24 hours (low dose phase) after the previous oral dose. Blood was collected into EDTA anticoagulant tubes, and analyzed within 24 hours using a modification of the HPLC assay used for therapeutic drug monitoring at the University of California at Davis (John D Patz, personal communication, 2008). Standard curves were made using blank EDTA anticoagulated whole blood and cyclosporine[s] at 0, 200, 400, 800, and 1600 ng/mL. The extraction procedure used 2 mL of whole blood sample mixed with 6 mL of a protein precipitating solution consisting of 5% zinc sulfate, 20% acetonitrile, 30% methanol, and water. This

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solution also contained 400 ng/mL of cyclosporine D[t] as an internal standard. After vortexing and subsequent centrifugation, the supernatant was added to a prepared C18 solid phase extraction column[u]. After filtration of the sample by vacuum, the solid phase extraction column was washed with 5 mL of 50% acetonitrile followed by 1 mL of 100% methanol for elution of the drug. To the methanol eluent 200 µL of water was first added followed by 300 µL of hexane. This fluid was then vortexed and centrifuged with 200 µL of the aqueous layer extracted and placed in vials for subsequent HPLC analysis using a sample injection volume of 100 µL.

An 1100 HPLC system[v] with degasser, quaternary pump, autoinjector, and diode array detector was used. The reverse phase column was a Phenomenex Luna 5u C18(2) with guard cartridge. The column was maintained at 75 degrees C. A gradient mobile phase at 1 mL per minute was used consisting of acetonitrile (A) and water adjusted to pH 3.1 (B). The gradient was initially 65% A and 35% B that transitioned linearly over 5 minutes to 70% A and 30% B. This ratio was then held for 15 minutes. A 5 minute re-equilibration time followed each injection. Detection was at 200 nm. The retention time for cyclosporine was 4.2 min versus 5.6 minutes for cyclosporine D. The assay was linear over the standard curve range of 200 to 1600 ng/mL with an r^2 of 0.9889. Based on the method described by Taylor, the limit of quantification (LOQ) was 189.93 ng/ml and the limit of detection (LOD) was 56.97 ng/ml (37). The assay had an average coefficient of variation of 6.7% (range 3.7 to 9.9%) and an average accuracy of 94.4% (range 92 to 98%).

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Statistical Analysis

A paired comparison design was used in this study. The Wilcoxon signed rank test was used to compare the average baseline cytokine MFI values to the MFI values after treatment for each cytokine at the high dosage. The Wilcoxon signed rank test was also utilized to compare the average baseline cytokine MFI values to the MFI values after treatment for IL-2 and IFN-γ at the low dosage, to compare the washout cytokine MFI values to the MFI values after treatment for IL-2 and IFN-γ at the low dosage, and to compare the baseline cytokine MFI values to the washout cytokine MFI values for IL-2 and IFN-γ. Analyses were conducted for both cyclosporine dosages for IL-2 and INF-γ while only the effect of high dosage cyclosporine was analyzed for IL-4. The UNIVARIATE procedure in SAS for Windows® version 9.2[w] was used for statistical analysis. *P*-values ≤ 0.05 were considered significant.

The data was analyzed to determine if MFI and cyclosporine concentration were correlated using PROC CORR (SAS for Windows® version 9.2[w]). A separate analysis was conducted for each cyclosporine dosage and cytokine combination.

Results

Cytokine Analysis

Expression of both IL-2 and IFN-γ decreased significantly from baseline values following administration of high dosage cyclosporine (*P* for IL-2= 0.0156, *P* for IFN-γ= 0.0156), and cytokine expression decreased in all 7 dogs for both cytokines (Table 1). In contrast, IL-4 expression following administration of high dosage cyclosporine varied markedly between individual dogs, with no significant difference between baseline

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values and values after treatment (*P*-value of 0.2188). Due to the inconsistent and non-significant changes in T-cell IL-4 expression in dogs administered cyclosporine at a high dosage, this cytokine was not evaluated in the subsequent low dose trial.

