Assessment Of The Pharmacodynamic Effects Of Cyclosporine In Dogs

Claire Fellman

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Assessment of the pharmacodynamic effects of cyclosporine in dogs

By

Claire L. Fellman

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

Mississippi State, Mississippi
May 2016
Assessment of the pharmacodynamic effects of cyclosporine in dogs

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Cyclosporine is a commonly used immunosuppressive drug in dogs, but dosing is often empirical and based primarily on clinical response. Pharmacokinetic monitoring of blood drug concentrations can be performed, but target blood concentrations for various disease states in dogs are not well described. Pharmacodynamic assays measuring the effects of cyclosporine on target cells are being used to evaluate immunosuppressive effectiveness in humans, but have been minimally explored in veterinary medicine. This dissertation describes the development of pharmacodynamic assays for measuring the effects of cyclosporine on canine T cell cytokine production and surface antigen expression. Incubation with cyclosporine in vitro caused significant suppression of activated T cell production of interleukin-2 (IL-2), IL-4, interferon-gamma (IFN-gamma), CD25, and CD95 measured in peripheral blood mononuclear cells using flow cytometry. IL-2 and IFN-gamma were then evaluated using flow cytometry and quantitative reverse transcription polymerase chain reaction (qRT-PCR) in whole blood incubated with cyclosporine and dexamethasone in vitro. Cyclosporine caused concentration-dependent inhibition of both cytokines, and a greater degree of suppression was noted with qRT-PCR than flow cytometry. Dexamethasone caused concentration-dependent inhibition of
IFN-gamma with both methods, but IL-2 reduction was only significant for qRT-PCR. Both methods were then used to evaluate IL-2 and IFN-gamma after administration of high dose oral cyclosporine to dogs. Both qRT-PCR and flow cytometry identified marked cytokine suppression after cyclosporine dosing, but qRT-PCR was uniformly suppressed across the 12-hour dosing interval, while flow cytometry results were significantly higher at trough blood drug concentrations than at peak blood concentrations and subsequent post-dosing time points. Both flow cytometry and qRT-PCR are valid methods for evaluation of T cell cytokine expression in dogs. Further study at lower drug doses is needed to correlate pharmacodynamic results with pharmacokinetic drug concentrations, and to confirm the best method for cytokine monitoring. Studies in clinic patients are also needed to determine the level of cytokine suppression associated with clinical effectiveness in different disease states.

Pharmacodynamic evaluation of cyclosporine’s effects shows promise, and may allow for more individualized dosing of cyclosporine in dogs.
DEDICATION

To my Mom, Dad, and big brother, Zach, for teaching me what is important, and to Joseph for being there through it all.
ACKNOWLEDGEMENTS

I would like to acknowledge the help of several students that have contributed to cyclosporine pharmacodynamic assay development as part of the Mississippi State University College of Veterinary Medicine Summer Research Experience, including Dr. Courtney Bruner (2008), Dr. Rebecca Flores (2010), Dr. Jenica Haraschak (2011), and Dr. Joyce Follows (2012). This work has also been aided and further investigated by subsequent PhD students Caitlin Riggs and Charlee Mulligan. Invaluable technical assistance has been provided by John Stokes throughout my training, and by Dr. Lakshmi Narayanan more recently. Thank you to the members of my committee for their assistance during this process, especially to Dr. Todd Archer whose Master’s project initiated our cyclosporine studies, to Dr. Andrew Mackin for mentoring me in so many areas, and to Dr. Todd Pharr for introducing me to and guiding me through the intricacies of immunology research. An additional heartfelt thank you to Dr. Cory Langston at Mississippi State and Dr. Dawn Boothe at Auburn University for their assistance in the pharmacologic aspects of my training. A final thank you to the many research dogs including Brownie, Bonnie, Ella, Patty, Noodle, Stella, Sticky Buns and others that have made this work possible.
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<th>Full Form</th>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CM</td>
<td>complete media</td>
</tr>
<tr>
<td>Cmax</td>
<td>maximum drug blood concentration</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine</td>
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<tr>
<td>CYP3A</td>
<td>cytochrome P450 3A</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FPIA</td>
<td>fluorescence polarization immunoassay</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPLC-MS/MS</td>
<td>high performance liquid chromatography-tandem mass spectrometry</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration of drug that causes 50% reduction of the response tested</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IMHA</td>
<td>immune-mediated hemolytic anemia</td>
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<td>IMPDH</td>
<td>inosine-5′-monophosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>(k)Da</td>
<td>(kilo)dalton</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MUE</td>
<td>meningoencephalomyelitis of unknown etiology</td>
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<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
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<tr>
<td>OAT</td>
<td>organic anion transporter</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>RPE</td>
<td>R-phycoerythrin</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>SSC</td>
<td>side scatter</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>Tmax</td>
<td>time to maximum drug blood concentration</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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INTRODUCTION

Background

Cyclosporine is an important immunosuppressive drug, originally pioneered for human transplantation, but since employed for a variety of inflammatory and immune-mediated diseases in both human and veterinary medicine. In veterinary medicine, cyclosporine is approved for the treatment of atopic dermatitis in dogs, and for inflammatory skin diseases in cats, at a relatively low dose of five and seven mg/kg/day in dogs and cats, respectively.\textsuperscript{1,2} Higher extralabel doses (up to 20 mg/kg/day) are used for transplantation and in the treatment of immune-mediated diseases such as inflammatory bowel disease, immune-mediated thrombocytopenia, and immune-mediated hemolytic anemia.\textsuperscript{3} Cyclosporine decreases adaptive immune responses by reducing cytokine release from T cells. Specifically, cyclosporine inhibits calcineurin, which is then unable to dephosphorylate nuclear factor of activated T cells (NFAT), a transcription factor normally involved in the production of pro-inflammatory cytokines, including interleukin 2 (IL-2), needed for the proliferation of T cells.\textsuperscript{4} Nephrotoxicity is the most significant adverse event in human medicine,\textsuperscript{5} while gastrointestinal adverse effects predominate in veterinary medicine.\textsuperscript{6} In both humans and companion animals, a primary difficulty in cyclosporine therapy is variability in patient response, with an associated need for individualized therapy.
Cyclosporine is a large hydrophobic molecule, and the first approved human formulation of the drug, Sandimmune®, suffered from poor bioavailability and highly variable absorption. Because the drug was used in transplant medicine, it was important that cyclosporine reach therapeutic levels to help prevent rejection, but that levels not be so high that the patient experienced nephrotoxicity. Pharmacokinetic monitoring of trough blood concentrations was used to monitor the cyclosporine concentrations being achieved in individual patients, but there was poor correlation between blood concentrations and either transplant rejection or nephrotoxicity. Pharmacodynamic measures, evaluating the effect of cyclosporine on the biological target, the T cell, were also developed, and included measuring T cell IL-2 production and mitogen-induced lymphocyte proliferation.

Introduction of the microemulsified formulation of cyclosporine, Neoral®, in 1995 reduced interpatient pharmacokinetic variability. Additional pharmacokinetic monitoring strategies were investigated including area under the curve (AUC) and peak cyclosporine blood concentration two hours after dosing, both of which showed improved correlation to patient outcomes compared to trough concentration monitoring. Individual patients still experienced rejection and nephrotoxicity despite attainment of blood concentrations considered therapeutic in other patients, however, so pharmacodynamic assays have continued to be investigated as an alternative method for potentially better evaluating patient responses to cyclosporine by identifying when lymphocyte suppression has been adequately achieved.

The most common pharmacodynamic assays in human medicine include measuring calcineurin inhibition, activation-related cell surface antigens, cytokines, and
lymphocyte proliferation. Evaluation of NFAT-regulated cytokines, including IL-2, IL-4, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), is one of the most popular methods of cyclosporine pharmacodynamic monitoring, with cytokines measured using enzyme-linked immunosorbent assays (ELISAs), flow cytometry, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Serial monitoring of IL-2 is performed by some groups, while others evaluate the reduction in cytokine production at peak cyclosporine blood concentrations relative to trough, termed “residual gene expression”. Higher IL-2 levels have been correlated with higher risk of transplant rejection, and lower gene expression has been correlated with a higher risk of neoplasia and potentially greater immunosuppression than is needed clinically. In human medicine, pharmacodynamic studies are performed in conjunction with pharmacokinetic studies to help monitor patients after transplantation. No pharmacodynamic measure is well accepted to improve patient outcome, and none is standardly performed.