Expression of IFN-γ decreased significantly from baseline values (*P*= 0.0156) as well as washout values (*P*= 0.0156) following administration of low dosage cyclosporine, and cytokine expression decreased in all 7 dogs (Table 2). There was not a significant difference between the IFN-γ baseline values and the IFN-γ washout values (*P*= 0.9375). Expression of IL-2 at the low cyclosporine dosage had more variability in individual dogs compared to expression of IFN-γ at the same dosage, and compared to expression of IL-2 at the high cyclosporine dosage, with only 5 of 7 dogs showing moderately suppressed IL-2 expression on the lower dosage. Expression of IL-2 following administration of low dosage cyclosporine was not significantly different from baseline values (*P*= 0.1094) nor from washout values (*P*= 0.6875). IL-2 washout values were found to be significantly lower than the baseline IL-2 values (*P*= 0.0469).

Cyclosporine Blood Concentrations

The high cyclosporine dosage consisted of a starting dosage of 10 mg/kg every 12 hours, with the 12 hour doses adjusted upwards as needed to ensure trough blood drug concentrations of at least 600 ng/mL. One dog achieved the minimum target trough concentration on the starting dose, while the other 6 dogs needed doses titrated up and had confirmed cyclosporine trough concentrations above 600 ng/mL by day 8. The low cyclosporine dosage was 5 mg/kg every 24 hours. After 8 days of dosing at the low cyclosporine dosage, blood was drawn for measurement of trough concentrations. Each trough concentration was drawn just prior to administration of the next cyclosporine

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dosage. Trough blood cyclosporine concentrations for high dosage cyclosporine ranged from 728 ng/mL to 1330 ng/mL, with a median value of 1005 ng/mL (Table 1). Trough blood cyclosporine concentrations for low dosage cyclosporine ranged from below the level of detection (57 ng/mL) to 145 ng/mL, with all values being below the limit of quantification (190 ng/mL) (Table 2).

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Table 1

Flow cytometric measurement of activated T-cell expression of interleukin-2 (IL-2), IL-4, and interferon-gamma (IFN-γ) before (baseline) and after (day 8) administration of high dosage oral cyclosporine. Accompanying trough blood cyclosporine concentrations during the administration of high dosage cyclosporine are shown.

	High Dosage Cyclosporine									
	IL-2 (MFI)			IL-4 (MFI)			IFN-γ (MFI)			
	Baseline	Post-treatment	Δ from baseline	Baseline	Post-treatment	Δ from baseline	Baseline	Post-treatment	Δ from baseline	Cyclosporine trough concentration (ng/mL)
Median	60	28.9	− 36.4	8.33	11.02	3.03	29.9	12.9	−19.7	1,005
Range	(51.1–84.5)	(23.6–34.1)	(−54.3–−22.3)	(6.29–10.62)	(7.63–11.81)	(−0.41–4.73)	(19.1–36.3)	(6.9–14.2)	(−23.3–−7)	(728–1,330)

Table 2

Flow cytometric measurement of activated T-cell expression of interleukin-2 (IL-2) and interferon-gamma (IFN-γ) before (baseline), after washout, and after day 8 administration of low dosage oral cyclosporine. Accompanying trough blood cyclosporine concentrations during the administration of low dosage cyclosporine are shown.

	Low Dosage Cyclosporine										
	IL-2 (MFI)					IFN-γ (MFI)					
	Baseline	Washout	Post-treatment	Δ from baseline	Δ from washout	Baseline	Washout	Post-treatment	Δ from baseline	Δ from washout	Cyclosporine trough concentration (ng/ml)
Median	60	54.8	53.9	−14.5	−1.6	29.9	28.1	23.4	−6.2	−6.6	63
Range	(51.1–84.5)	(47.1–78.4)	(45.5–65.3)	(−30.6–12.9)	(−24.5–7.9)	(19.1–36.3)	(18.9–50.6)	(14.1–25.5)	(−14.1–−4.4)	(−27.2–−3.4)	(BDL to 145)

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Cyclosporine Blood Concentration and Cytokine Correlation

IL-2 MFI was not significantly correlated with cyclosporine concentration for either high dosage (r=-0.16, p=0.7317) or low dosage (r=-0.22, p=0.6293) cyclosporine administration. Similarly, IFN-γ MFI was not significantly correlated with cyclosporine concentration for either high dosage (r=-0.41, p=0.3619) or low dosage (r=-0.13, p=0.7858) cyclosporine administration.