Cyclosporine has a shorter history in veterinary medicine than in human medicine, having only been approved in 2003 in dogs, and 2011 in cats. It has been in extralabel use before and after approval for atopy, however, and there is substantial clinical experience documenting the lack of nephrotoxicity in companion animals. Given this, pharmacokinetic monitoring is arguably less essential (since unexpectedly high concentrations have less major potential adverse reactions), but monitoring is still performed in cases where achieving a certain blood concentration is important. This includes the relatively uncommon (in veterinary medicine) scenario of transplantation, as well as life-threatening immune-mediated disease. Another common indication for
blood cyclosporine measurement is when cyclosporine is paired with another drug, most often ketoconazole, which impairs the metabolism of cyclosporine and thus allows less cyclosporine to be given while still attaining target blood concentrations.\textsuperscript{28-30} Cyclosporine is an expensive drug, and pairing with ketoconazole is most often attempted for treatment of anal furunculosis (also known as perianal fistulas), when long-term therapy is needed and patients are often large breed dogs. Target trough concentrations are most clearly established for transplantation, and little evidence exists to support the need to monitor trough concentrations for other inflammatory and immune-mediated conditions. The author is not aware of any published literature investigating peak or AUC values and their correlations to clinical outcome in veterinary medicine.

Pharmacodynamic evaluation of cyclosporine in veterinary medicine is in its infancy. Most work has been done \textit{in vitro}, and has documented decreased production of cytokines by canine and feline peripheral blood mononuclear cells incubated with cyclosporine.\textsuperscript{31,32} Two studies have evaluated the effects of topical ophthalmic cyclosporine, administered for the treatment of keratoconjunctivitis sicca (dry eye) on the systemic immune system, with one identifying decreased lymphocyte proliferation,\textsuperscript{33} and the other more recent study showing no effect and very low systemic blood cyclosporine concentrations.\textsuperscript{34} An additional study investigated cytokine levels in tissue biopsies taken from the area of anal furunculosis lesions, and showed significantly decreased intralesional expression of IL-2 after cyclosporine treatment.\textsuperscript{35} Despite the frequent use of cyclosporine in clinic patients, very little work has been done to investigate individual immune responses to oral cyclosporine.
Variable individual patient immune responses to cyclosporine are strongly suggested by the reports of animals on cyclosporine developing serious, and sometimes fatal, infectious diseases while receiving atopy doses not typically considered likely to cause significant immune suppression. There are also clinically-apparent differences in responses to cyclosporine for other immune-mediated diseases, with some animals showing poor response to therapy despite the use of a dose effective in other patients. It is likely some of these clinical differences can be attributed to variable lymphocyte responses to cyclosporine. It is also possible some clinical variability is caused by failure to achieve sufficient blood concentrations, but again, without well-established target blood concentrations, this is difficult to definitively determine.

**Hypothesis and Chapter Overview**

The overall hypothesis of our research laboratory is that a comprehensive panel of biomarkers of immunosuppression can be used to objectively and accurately establish target doses and drug concentrations of cyclosporine in the dog. This dissertation describes the development of a panel of biomarkers, and provides *in vitro* and *in vivo* evidence of their ability to reflect the effects of cyclosporine on canine lymphocytes. Chapter II gives an overview of the relevant literature pertaining to cyclosporine and its pharmacokinetic and pharmacodynamic monitoring. Chapter III describes an effort to identify biomarkers of cyclosporine immunosuppression in dogs, and to develop assays for their measurement in canine blood samples using flow cytometry. Chapter IV then evaluates the effects of *in vitro* cyclosporine and dexamethasone on a biomarker panel using flow cytometry and a previously validated qRT-PCR assay. In Chapter V, these biomarkers are then evaluated after oral cyclosporine to assess cyclosporine’s
pharmacokinetic/pharmacodynamic relationship with concurrent cyclosporine blood concentration determination and biomarker measurement using flow cytometry and qRT-PCR. Chapter VI summarizes findings and discusses future research directions.

**Project Significance**

At present, veterinary medicine lacks effective means for monitoring patient response to cyclosporine. This dissertation describes initial investigations into the pharmacodynamic evaluation of oral cyclosporine in dogs using both flow cytometry and qRT-PCR. Since glucocorticoids are often co-administered with cyclosporine, their effect on the biomarkers is also evaluated.

Veterinary medicine has made tremendous advancements in recent decades. The American College of Veterinary Internal Medicine was recognized by the American Veterinary Medical Association in 1980, and since that time specialty medicine has evolved into a growing industry that has revolutionized the standard of care and ability to provide quality medicine for companion animals. That said, veterinary medicine still largely relies on knowledge gleaned from human medicine for pioneering new treatments and diagnostic modalities. Although this approach is understandable, as a profession veterinarians must continue to seek opportunities for innovative and original animal research.

Pharmacodynamic assessment of cyclosporine’s effects is an example of how human medicine is becoming individualized to tailor treatment plans to the patient. Despite being increasingly published, however, definitive cyclosporine pharmacodynamic standards still remain elusive, with several monitoring strategies suggested by different groups.\textsuperscript{17,22,23,38} This dissertation describes a pharmacodynamic
monitoring strategy for dogs treated with cyclosporine. Much human cyclosporine pharmacodynamic research involves transplant monitoring, while this is a much smaller market for veterinary use. There are, however, a multitude of inflammatory and immune-mediated conditions in dogs that would benefit from enhanced cyclosporine monitoring and, significantly, different degrees of immune suppression are needed for these diseases. Veterinary medicine offers an excellent opportunity to enhance the understanding of mammalian immune responses to cyclosporine across a wide variety of doses and dosing intervals. The biomarkers used to evaluate cyclosporine may also be useful to monitor overall T cell responsiveness and patient response to other immunosuppressive drugs. Cyclosporine pharmacodynamics is a step towards individualized veterinary medicine, and is an important area for research with the potential to improve the standard of care for veterinary patients with inflammatory and immune-mediated diseases.
References


Cyclosporine is an 11 amino acid cyclic polypeptide with a molecular weight of 1203.63 daltons (Da). It was originally isolated from a soil sample collected by H.P. Frey, a Sandoz employee, while vacationing in Norway. Compound 24-556, a metabolite of the soil fungus *Tolypocladium inflatum*, also known as *Beauveria nivea*, was recognized in 1971 for its strong immunosuppressive properties in the absence of significant cytotoxicity. Compound 24-556 also lacked the myelotoxicity caused by azathioprine, the other major non-glucocorticoid immunosuppressive drug in use at the time. Stähelin and Borel were the primary discoverers of the molecule working in the Sandoz laboratory in Switzerland, and it was named cyclosporine because it was a cyclical peptide derived from a spore.

Early issues with cyclosporine included its hydrophobicity, and difficulty in obtaining sufficient quantities for testing. Cyclosporine showed significant promise as an immunosuppressive agent, however, in skin transplantation and autoimmune models. This early in vitro and in vivo work was first published as an abstract in *Experientia*, and was then described by Borel at the 1976 British Society of Immunology meeting in London. Borel’s presentation sparked interest in the drug as an aid to transplant
immunosuppression. Early work was done investigating the drug with heart and renal allografts in rats and rabbits,\textsuperscript{12-15} and progressed to work involving dogs and pigs.\textsuperscript{16-18} These studies demonstrated improved graft function and survival, with minimal side effects.