Discussion

Our study has established that activated T-cell expression of two cytokines, IL-2 and IFN-γ, is reliably suppressed in dogs receiving cyclosporine at established immunosuppressive dosages, and that these cytokines are therefore strong candidates for development as biomarkers of immunosuppression for subsequent pharmacodynamic assays. There is clearly a need to develop assays that have the potential to more accurately predict the immunosuppressive effects of cyclosporine in the individual dog, and that allow dosage adjustments that improve clinical outcomes. Such assays have the potential to provide individualized therapy in dogs suffering from severe and life-threatening immune mediated diseases, as blood concentrations do not always correspond to clinical response (19).

Our study utilized flow cytometry to evaluate T-cell expression of cytokines to quantitate the biological effects of cyclosporine on T-cells in healthy dogs. Many human studies include flow cytometric analysis of T-cell cytokine and surface molecule expression as biomarkers when investigating the immunosuppressive effects of drugs such as cyclosporine (28, 38–40). Few such studies are found in the veterinary literature, though one study did demonstrate suppression of lymphocyte proliferation via flow

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cytometry after the use of topical cyclosporine for the treatment of keratoconjunctivitis sicca in dogs (41). Cytokine analysis in veterinary immunosuppression research more commonly utilizes quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) assays to measure cellular messenger ribonucleic acid (mRNA) expression (42-46). One recent study investigating the effects of cyclosporine on canine mononuclear cells *in vitro,* for example, showed a concentration-dependent reduction in IL-2, IL-4, and IFN-γ mRNA expression via QRT-PCR (43). Another recent study evaluated cellular IL-2 and IFN-γ mRNA expression within lesional biopsies from German shepherd dogs with anal furunculosis before and after therapy with cyclosporine, and demonstrated a significant reduction in IL-2 expression after treatment (42). Previous *in vitro* work in our laboratory established the utility of flow cytometric techniques for monitoring the effects of cyclosporine on activated T-cell expression of cytokines and surface molecules (35), and we therefore elected to use this method for our current study. This study has established that activated T-cell expression of the cytokines IL-2 and IFN-γ shows promise as a potential biomarker of immunosuppression for pharmacodynamic monitoring.

Although activated T-cell expression of IL-2 and IFN-γ was reliably suppressed by high dosage cyclosporine therapy in the dogs in our study, expression of IL-4 was not similarly affected. We included IL-4 in our high dosage study because previous work in our laboratory showed that activated T-cell expression of IL-4 was suppressed by *in vitro* exposure to cyclosporine (35), and because IL-4 was used in human studies as a biomarker of immunosuppression (28). Because IL-4 did not reliably suppress during the high dosage cyclosporine phase of our current study, we concluded that further

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exploration of IL-4 as a potential biomarker was not warranted, and elected not to include this cytokine in the low dosage phase of our study. Failure of T-cell expression of IL-4 to suppress after administration of oral cyclosporine may be due to a dog-specific difference in *in vivo* responses compared to *in vitro* responses. One *in vivo* study of cyclosporine in dogs showed a drug-associated reduction in IL-2 but not in antibody (IgA, IgG, and IgM) production, a presumed Type 2 helper T-cell-dependent response which utilizes IL-4 for up-regulation (47). Although the researchers did not directly measure IL-4, they concluded that cyclosporine had a negligible effect on humoral immunity (47).

In our study, we used two extremes of cyclosporine dosing, a high dosage adjusted upwards as needed to meet target trough blood concentrations of 600 ng/mL, and a much lower fixed dosage. We elected to use our high dosage protocol in order to be as certain as possible that the treated dogs were reliably immunosuppressed, and thereby establish the degree of suppression of cytokine biomarkers associated with immunosuppression. With the high dosage protocol, activated T-cell expression of IL-2 and INF-γ was significantly reduced, consistent with findings in pharmacodynamic studies in people (28, 29, 48). The lower fixed cyclosporine dosage utilized in our study is the labeled dosage approved by the United States Food and Drug Administration for the treatment of canine atopy (24). The atopy dosage of cyclosporine has not been previously definitively documented to cause significant suppression of the canine immune system, and whether or not this low dosage of cyclosporine can cause clinically relevant immunosuppression remains controversial among veterinary dermatologists. Individual dogs on the atopy dosage of cyclosporine long term have however been anecdotally reported to develop secondary infections, suggesting the possibility that even