Calne and White were two of the leaders in the field, and performed the first human renal cadaveric transplantations with cyclosporine as the sole immunosuppressant.\textsuperscript{19,20} Early trials in humans revealed unacceptable renal toxicity and a high incidence of lymphoma and secondary infections, but this was improved through cyclosporine dose reduction.\textsuperscript{10,20,21} Cyclosporine was developed for the human market, and the first multicenter trial in Europe documented 73\% graft survival for patients receiving cyclosporine alone, versus 53\% for steroid and azathioprine-treated controls.\textsuperscript{22} This information led to formal approval of the drug by the Food and Drug Administration (FDA) in 1983, and to cyclosporine even being called “the penicillin of transplantation.”\textsuperscript{23}

Combining cyclosporine with methylprednisolone led to further improvements in graft survival, up to 88.2\% at six months, compared to 53.2\% for azathioprine and steroid-treated controls.\textsuperscript{24} Cyclosporine was incorporated into protocols for transplantation of other organs including heart, liver, and pancreas, and immunosuppressive protocols were further modified to include azathioprine as part of “triple-therapy” regimens (steroid + azathioprine + cyclosporine).\textsuperscript{8} More recently, mycophenolate mofetil, an inhibitor of inosine-5’-monophosphate dehydrogenase (IMPDH) that decreases lymphocyte proliferation, has often been included as a replacement for azathioprine.\textsuperscript{3}
Cyclosporine is also used in veterinary medicine for renal transplantation,25-28 but is more commonly used for the treatment of a variety of inflammatory and immune-mediated diseases. Atopica®, a microemulsified formulation of cyclosporine, was approved for the control of atopic dermatitis in dogs in 2003.29 A liquid solution, Atopica® for cats, was approved in 2011 for the control of feline allergic dermatitis, including military dermatitis, eosinophilic plaques, and self-induced alopecia.30 There is also an approved topical formulation of cyclosporine, Optimmune®, used for the management of chronic keratoconjunctivitis sicca and chronic superficial keratitis in dogs.31 Cyclosporine undergoes frequent extralabel use in dogs and cats for a number of conditions, including but not limited to the treatment of immune-mediated hemolytic anemia, inflammatory bowel disease, anal furunculosis (dogs only), and immune-mediated skin diseases.32,33

**Chemistry and Clinical Pharmacology**

**Molecule**

Cyclosporin A, commonly called cyclosporine, is produced by the 800 kDa multifunctional enzyme cyclosporin synthetase.34 The cyclosporine molecule is notable for having a D-amino acid and only hydrogen bonded or methylated amide nitrogens, which makes it resistant to degradation in the gastrointestinal tract (Figure 1).3,35 Its chemical formula is C₆₂H₁₁₁N₁₁O₁₂,¹ and chemical designation is [R-[R*,R*-](E)]-cyclic (L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl-3-hydroxy-N,4-dimethyl-L-2-amino-6-octenoyl-L-α-amino-butyrylN-methylglycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl).36 Cyclosporine is large, lipophilic, and hydrophobic, and thus formulation has significant effect on bioavailability.
Formulations

The original formulation of cyclosporine, Sandimmune®, is an oil and alcohol solution, and is available as a soft gelatin capsule, oral solution, and intravenous solution. According to the prescribing information, the injectable formulation is in a polyoxethylated castor oil vehicle, Cremophor® EL, which has been associated with anaphylactic reactions. The oral liquid is dissolved in an olive oil vehicle, and the gelatin capsule contains corn oil and linoleoyl macrogolglycerides. Sandimmune®’s absolute bioavailability is approximately 30%, and the capsule and oral formulations are bioequivalent.

A modified microemulsion was approved in July 1995 with the trade name Neoral®, and has a surfactant, lipophilic and hydrophilic solvents, and ethanol. This formulation comes as a soft gelatin capsule and oral solution and, for both, corn oil-
mono-di-triglycerides, polyoxyl 40 hydrogenated castor oil NF, DL-α-tocopherol and propylene glycol are inactive ingredients. Neoral® forms a microemulsion in an aqueous environment, and has higher bioavailability (relative bioavailability of 174-239% depending on the dose) and dose proportionality, as well as reduced variability in pharmacokinetics, compared to Sandimmune®. While for both Sandimmune® and Neoral® the respective oral solutions and capsules are bioequivalent, the two formulations are not bioequivalent. The veterinary approved product Atopica® is also the microemulsified (modified) cyclosporine. Human generic formulations are available for Neoral® and Sandimmune®.

**Absorption**

Cyclosporine is emulsified in the small intestine, and Sandimmune® is affected by bile flow, intestinal motility, and the presence of food, while the microemulsified formulation is not affected by bile secretion. Bioavailability in dogs is approximately 20-27% for dogs with Sandimmune®, and 35% for Atopica®, which is largely due to the molecule size and poor solubility, even with the microemulsified formulation. In humans, meal consumption within 30 minutes of taking cyclosporine decreases the pharmacokinetic parameters area under the curve by 13% and peak concentration by 33%. In dogs, administration with food decreases bioavailability by 22%, and causes higher interindividual variation in drug absorption. Therefore, in dogs it is recommended to administer Atopica® at least one hour before or two hours after a meal. In contrast, Atopica® for cats is recommended to be administered with food, which results in slightly higher peak concentrations and area under the curve than when
given fasted. In dogs, peak cyclosporine blood concentrations are generally reached between 1-2 hours after oral administration of the microemulsified formulation.44

Active efflux from enterocytes back to the intestinal lumen through the action of the efflux pump P-glycoprotein, a multidrug transporter, is suggested as a potential contributor to the variability in absorption found with cyclosporine.46 Lower P-glycoprotein function may also increase the risk of nephrotoxicity in humans.47,48 P-glycoprotein-deficient mice have been shown to have higher brain concentrations of cyclosporine, and to also have slower drug elimination.49 In dogs, however, although Collies commonly possess a mutation in the gene encoding P-glycoprotein (ABCB1), no difference has been found in cyclosporine pharmacokinetics for P-glycoprotein-deficient versus wild-type dogs.50 In a study by Mealey, no significant bioavailability differences were identified for cyclosporine and other P-glycoprotein substrates (quinidine, loperamide, or nelfinavir), despite dogs given loperamide displaying excessive sedation consistent with an affected phenotype.50 Mealey suggested that intestinal P-glycoprotein may be saturated at doses used clinically, and thus have little influence on substrate bioavailability. Since the tested drugs were also substrates of cytochrome P450 3A (CYP3A) metabolizing enzymes, however, which are also found in enterocytes, the roles of P-glycoprotein versus CYP3A could not be definitively identified. Nonetheless, the lack of significant differences in pharmacokinetics for cyclosporine in ABCB1 deficient dogs versus normal dogs, along with the fact that polymorphisms in CYP3A4, CYP3A5 and ABCB1 are not associated with significant alterations cyclosporine pharmacokinetics in humans,51 makes P-glycoprotein a less likely cause of variable intestinal absorption.
Cytochrome enzymes are located in both enterocytes and the liver, and have the potential to influence cyclosporine bioavailability through a first pass effect, as well as by affecting cyclosporine clearance. The role of both P-glycoprotein and intestinal CYP3A activity in cyclosporine pharmacokinetics is controversial, but one study showed despite a 10-fold variation in intestinal CYP3A4 activity, there was no significant association between enzyme activity and cyclosporine pharmacokinetics in humans. In this study, 32% of peak drug concentration variability and 56% of oral clearance variability were attributed to hepatic CYP3A4 activity, compared to 30% of peak and 17% of clearance variability attributed to intestinal P-glycoprotein expression. The author is not aware of any studies in veterinary medicine examining intestinal CYP3A activity and Atopica® bioavailability, but it is possible factors like P-glycoprotein and CYP3A expression may influence local drug concentrations and thus cyclosporine effectiveness for conditions like inflammatory bowel disease, where more studies are needed to determine if there is a correlation between blood concentrations and clinical outcome.

**Distribution**

Being highly lipophilic, cyclosporine is extensively distributed to body tissues other than the central nervous system, which is protected by transporters such as P-glycoprotein. Cyclosporine’s volume of distribution is 3-5 L/kg, and the drug is 90% protein bound in blood, primarily to lipoproteins, and only 8% to other blood proteins like albumin. According to Novartis, in blood approximately 33-47% of the drug is found in plasma, 41-58% is in erythrocytes, 5-12% is in granulocytes, and the remaining 4-9% is in lymphocytes. Within tissues, a study of autopsied humans on cyclosporine for organ transplantation revealed the highest amount of cyclosporine and metabolites per
kilogram of tissue in pancreas, followed by spleen, liver, fat, kidney, lung, bone marrow, muscle (heart) and whole blood.52 Also in this study, fat was found to contain almost exclusively unmetabolized cyclosporine, suggesting a possible role for obesity in cyclosporine pharmacokinetic variability, and the need to dose cyclosporine on lean body weight.