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this low dosage can sometimes cause immunosuppression. Our study provides preliminary supportive evidence that low dosages of cyclosporine may have immunosuppressive effects in dogs, in that even the atopy dose used in our low dose phase was associated with a significant decrease in activated T-cell expression of IFN-γ. Remarkably, T-cell expression of both IFN-γ and IL-2 was observed to decrease in individual dogs even when trough blood cyclosporine concentrations were far below published target trough concentrations and were below the limit of quantification, and in two instances, below the level of detection by HPLC. That some dogs showed substantial decreases in T-cell cytokine production despite having extremely low trough drug concentrations suggests that therapeutic drug monitoring of trough blood cyclosporine concentrations as a means of predicting immunosuppression in individual dogs is of questionable reliability. Our low dosage study demonstrated that blood drug concentrations and T-cell suppression were both highly variable among individual dogs treated with the same oral dosage of cyclosporine, supporting the proposition that, in a clinical setting, pharmacodynamic assays may be needed in order to determine individual patient responses to immunosuppressive therapy. Comparison of the cyclosporine concentrations and MFI values for each cytokine did not demonstrate a correlation between the two measures at either dosage. This further calls into question the utility of blood drug concentrations and supports the need for a more individualized, patient specific approach when monitoring cyclosporine therapy in dogs.

One weakness of our study is that, because of the lack of a cross-over design, all dogs received high cyclosporine dosages before subsequently receiving low dosages. A possible residual drug effect following high dosage cyclosporine could have played a role

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in our IL-2 analysis because a statistically significant reduction in values was seen between original baseline and washout values. This effect was not appreciated with the analysis of the IFN-γ data, a difference that could be due to a possible prolonged post-treatment effect of cyclosporine on the expression of IL-2 but not IFN-γ.

Our study was performed in healthy research dogs, with demonstration of suppression of T-cell expression of cytokines in response to oral dosing of cyclosporine. Clinical dosing recommendations cannot be made at this time based on our assay, since we do not as yet know whether cytokine expression corresponds with clinical efficacy in dogs as it does in humans. Our study only incorporated two extreme dosages of cyclosporine, and did not evaluate alterations in activated T-cell expression of cytokines associated with the mid-range cyclosporine dosages that are often used for treating inflammatory and immune-mediated diseases in the dog. Performing similar analyses at cyclosporine dosages between the two dosages used in our study would further clarify the potential clinical utility of flow cytometric measurement of T-cell cytokine expression as a biomarker of immunosuppression. Finally, clinical studies in clinical patients at dosages approximating those typically used for immunosuppressive therapy will help determine this assay's ability to predict immunosuppression and allow for dose adjustments in the individual dog.

Endnotes

[a] Atopica, Novartis Animal Health, Basel, Switzerland

[b] Histopaque®-1077, Sigma Aldrich, St. Louis, MO

[c] RPMI 1640 Medium, 21870-084, Invitrogen, Carlsbad, CA

[d] 10% Heat-inactivated fetal bovine serum, 10438-026, Invitrogen, Carlsbad, CA

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[e] GlutaMAX, 35050, Invitrogen, Carlsbad, CA

[f] Sodium Pyruvate, 11360, Invitrogen, Carlsbad, CA

[g] 2-mercaptoethanol, 21985-023, Invitrogen, Carlsbad, CA

[h] Gentamicin, 15750-060, Invitrogen, Carlsbad, CA

[i] MEM Amino Acids Solution without L-glutamine, 11130, Invitrogen, Carlsbad, CA

[j] Phorbol-12-myristate-13-acetate, P-8139, Sigma Aldrich, St. Louis, MO

[k] Ionomycin, I-0634, Sigma Aldrich, St. Louis, MO

[l] Anti-canine CD3 antibody, MCA1774F, AbD Serotec, Raleigh, NC

[m] BD Cytofix/Cytoperm Plus Kit, Becton Dickinson, San Jose, CA

[n] Anti-bovine IL-4 antibody, MCA1820PE, AbD Serotec, Raleigh, NC

[o] Anti-bovine IFN-γ antibody, MCA1783PE, AbD Serotec, Raleigh, NC

[p] Biotinylated anti-canine IL-2, BAF1815, R&D Systems, Minneapolis, MN

[q] Streptavidin, #60669, Anaspec, San Jose, CA

[r] FACSCalibur Flow Cytometer and CellQuest Pro software, Becton Dickinson, San Jose, CA

[s] Cyclosporine, Sigma-Aldrich, St. Louis, MO USA

[t] Cyclosporine D, Novartis Pharmaceuticals, East Hanover, NJ USA

[u] Varian Bond Elut 1 cc 100mg solid phase extraction column, Varian Incorporated, Walnut Creek, CA USA

[v] Agilent 1100 HPLC system, Agilent Technologies, Santa Clara, CA USA

[w] SAS for Windows® version 9.2, SAS Institute Inc., Cary, NC

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CHAPTER IV

CONCLUSION

Our preliminary work and *in vivo* study documented measurable suppression of selected biomarkers of immunosuppression in T-cells, more specifically the cytokines IL-2 and INF-γ, when T-cells were exposed to cyclosporine. The *in vivo* study documented that high doses of cyclosporine reliably suppressed T-cell expression of both IL-2 and IFN-γ, with suppression of IFN-γ even at the much lower cyclosporine dosage commonly used for atopic dermatitis. During the *in vivo* study, measurable suppression of T-cell cytokine expression was noted to occur even at very low trough blood cyclosporine concentrations that were well below the target immunosuppressive ranges recommended by most reference laboratories, calling into question the ability of pharmacokinetic assays to be the sole technique for assessing the efficacy of cyclosporine therapy. Therapeutic drug monitoring may not necessarily be able to predict the pharmacological effects of the drug on the immune system. There is clearly the need to develop pharmacodynamic assays which can assess the effects of cyclosporine on the immune system during therapy and allow for more accurate dose adjustments to improve clinical outcome.

Although cyclosporine is gaining favor for treating a wide and increasing number of inflammatory and immune-mediated diseases in veterinary medicine, optimal oral dosing protocols in dogs are still unclear. For many dermatologic and mild gastrointestinal disorders, cyclosporine is often used at the lowest effective dose that

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allows continued resolution of clinical signs, without the need for measurement of blood drug concentrations. Even at the low atopic dose traditionally not thought to induce immunosuppression, some dogs in our study showed clear suppression of biomarker expression. This phenomenon occurred at very low trough cyclosporine concentrations. Explanations for this effect are most likely multifactorial. In human medicine, peak cyclosporine concentrations have been shown to be consistently a better predictor of both the area under the curve and immunosuppressive efficacy compared to trough levels. Also, pharmacodynamic monitoring in human medicine has shown individual-to-individual variability in calcineurin inhibition in response to comparable blood concentrations of cyclosporine, demonstrating individual sensitivity to the effects of the drug. Our findings provide evidence supporting the concept of individual drug sensitivities. Veterinary dermatologists have debated for years about whether the low atopy dose of cyclosporine could explain secondary infections sometimes seen in dogs treated with cyclosporine for atopy. Our findings strongly suggest that the immune system in some dogs on the low atopy dose of cyclosporine can still be significantly affected, potentially leading to clinically relevant immunosuppression. Clinically, a pharmacodynamic assay such as ours could help to individually adjust cyclosporine dosages, and to explore sensitivity to cyclosporine as a potential cause in those patients being treated for canine atopy that appear to develop immunosuppression secondary to treatment.

With more severe and immediately life-threatening immune-mediated diseases, it is currently recommended that cyclosporine dosages be adjusted based on the monitoring of blood cyclosporine concentrations. Typically, trough blood concentrations

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are used to adjust drug dosages, but recommendations vary widely depending on the disease treated and on the assay utilized to measure drug concentrations. Monitoring cyclosporine blood concentrations is recommended in patients with severe diseases because, in the individual patient, cyclosporine pharmacokinetics are inherently highly variable and unpredictable, largely due to issues with oral bioavailability. However, even if therapeutic drug monitoring is used to adjust therapy, patients with comparable drug blood levels may have quite different outcomes, as blood levels do not always correspond to clinical response. This may lead us to question whether cyclosporine blood concentrations should be measured at all. We generally can reasonably estimate starting immunosuppressive cyclosporine dosages for the treatment of immune-mediated blood disorders. If there is a good clinical response, blood concentrations are generally not measured, and therapy is tapered very slowly over time in a controlled fashion. When a good clinical response is not achieved, measurement of blood concentrations may not help with adjustment of therapy, as some dogs on very low doses of cyclosporine, with low trough concentrations, still have measurable T-cell cytokine suppression. With further investigation focusing on peak cyclosporine concentrations, target concentrations may be identified which better predict adequate immunosuppression and ascertain ranges which put patients at risk for toxicity.