Cyclosporine is also recognized to concentrate in the skin, and a trial of human psoriasis patients found skin concentrations similar to those found in blood at peak cyclosporine levels, approximately 10 times greater than trough cyclosporine blood concentrations.53 Steffan and others at Novartis showed that cyclosporine also concentrates in the skin of dogs. After 14 days of administration, canine skin concentrations were 2.5-6.4 times greater than the blood cyclosporine concentration at 4 hours, and at 24 hours blood concentrations were < 25 ng/mL, while skin concentrations were still 206 ng/g. The authors suggested that once daily dosing is effective for atopic dermatitis in dogs because of cyclosporine’s concentration in, and slow depletion from, skin.54 Another study by Gray and others also showed that cyclosporine accumulates in the skin of dogs dosed for seven days, and only a moderate correlation was noted between skin and blood cyclosporine concentrations.55

**Metabolism**

Cyclosporine is extensively metabolized, and less than 1% of the drug is eliminated unchanged in the feces,56 with a similar proportion in urine.57,58 Cyclosporine is metabolized by CYP3A enzymes, primarily through N-demethylation and hydroxylation.59 There are various reports of the number of metabolites, with most sources listing greater than 25 identified.1,60,61 Metabolites are named “A” for being
metabolites of cyclosporine A, “M” for metabolite, and then a number based on the amino acid hydroxylation site, or the amino acid number and “N” if the metabolite is modified by demethylation.62 There are three primary metabolites, considered first generation metabolites produced through phase I metabolism, that are the most biologically active.1,62 These are: AM1 (formerly M-17), 1-beta (8’) hydroxylated; AM9 (formerly M-1), 9-gamma hydroxylated; and AM4N (formerly M-21), 4-N-demethylated. The cyclized metabolite AM1c (formerly M-18) 1-beta 1-epsilon is also commonly measured.60,62

AM1 is considered the most biologically active metabolite, having 10-20% the activity of the parent cyclosporine molecule.62 In a study by Venkataramanan and others, cyclosporine metabolites were collected via choledochoureterostomy in Beagles, and results suggested that AM9 and AM4N were the primary metabolites in dogs, with lesser amounts of AM1 and AM1c. Cats also produce primarily AM9 and AM4N.58 Humans primarily excrete AM1 in bile, followed by AM9, AM4N and AM1c.58 Clear differences in metabolic pathways are present between species, with dogs recognized to have high hepatic enzyme activity and higher concentrations of the metabolite AM4N than humans.60

The different metabolites have different affinities for blood components, with some found in the plasma, and others, like the parent cyclosporine, associating more with cellular elements.1 The metabolites also have different affinities for cyclophilin, with cyclosporine having the greatest affinity, followed by AM1 and AM9.63 The ability to bind cyclophilin has been reported, fairly logically, as a requirement for the immunosuppressive action of metabolites.64
As described in the section on absorption, both the liver and intestines contain significant amounts of CYP3A enzymes that contribute to the first pass effect and reduced bioavailability of cyclosporine.\textsuperscript{1,65} In humans, cyclosporine is also metabolized to AM1 in the kidney, as shown using organ slice cultures, but this is not apparent for canine kidney slices.\textsuperscript{66} The toxicity of individual metabolites is contested, but it is possible metabolite formation differences in the kidney could contribute to the greater nephrotoxicity caused by cyclosporine in humans than dogs.

Since cytochrome P450 activity is so important to cyclosporine disposition in patients, various attempts have been made to predict patient response to cyclosporine based on CYP activity. Originally, an erythromycin breath test was developed by Watkins. After giving intravenous $^{14}$C-N-methyl erythromycin, $^{14}$C-containing carbon dioxide ($^{14}$CO\textsubscript{2}) was measured in breath, and a negative correlation was shown between cyclosporine blood concentration and the breath level of $^{14}$CO\textsubscript{2}.\textsuperscript{67} More recently, extensive efforts have been made in pharmacogenetic assessments of different CYP single nucleotide polymorphisms (SNPs). CYP3A4 is the primary cyclosporine metabolizing enzyme in humans, with a smaller role for CYP3A5.\textsuperscript{68} The analogous CYP in dogs for CYP3A4 is considered to be CYP3A12.\textsuperscript{69} A CYP3A5*3 SNP has been found to influence tacrolimus pharmacokinetics, but has less convincing influence on cyclosporine.\textsuperscript{68} The low functioning CYP3A4*22 mutation has recently been discovered, however, and has been found to decrease clearance and increase peak cyclosporine concentrations, and is a risk factor for worse renal function in human patients.\textsuperscript{70-72} Pharmacogenetics is a new field in veterinary medicine, but future investigations could
evaluate the influence of cytochrome expression in canine cyclosporine pharmacokinetics.

**Elimination**

Cyclosporine has a low to moderate hepatic extraction ratio,\textsuperscript{73,74} and thus hepatic blood flow variation has less influence on clearance, while metabolizing enzyme activity more significantly impacts cyclosporine clearance. Drug interactions are common because of the multitude of CYP3A substrates, and drugs that impact CYP3A enzyme activity can have a significant impact on cyclosporine pharmacokinetics (see drug interactions). Cyclosporine is primarily eliminated through the bile, with less than 1% as parent compound, and the majority as metabolites.\textsuperscript{56} Renal elimination of the parent molecule and metabolites is less than 6% of the administered dose in humans.\textsuperscript{57,75} Most sources report similar (minimal) renal excretion for dogs,\textsuperscript{32,44,76} though one early radiolabeled study reported 10.4 ± 5.7% urinary radioactivity excretion after oral dosing, and 17.5 ± 3.2% urinary excretion after intravenous dosing when collected for 96 hours.\textsuperscript{75} The half-life is variable, but for dogs given Atopica\textsuperscript{®} cyclosporine half-life is reported as 4.5 hours.\textsuperscript{39}

**Mechanism of Action**

After entering the cell though passive diffusion,\textsuperscript{35} cyclosporine binds to the cytoplasmic protein cyclophilin A.\textsuperscript{77} Cyclophilins are a group of proteins with peptidyl-prolyl isomerase activity conserved across mammals, plants, fungi, and insects. They help stabilize the *cis-trans* transition, which is important for protein folding and multidomain protein assembly.\textsuperscript{78} Cyclophilins are considered immunophilins, a protein
group that includes cyclophilin A, the binding target for cyclosporine, and FK506 binding proteins (FKBPs) for tacrolimus. Cyclophilin A is found in the largest amounts in T cells, erythrocytes, colonic epithelium, kidney and brain cells, and is widely distributed in other body tissues at lower concentrations. Cyclosporine’s binding to cyclophilin is highly specific, and is not displaced by co-incubation with a number of drugs including other P-glycoprotein substrates such as rhodamine and vincristine, steroids such as triamcinolone and dexamethasone, or the enzyme inducer rifampin. Indeed, in the initial report by Handschumacher describing cyclophilin as the cytoplasmic binding target for cyclosporine, only natural and synthetic cyclosporine analogs able to inhibit mixed lymphocyte reactions were able to displace cyclosporine from binding cyclophilin.

The cyclosporine-cyclophilin complex then inhibits calcineurin, a serine-threonine phosphatase. Like cyclophilin, calcineurin is widely distributed in tissues (most concentrated in the brain) and is found in mammals, plants, and fungi. Calcineurin has a catalytic A subunit, and a calcium-binding B subunit, and after binding both calcium and calmodulin, the enzyme becomes active through either loss of inhibition from the autoinhibitory domain, or changes in catalytic site affinity depending whether calcium binds the A subunit, or B subunit, respectively. Though not structurally similar, cyclosporine bound to cyclophilin A and tacrolimus bound to FKBPs both bind the B subunit, B subunit binding helix, and part of the calcineurin heterodimer substrate-binding cleft, and impair calcineurin’s ability to bind substrates, with greater effect for larger substrates. The difference in structure between tacrolimus and cyclosporine likely accounts for their calcineurin inhibition abilities, with 10 times lower
concentrations of tacrolimus needed to inhibit calcineurin and decrease IL-2 production by 50%.

In addition to its role in T cell activation, calcineurin has roles in calcium-dependent activation processes in a variety of tissues including pancreatic beta cell insulin secretion, skeletal and cardiac muscle hypertrophy, and multiple functions in the brain associated with memory, ion channel regulation, and neuritogenesis.

In normal T cell activation, signal transduction through the T cell receptor in response to the cognate antigen provides signal 1. Binding of the CD28 receptor by CD80 or CD86 on antigen presenting cells provides signal 2 (costimulation). This begins an intracellular signaling cascade with the activation of phospholipase C-γ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol and inositol 1,4,5 trisphosphate (IP3). Diacylglycerol and IP3 are second messengers, with diacylglycerol activating protein kinase C, and IP3 triggering release of calcium from the endoplasmic reticulum. This influx of calcium allows activation of calcineurin, whose normal role in T cells is to dephosphorylate the transcription factor NFAT. Phosphorylation of NFAT keeps it in the cytosol because the nuclear localization sequence cannot be recognized by nuclear transporters. Once dephosphorylated, NFAT translocates to the nucleus and increases the expression of pro-inflammatory genes like IL-2, IL-4, IFN-γ, and TNF-α. Calcineurin inhibitors like cyclosporine and tacrolimus block the dephosphorylation of NFAT, and thus reduce production of cytokines, like IL-2, that normally drive the proliferation of T cells. T cells are particularly sensitive to cyclosporine because they have naturally low expression of calcineurin.

Cyclosporine has minimal effects on humoral immunity, and has been shown to have no effect on IgG, IgM, or IgA secretion after intravenous dosing of 2 and 5 mg/kg.
intravenously once daily for 21 days in dogs. Both proliferative responses to T cell mitogens and cytotoxic activity are impaired, however, reflecting cyclosporine’s primary action against T lymphocytes.\textsuperscript{86} Cyclosporine has less effect on secondary responses from lymphocytes that are already activated.\textsuperscript{87} Cyclosporine does have effects on other cells that may influence its therapeutic efficacy, particularly for skin disease. Cyclosporine has, for example, been shown to decrease histamine release from mast cells both \textit{in vitro} and \textit{in vivo} in dogs.\textsuperscript{88,89} Various other effects have been reported in humans and rodents, including reduced eosinophil numbers and granule release, as well as decreased epidermal Langerhans cells.\textsuperscript{44}

\textbf{Adverse Effects}

Cyclosporine’s most well recognized adverse reaction is nephrotoxicity. Cyclosporine is well documented as potentially causing both acute and chronic renal damage after transplantation in humans, and is especially recognized as a factor limiting long-term survival of renal allograft recipients.\textsuperscript{8,47,90-92} Cyclosporine nephrotoxicity is suggested to have multiple mechanisms including activation of the renin-angiotensin-aldosterone system, increased endothelin-1, loss of sympathetic regulation, increased production of transforming growth factor (TGF)-\(\beta\) leading to fibrosis, and direct inhibition of calcineurin in the kidney.\textsuperscript{47} Cyclosporine is also recognized to alter the production of thromboxanes and prostaglandins in the kidney. There is a binding site for NFAT on the cyclooxygenase-2 (COX-2) promoter, and NFAT inhibition by cyclosporine reduces the downstream production of vasodilators like prostaglandin E\(_2\), and may therefore contribute to renal vasoconstriction.\textsuperscript{93} Loss of immunophilin function because of calcineurin inhibitor binding has also been investigated as a reason for
calcineurin inhibitor nephrotoxicity. Transgenic mice with lower cyclophilin A, the cytoplasmic binding target for cyclosporine, have been shown to experience more severe cyclosporine nephrotoxicity than mice with normal amounts of cyclophilin A.\textsuperscript{94} In contrast, reduced FKBP immunophilin, the binding target for tacrolimus, has not been shown to increase the risk for tacrolimus nephrotoxicity.\textsuperscript{95} These findings suggest that cyclophilin A isomerase action, but not FKBP, is needed for normal kidney function. This difference may account for the greater risk of acute tubular damage with cyclosporine than tacrolimus.\textsuperscript{47}

Acute cyclosporine nephrotoxicity generally develops within a few days of transplantation, is reversible upon drug discontinuation, and is considered to be dose-dependent and thus is reduced through therapeutic drug monitoring.\textsuperscript{47,92} Chronic calcineurin inhibitor nephrotoxicity develops even with careful drug concentration monitoring and drug minimization protocols, and is considered to be multifactorial.\textsuperscript{92,96} In renal transplantation, there is some controversy regarding how much of cyclosporine nephrotoxicity is simply related to chronic graft rejection. There is no defined lesion associated with calcineurin nephrotoxicity, but associated histopathological changes are arteriolar hyalinosis and interstitial (also known as striped) fibrosis, although these findings are nonspecific.\textsuperscript{91} Renal damage has also been documented in heart, lung, and liver allograft patients, however, and demonstrates the reality of calcineurin inhibitor nephrotoxicity. Calcineurin inhibitor minimization protocols do not eliminate the progression of chronic nephrotoxicity, but early withdrawal protocols are being investigated in an attempt to provide the benefit of initial graft rejection prevention using calcineurin inhibitors, while reducing long-term exposure and chronic nephrotoxicity.\textsuperscript{96}
Fortunately, in veterinary medicine, nephrotoxicity does not seem to be a major concern. In some of the original canine transplantation work employing very high doses of cyclosporine (maximum 50 mg/kg/day), 5 of 34 (14%) dogs became jaundiced, including one dog with toxoplasmosis and the remaining with hepatic necrosis. An additional 35% died of infection, and 23% from organ rejection, but no renal changes were reported. In the Atopica® approval studies, at the much lower dose of 5 mg/kg/day, vomiting (30.9%) and diarrhea (20%) were the most common adverse effects, followed by persistent otitis externa, urinary tract infection, anorexia, lethargy, gingival hyperplasia, and lymphadenopathy, all with less than 10% incidence. Bloodwork changes that were noted included an elevated creatinine, globulin, phosphorus, protein, cholesterol, and blood urea nitrogen, and decreased albumin and calcium.

According to a recent review of cyclosporine adverse events, 55% of treated dogs experienced an adverse event, but only 4% of events warranted drug discontinuation. Nuttall and others confirm gastrointestinal effects as the primary adverse events associated with cyclosporine use in dogs, occurring in up to 46% of patients. Gingival hyperplasia, thought to be caused by increased TGF-β production, which in turn induces fibroblast proliferation, occurred in 1% of patients in the studies evaluated. Other less common adverse events include verrucous dermatitis or papillomatosis, hirsutism, and predisposition to infection. These authors found no risk of renal damage and minimal risk of neurotoxicity, and suggested a possible risk for malignancy associated more with the level of immunosuppression than cyclosporine itself. Cyclosporine is also recognized as a contributor to insulin resistance.
**Drug Interactions**

As discussed under metabolism, cyclosporine is a substrate of both hepatic and intestinal CYP3A enzymes and P-glycoprotein, making it highly susceptible to drug interactions. Other drugs metabolized by CYP3A4 can compete for metabolism with cyclosporine, and drugs that affect the activity of these enzymes can have significant effects on cyclosporine blood concentrations. Many drugs are recognized to affect cyclosporine metabolism, but the one that has been used specifically for that purpose is ketoconazole. The azole antifungals are known inhibitors of CYP3A enzymes, and co-administration of cyclosporine and ketoconazole allows oral cyclosporine dose reductions of up to 75% at the higher cyclosporine doses needed to maintain trough concentrations sufficient to prevent rejection after renal transplantation in dogs,\(^{100}\) and up to 70-90% at the lower doses needed for treatment of anal furunculosis.\(^{101,102}\) A similar effect is noted for fluconazole, although maximal cyclosporine dose reductions of only approximately 50% are achieved in both healthy dogs and renal transplant patients.\(^{103,104}\)

A listing of drugs with the potential to increase or decrease cyclosporine blood concentrations is found Table 2.2. In addition to the azoles, other important drugs with the potential to increase cyclosporine blood concentrations include the calcium channel blockers, verapamil and diltiazem, and macrolide antibiotics.\(^{76,105}\) Drugs that act as CYP3A inducers and have the potential to reduce cyclosporine blood concentrations through increased metabolism include phenobarbital, rifampin, phenylbutazone, phenytoin, and carbamazepine.\(^{35}\) Different glucocorticoids also have the potential to either increase or decrease cyclosporine blood concentrations. Prednisone and methylprednisone are CYP3A inhibitors, while dexamethasone is a CYP3A inducer.\(^{105}\)
Natural biological products have also been found to affect CYP3A, and thus cyclosporine blood concentrations. Grapefruit contains compounds called furanocoumarins that inhibit intestinal CYP3A enzyme activity.\textsuperscript{106} This effect was shown in a study that documented increased peak blood cyclosporine concentrations and area under the curve (AUC) for human subjects that drank grapefruit juice before and after oral cyclosporine dosing. These subjects experienced increased cyclosporine bioavailability after oral dosing, but no change in drug clearance. No effect on intravenous cyclosporine pharmacokinetics was noted, suggesting the effect to be mediated during drug absorption, likely by alteration in intestinal CYP3A activity.\textsuperscript{107} In dogs, cyclosporine administered with 100 mL of liquid grapefruit juice or 10 g of freeze-dried grapefruit juice approximately doubled the maximum drug concentration (Cmax) in 3 dogs, and AUC increased by 25\% and 27\% for liquid and freeze-dried grapefruit, respectively.\textsuperscript{108} In another study of 6 dogs, powdered grapefruit significantly increased AUC by 54\%, but utilized a large dose of grapefruit powder (10 g) that was not considered to be cost-effective.\textsuperscript{109} In contrast, St. John’s Wort, an herb used for depression in people, has been shown to induce CYP3A enzymes and P-glycoprotein in humans and rats.\textsuperscript{110} Consistent with these findings, St. John’s Wort decreased cyclosporine AUC and Cmax after 7 days of co-administration in dogs, and increased cyclosporine clearance.\textsuperscript{111} The role of P-glycoprotein in drug interactions is less clear, but many of the drugs that affect CYP3A function also have a parallel effect on the function of P-glycoprotein. Ketoconazole and the other azole antifungals inhibit both CYP3A and P-glycoprotein, while St. John’s Wort and rifampin induce both CYP3A and P-glycoprotein. Other drugs
recognized as inhibitors of P-glycoprotein include verapamil, diltiazem, erythromycin, quinidine, fluoxetine, and spinosad.\textsuperscript{112} Other drugs that are substrates, and thus could compete with cyclosporine for efflux pumps such as P-glycoprotein, include digoxin, colchicine, and fexofenadine.\textsuperscript{113} Despite the number of shared inhibitors, studies have found poor correlations between the concentration of drug that inhibits P-glycoprotein and CYP3A4 function by 50\% (IC\textsubscript{50}) for the multiple inhibitors tested, possibly because of variability in \textit{in vitro} measurements of P-glycoprotein activity.\textsuperscript{114-116}