In human medicine, studies have shown patient-to-patient variability in the effects of cyclosporine on immune function even when there are comparable blood cyclosporine levels, demonstrating the need for a better assay for assessing the immune system during cyclosporine therapy. Pharmacodynamic monitoring in conjunction with pharmacokinetic monitoring is being investigated in human medicine as a means to

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provide a better overall assessment of immunosuppression and predictor of clinical outcome in response to cyclosporine therapy. Our research has developed a pharmacodynamic assay that utilizes cytokines as biomarkers of immunosuppression to quantitate the biological effects of cyclosporine on T-cells in healthy dogs. The developed pharmacodynamic assay would be relevant for assessing and monitoring the immune system during therapy with any medication which down-regulates IL-2 and INF-γ cytokine production. This would include drugs which target any part of the cascade from the cell surface signal all the way down into the nucleus for gene expression. Most notably are those medications targeting calcineurin with subsequent down-regulation of nuclear factors of activation of T-cells (NFAT), leading to decreased cytokine gene expression. Beyond cyclosporine, medications for which our pharmacodynamic assays might be applicable include glucocorticoids and tacrolimus. Our assay may not be directly applicable to assessing disease-induced immunosuppression, as often this is due to decreased populations of white blood cells allowing secondary infection to occur, rather than decreased T-cell function.

Our study utilized flow cytometry to evaluate T-cell expression of cytokines. Flow cytometry, which requires rapid and specialized sample processing, may not however be the ideal tool the pharmacodynamic monitoring of clinical patients, and a quantitative real-time PCR (QRT-PCR) assay may in the long term hold more promise as a method for clinical pharmacodynamic monitoring of patients. Our study has established that, regardless of the method eventually developed for clinical use, activated T-cell expression of the cytokines IL-2 and IFN-γ shows promise as a potential biomarker of immunosuppression for pharmacodynamic monitoring of cyclosporine.

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Human studies have investigated a number of differing measures of the immune system to assess the pharmacodynamic effects during cyclosporine therapy. We chose our biomarkers of immunosuppression based on previous human studies and the availability of antibodies which were canine specific or cross reactive to dogs. Calcineurin activity is an assay used commonly in human studies, directly assessing the effects of cyclosporine in its cellular target. In our initial investigation towards pharmacodynamic monitoring, measurement of calcineurin activity was not widely used, and procedures such as flow cytometry were more readily utilized. In the process of developing a clinical assay, the time and manpower needed to process samples has to be considered. Quantifying calcineurin activity could be an alternative assay to measure the pharmacodynamic effects of cyclosporine on canine T-cells. This assay has never been published in dogs, and would have to be thoroughly investigated before potential development as a viable canine specific assay. In contrast, our ability to quantify changes in IL-2 and IFN-γ expression when T-cells are exposed to cyclosporine both *in vitro* as well as *in vivo* has already been established as repeatable, and appears to be a valid and viable assay for further development in the clinical monitoring of patients.

Our *in vivo* study only incorporated two extreme doses of cyclosporine, and did not evaluate activated T-cell expression of cytokines at dosages which are often employed for treating inflammatory and immune-mediated diseases in the dog. Performing similar analyses at cyclosporine dosages between the two extreme dosages used in our study would further clarify the potential clinical utility of flow cytometric measurement of T-cell cytokine expression as a biomarker of immunosuppression. Additionally, comparison of flow cytometry versus QRT-PCR as a means of monitoring

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T-cell cytokine expression in dogs receiving cyclosporine may help identify which method is likely to be of more value for clinical pharmacodynamic monitoring. Finally, clinical trials in canine patients with naturally-occurring disease will be needed to establish the clinical utility of adjusting therapy based on measurement of potential biomarkers of immunosuppression, both with cyclosporine and eventually with other immunosuppressive agents that affect T-cell function. With further investigation into the pharmacokinetics and pharmacodynamics of cyclosporine in veterinary medicine, a better assessment of the immune status may be gained when combining these two techniques and allow for optimization of therapy and delivery of individualized treatment.

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