Table 2.1  Drugs potentially affecting cyclosporine blood concentrations

<table>
<thead>
<tr>
<th>Increase cyclosporine</th>
<th>Decrease cyclosporine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetazolamide</td>
<td>Erythromycin</td>
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<tr>
<td>Allopurinol</td>
<td>Estrogens</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>Fluvoxamine</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>Glipizide/glyburide</td>
</tr>
<tr>
<td>Azole antifungals</td>
<td>Grapefruit juice/powder</td>
</tr>
<tr>
<td>Bromocriptine</td>
<td>Imipenem</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>Losartan/valsartan</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>Medroxyprogesterone</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>\textit{Methylprednisolone}</td>
</tr>
<tr>
<td>\textit{Cimetidine}</td>
<td>\textit{Metoclopramide}</td>
</tr>
<tr>
<td>Ciprofloxacin/enrofloxacin</td>
<td>Metronidazole</td>
</tr>
<tr>
<td>Cisapride</td>
<td>Midazolam</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Omeprazole</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>Prednisone/prednisolone</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Sertraline</td>
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<tr>
<td>Danazol</td>
<td>Tinidazole</td>
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<tr>
<td>Digoxin</td>
<td>Vitamin E</td>
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</tbody>
</table>

Table showing drug interactions affecting cyclosporine blood concentrations. This list includes drugs affecting both CYP3A and P-glycoprotein, and others with unconfirmed mechanisms. Items listed in italics have been shown to be of limited relevance for dogs.\textsuperscript{44,97,105,117}

Despite a multitude of possible drug interactions, several studies have shown the more minor drug interactions (that is, not azoles) to have a limited effect on cyclosporine
pharmacokinetics. The clinical significance of increased cyclosporine blood concentrations caused by drug interactions is also less in dogs than in people because elevated cyclosporine concentrations do not carry the risk of nephrotoxicity found in human medicine.44 No interaction was shown between methylprednisolone (1 mg/kg/day) and high dose cyclosporine (20 mg/kg/day) when the drugs were co-administered to dogs for 2 weeks in a drug safety study, though full pharmacokinetic details were not described.29,44 Metoclopramide is reported to increase cyclosporine AUC in humans, but a similar finding was not reported in a study of 8 dogs.109 Similarly, cimetidine is a potent hepatic microsomal enzyme inhibitor, but only delayed the absorption of cyclosporine in dogs, and did not affect total drug exposure.118 Individual dog variability in blood concentrations at similar drug doses is consistently high, however, even with the microemulsified formulation of cyclosporine.44,97,118 Therefore, blood cyclosporine concentration monitoring should be considered with co-administration of potentially interacting drugs if drug interaction is suspected based on therapeutic failure or adverse events, or when treating more life-threatening diseases where obtaining adequate cyclosporine blood concentrations has greater clinical significance.

In addition to being subject to a number of drug interactions, cyclosporine can also alter the blood concentrations of other medications. Cyclosporine is a competitive inhibitor of P-glycoprotein.119 Cyclosporine also inhibits the same CYP enzymes it is metabolized by,120,121 and has been shown to reduce the activity of the organic anion transporter (OAT) 1B1 (also known as OATP2) located on the hepatocyte basolateral membrane.122,123 Inhibition of P-glycoprotein, CYP enzymes and OAT1B1 is suspected
to, in combination, contribute to the increased blood concentrations of statins found in patients also treated with cyclosporine. Similar concentration increases for the antidiabetic drug repaglinide were noted in humans given cyclosporine, and this was attributed to cyclosporine-mediated inhibition of OAT1B1 and CYP3A4. These effects are drug specific, however, as cyclosporine co-administered with diltiazem, another CYP3A and P-glycoprotein substrate, does not affect diltiazem pharmacokinetics in people, although blood cyclosporine AUC and peak concentrations are increased.

Drug-drug interactions are even more difficult to predict in veterinary medicine, where less is known about cytochrome metabolic pathways and variations in P-glycoprotein or OAT affinity. One drug-drug interaction investigation using canine hepatic microsomes showed some CYP2B11 inhibition by cyclosporine, and a greater effect on CYP3A12 and CYP3A26, but all the substrates of these cytochromes in dogs have not been determined. Caution should be taken when administering cyclosporine with other drugs metabolized and excreted through similar pathways, especially those with narrow therapeutic indexes such as doxorubicin, vincristine, and digoxin, which all require P-glycoprotein for excretion.

Monitoring

Since cyclosporine has a narrow therapeutic index, monitoring of blood drug concentrations has been a priority since the drug was introduced to human medicine. Interestingly, since chemical assays or immunoassays were not initially available, the first assays of cyclosporine’s effects were bioassays measuring the drug’s effects on lymphocyte responses to mitogens. In the first report of human renal cadaveric transplants (1978), Calne and others reported taking weekly serum samples and
incubating transplant patient serum with healthy lymphocytes to determine the serum’s ability to inhibit proliferation induced by phytohemagglutinin (PHA). Analytical techniques were soon developed, and a tremendous amount of literature is published regarding different methods and sample timings in an effort to correlate blood concentrations with clinical outcome. Throughout the drug’s use, there have also been studies evaluating pharmacodynamic assays as a way to investigate immune responses to cyclosporine to possibly explain some of the variability in clinical outcomes that has been appreciated despite attainment of similar blood concentrations. This section will first review assays used for blood concentration determination and strategies for cyclosporine monitoring, and will then proceed to a discussion of pharmacodynamic monitoring efforts in humans and animals.

**Pharmacokinetics**

**Analytical Methods**

High performance liquid chromatography (HPLC) was the first technique published for cyclosporine quantitation, and is still considered the gold standard. HPLC is able to individually identify cyclosporine and its metabolites, and the metabolites were originally named in part by their elution times on the chromatograph. As the metabolite chemical structures have been elucidated, metabolite names have since changed to reflect the position of chemical modification and the action performed (hydroxylation or N-demethylation). According to Wang and others, “HPLC is considered to be the most difficult to perform, the most accurate, the least precise, and the most time consuming” assay.
Shortly after the development of an HPLC assay, a radioimmunoassay (RIA) was created with polyclonal antibodies harvested from rabbits inoculated with cyclosporin C. Significant cross-reactivity with the parent cyclosporin A and its metabolites was recognized. Other polyclonal immunoassays were developed, and though easier to perform, the results were consistently higher than those found with HPLC, and sometimes led to clinically significant differences. This was noted by Burckart and others in a study of pediatric liver transplant patients, where poor liver function in the first week after transplantation led to lower metabolite excretion and much higher RIA results than those found with HPLC.

A more specific monoclonal antibody was then developed by Quesniaux and others at Sandoz, and was incorporated into both radioimmunoassays and a fluorescence polarization immunoassay (FPIA). These more specific RIAs and the FPIA showed improved correlation with HPLC, and were simpler to perform. The TDx FPIA developed by Abbott performed particularly well, and decreased sample processing time for 20 samples to approximately 30 minutes (versus 2-4 hours for the monoclonal RIA), and lowered detection limits, with cross-reactivity with metabolites of 15.3% for AM9, 8.2% for AM1, 3.7% for AM4N, and less than 3% for the other metabolites. The TDx FPIA had values approximately 24% higher than those evaluated with HPLC in one study. Newer assays have since been developed including a chemiluminescent polarization immunoassay (ARCHITECT, Abbott) that has replaced the TDx assay, and a chemiluminescence immunoassay (ADVIA Centaur®, Siemens), as well as a magnetic particle immunoassay with photometric detection (ACMIA). These assays report even better correlation with HPLC, and limited metabolite cross-
reactivity. One study showed ACMIA results to be only 1.7% higher than those determined with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Interference is still a potential issue for immunoassays, however, as was noted for the ACMIA assay, when a patient’s blood drug concentrations were still in the therapeutic range after stopping cyclosporine treatment. The issue was suspected to be interference from anti-animal antibodies that may have developed while the patient was being treated with thymoglobulin, which contains rabbit immunoglobulin. Immunoassay interference, though uncommon, is most often the result of circulating anti-animal antibodies and lower affinity heterophile antibodies, and can be blocked by incubating the serum with a heterophilic blocking reagent.

Presently, in veterinary medicine, both HPLC and immunoassays are reported. HPLC seems more common for post-transplant monitoring and some research projects, while the large reference laboratory that is currently measuring cyclosporine blood concentrations uses a magnetic particle immunoassay. Importantly, as described above, different assays will have different levels of cross-reactivity with cyclosporine metabolites. The impact of cross-reactivity is less significant for newer methods, but with the old TDx assay, Cmax and AUC were 1.5-1.7 times higher than results found with HPLC for dogs dosed with oral cyclosporine, and the assay was reported to cross react with AM1 and AM9 in dogs. It is recommended to refer to the reference ranges provided by the individual laboratories when interpreting cyclosporine blood concentrations, but variability in blood concentrations achieved through different methods has long been recognized as a significant contributor to the difficulty in establishing a therapeutic range for cyclosporine.
Sample/Matrix

Initially, there was controversy regarding which sample was best for measuring cyclosporine concentrations. Whole blood and plasma were used, but the amount of cyclosporine found in plasma was quickly recognized to vary with temperature, with lower plasma concentrations at lower temperatures.\textsuperscript{144} A 30-minute equilibration at 37°C was recommended prior to separating the plasma, but even this did not solve the issue of poor correlation between blood and plasma drug concentrations.\textsuperscript{145} Plasma concentrations also varied inversely with hematocrit,\textsuperscript{146} which is logical given that 41-58% of blood cyclosporine is in erythrocytes, while only 33-47% of the drug is found in plasma.\textsuperscript{36,41} Proponents of using plasma cyclosporine concentrations proposed that results were more representative of the free drug concentration, and potentially more likely to correlate with toxicity and efficacy.\textsuperscript{87,147} Whole blood concentrations correlated better with kidney concentrations than plasma,\textsuperscript{148} however, and ultimately to better standardize measurements and reduce variation across monitoring centers, whole blood measurement became recommended.\textsuperscript{129,149}

More recently, researchers have quantified intracellular cyclosporine in lymphocytes and peripheral blood mononuclear cells (PBMC).\textsuperscript{150-153} Crettol and others showed that lymphocyte cyclosporine concentrations were only moderately correlated with whole blood concentrations. P-glycoprotein SNPs have a stronger effect on lymphocyte cyclosporine concentration than whole blood drug concentration, suggesting P-glycoprotein activity as potentially an important factor in individual immune responses to cyclosporine.\textsuperscript{154} Supporting the importance of lymphocyte cyclosporine concentrations, decreased intracellular cyclosporine was identified by Falck and others 3
days before traditional measures indicated kidney rejection, while whole blood levels were unchanged. In addition, patients that experienced organ rejection within the first three months also had lower intracellular cyclosporine AUC₀₋₁₂ than patients not experiencing rejection. Measurement of intracellular cyclosporine, however, shows high inter- and intra-patient variability.¹⁵⁵

**Trough**

The most traditional method of cyclosporine monitoring is through trough drug concentration evaluation before the next dose is administered. One of the individuals most integral to optimizing cyclosporine monitoring strategies is Barry Kahan. Initially, Kahan proposed the trough concentration as the preferred time for measurement because it was the most reproducible value and less likely to be affected by variability in drug absorption and distribution,⁸⁷ which was a particular issue for the Sandimmune® formulation. In a study of 118 patients, Kahan found trough concentrations to be better correlated with clinical events than peak concentrations.¹⁵⁶ He proposed that peak concentrations were not well correlated with toxicity, but acknowledged it was yet to be determined if achieving a certain peak was needed to prevent rejection.⁸⁷,¹⁵⁶ Two other early studies showed reasonable to good correlations between cyclosporine plasma concentrations and both rejection and toxicity,¹⁴⁷,¹⁵⁷ but most other efforts struggled to find a clearly defined therapeutic range for cyclosporine.¹³⁵ Indeed, despite significant investigation into different pharmacokinetic parameters, frustration with poor correlations with both nephrotoxicity and rejection led The Ohio State University to publish an article titled “Cyclosporine levels are not helpful.”¹⁵⁸,¹⁵⁹
As described below, Kahan and others went on to investigate other monitoring strategies, including AUC and peak concentrations. These measures, and the development of the microemulsified cyclosporine formulation, led to improved pharmacokinetic-clinical correlations. Debate persists today, however, regarding the best strategy for therapeutic drug monitoring, and trough drug concentrations are still frequently used.140,160,161

Trough concentrations are also the only reasonably well-described target in veterinary medicine. Whole blood trough concentrations between 300-500 ng/mL27,141 and 400-700 ng/mL26 are recommended by different authors for renal transplantation in dogs, while target feline post-transplant concentrations are lower at 250-400 ng/mL162 and 300-500 ng/mL.25 Therapeutic drug monitoring is also proposed when treating anal furunculosis in dogs. Initial recommendations were trough whole blood concentrations between 400-600 ng/mL as measured by monoclonal RIA,163 but subsequent efforts to reduce cost through combined treatment with ketoconazole achieved success by targeting trough whole blood concentrations of at least 200 ng/mL as measured by HPLC.101 Less information is available for other inflammatory diseases, but Dawn Boothe’s clinical pharmacology laboratory at Auburn University recommends trough concentrations of 400-600 ng/mL for immune-mediated diseases and 250 ng/mL for chronic inflammatory disorders like inflammatory bowel disease39 Trough blood concentrations, however, have not been shown to correlate with clinical efficacy in cases of atopic dermatitis or anal furunculosis.45,164
**Area Under the Curve**

Subsequent studies in human medicine showed that multiple blood samplings and calculation of the AUC or average steady state concentration (AUC/dosing interval in hours) was more highly correlated with clinical outcomes, and the dose administered, than trough sampling.\(^{165-168}\) Unfortunately, AUC measurements require repeated sampling, which is more labor intensive, expensive, and uncomfortable to the patient. Significant effort (38 papers as of 2001)\(^{169}\) was therefore directed at establishing limited sampling strategies that still correlated with AUC. Measurements at 2, 6, and 24 hours,\(^ {170}\) 2, 6, and 14 hours,\(^ {171}\) 3.5, 8, and 10 hours,\(^ {172}\) and others were proposed, and equations derived to approximate full measurement AUC values.

The introduction of the Neoral\(^ \text{®}\) formulation in 1995 improved cyclosporine absorption and reduced pharmacokinetic variation. Shorter sampling strategies such as the AUC\(_{0-4}\) were investigated and found to correlate well with both rejection and toxicity.\(^ {173-175}\) Indeed, in a prospective trial, Mahalati and others were able to propose a therapeutic window (AUC\(_{0-4}\) 4400 to 5500 μg/h per L) where only 1/33 (3%) patients that achieved the target blood concentration experienced rejection, while 10/22 (45%) patients lower than the window experienced acute rejection. In addition, no patients in the therapeutic range experienced cyclosporine nephrotoxicity, while four patients above the range experienced nephrotoxicity. Trough concentrations and weight-adjusted cyclosporine dose both correlated poorly with AUC\(_{0-4}\).\(^ {174}\)

AUC is still considered the most reliable method for cyclosporine monitoring in human medicine,\(^ {161,176}\) but is infrequently used because of cost and inconvenience. No
veterinary studies have attempted correlating cyclosporine AUCs to clinical response to the author’s knowledge, likely for similar reasons.

**Peak**

Once troughs were recognized to provide an inaccurate predictor of response to cyclosporine, single samples at times other than trough began to be investigated in parallel with the above described AUC limited sampling methods. Initial investigations for Sandimmune® suggested sampling times of 5 or 6 hours after dosing as being better at reflecting AUC and clinical outcomes. With Neoral®, in contrast, peak blood drug concentrations reliably occur between 1-2 hours after oral dosing. In his review on the progress of therapeutic drug monitoring of cyclosporine, Kahan reminds the reader of the mathematical concept that the maximum concentration will most closely correlate with the AUC. This was more difficult to establish for Sandimmune® where the time to maximum blood concentration (Tmax) can be from 2 to 6 hours (and likely why single peak sampling times as late as 5-6 hours post-dosing were proposed as best in earlier studies), but application of this principle suggested 2 hours as potentially the most optimal time for Neoral® monitoring.

In support of this, peak (2 hours post oral administration) blood concentrations were found to better correlated with AUC than trough levels in multiple studies. Many studies have focused on the therapeutic benefit of C2 (peak) over C0 (trough) monitoring long term, with various studies showing equal to better performance for C2 monitoring. Citterio and others reported a clinical benefit to cyclosporine monitoring after heart transplantation for 69.3% of patients monitored with C2, versus 43.3% monitored with C0, though mortality was not different between the two periods.
study of liver transplant patients noted lower rates of acute rejection in a C2 monitored group, lower incidence of rejection in patients that reached target C2 levels by 3 days, and a lower incidence of moderate to severe changes in biopsy-proven acute rejections than with C0 monitored patients. In renal transplants, C2 monitoring resulted in a low incidence of biopsy-proven acute rejection in the MO2ART study, and a long-term management study showed C2 monitoring allowed dose reductions in 49% of the assessed patients, and this improved serum creatinine and lowered blood pressure in 54% of that patient subset.

One limitation of peak sample evaluation is that some of the assays for cyclosporine blood concentration are not able to measure the high blood concentrations found at peak times, and thus require sample dilution. This is the case for the Abbott ARCHITECT assay, which requires samples to be diluted at concentrations greater than 1500 ng/mL. The ACMIA that is also commonly used requires samples to be diluted at concentrations higher than 2000 ng/mL. As an example of typical peak levels, the MO2ART study targeted cyclosporine peak concentrations of 1600-2000 ng/mL in the first month after transplant. Dilution protocols were evaluated in 2004, and were shown to increase variability in drug monitoring results. Dilution protocols are better described for more modern systems, but still have the potential to influence results.

In veterinary medicine, peak concentrations are just beginning to be evaluated. Little enough information is available regarding target trough concentrations for different disease states, and even less information is available to make peak recommendations. Dr. Boothe at Auburn recommends a target peak concentration of 800-1400 ng/mL or a trough concentration of 400-600 ng/mL for diseases where high immunosuppression is
required. She goes on to state that, based on the short half-life of cyclosporine in dogs (approximately 5 hours), peaks of 1600-2400 ng/mL may be required to maintain desired trough concentrations, or even as high as 2600-3000 ng/mL if a trough of 750 ng/mL is desired after transplantation.\textsuperscript{39} More work in veterinary medicine is needed to determine if peak or trough concentrations correlate with outcome in clinic patients treated with cyclosporine for non-dermatologic diseases.

Despite the strong evidence in humans that C2 blood concentrations are more representative of AUC and cyclosporine exposure than trough, and the growing evidence that C2 may be better correlated to outcome, as of 2011, Peter and others wrote “Most transplantation centers, including ours, use the whole-blood trough concentration to adjust cyclosporine dosing.”\textsuperscript{140} Another C0 supporter, Filler, commented that the better correlation of C2 with AUC did not imply good agreement, and cited substantial variation noted from a Bland-Altman analysis. Filler considered the precise sampling required for peak concentrations to be an issue in pediatric transplantation, and suggested trough monitoring to be more “robust.”\textsuperscript{188} A critical evaluation of the two randomized controlled trials evaluating C2 monitoring in liver transplant patients (including the study described above) present at the time of writing in 2006 noted several study limitations that limited the strength of the conclusions drawn, including low power of the studies, high study withdrawal, and lack of information on dose adjustments.\textsuperscript{189} Figures from a survey of monitoring practices in 2004 reported 97.3\% of laboratories were evaluating EDTA-anticoagulated whole blood, and that 66\% of centers were using C0 monitoring alone, 6\% C2 alone, and 24\% C2 in combination with C0 or AUC.\textsuperscript{187} Unfortunately,
more recent data cannot be found, possibly because of a recent trend in human transplant medicine to use tacrolimus instead of cyclosporine, especially in the United States.

**Pharmacodynamics**

Pharmacodynamic assays evaluate the influence of cyclosporine on its therapeutic target, the T cell. Achievement of pharmacokinetic targets may be less clinically important than attaining adequate suppression of T cell function, which can be measured with pharmacodynamic assays. Importantly, achieving a certain blood cyclosporine concentration does not guarantee sufficient immune suppression, and conversely a given concentration may cause excessive suppression in an individual, increasing the risk for infection and malignancy. The reasons for this variability in individual response are less understood, but may include factors such as variability in P-glycoprotein and calcineurin activity within lymphocytes among individuals. Pharmacodynamic assays are also useful because some are able to identify the cumulative effect of co-administered immunosuppressive medications, and have been used to evaluate drug mechanisms and interactions.190-193

As mentioned above, it is interesting that the initial method for blood cyclosporine determination was a pharmacodynamic assay evaluating lymphocyte proliferation.2,19 There are many types of pharmacodynamic assays available to evaluate cyclosporine response, with most measuring some aspect of lymphocyte activation and cytokine response, since impaired IL-2 production (necessary for T cell activation and proliferation) is the primary mechanism of action of cyclosporine. Lymphocyte proliferation, surface antigen expression, cytokine production, and calcineurin activity
have all been evaluated in human clinical patients. These investigations and the preliminary work available in veterinary medicine are described below.

**Proliferation Assays**

The first bioassays for cyclosporine blood concentration determination measured proliferative responses of lectin-stimulated lymphocytes treated with cyclosporine via $[^3\text{H}]$-thymidine incorporation. Greater inhibition of proliferation corresponded to a higher cyclosporine blood concentration. Subsequent work confirmed that cyclosporine inhibited proliferation induced not only by the lectin PHA, but also by OKT3 (an anti-CD3 monoclonal antibody) and mixed lymphocyte culture. Spontaneous blastogenesis (proliferation) assays were also used in the 1980s to identify “strong immune responders”, that were shown to be at greater risk of transplant rejection than weak responders, when treated with azathioprine and prednisone. Cyclosporine decreased immune responses of strong responders to the level of weak responders, and decreased rejection episodes relative to azathioprine. Hibbins and others used a different approach and evaluated lymphocyte proliferation in patient lymphocytes activated *ex vivo* with PHA and incubated with varying concentrations of methylprednisolone and cyclosporine. A steeper gradient of inhibition indicated a greater response to the drug, and renal transplant patients with a steeper gradient were shown to be more responsive to methylprednisolone and cyclosporine after transplantation. The authors suggested this approach as a way to predict patient response, though gradients were not significantly different among patients that rejected their transplants and those that did not.

Proliferation assays are a commonly used *in vitro* technique to evaluate the effects of various immunosuppressive drugs, with many studies determining the IC$_{50}$, which in
this context reflects the concentration of drug required to inhibit cell proliferation by 50%. These *in vitro* proliferation assays have also been used to explore drug interactions, and evaluate the effects of combination immunosuppressive therapy at various drug concentrations. Similar to the approach of Hibbins, IC$_{50}$ determinations after concanavalin A-stimulated blastogenesis were used to identify resistance to cyclosporine and glucocorticoids in ulcerative colitis patients, and patients with low glucocorticoid sensitivity *in vitro* were found to require higher prednisone doses for control of clinical signs.

More recently, proliferation assays have been included in pharmacodynamic panels investigating patient immune responses after transplantation. Tritiated thymidine is still used occasionally to measure proliferation, but flow cytometry has become popular for its simplicity, with the most common assay using proliferating cell-nuclear antigen (PCNA) and propidium iodide. Studies have shown reduced lymphocyte proliferation in transplant patients relative to healthy controls, and reductions in lymphocyte proliferation at peak compared to trough cyclosporine levels. Barten and others used lymphocyte proliferation, surface antigens, and cytokines to monitor immune suppression in patients changed from cyclosporine to tacrolimus and sirolimus, and found proliferation to be among the pharmacodynamic parameters with the strongest correlation to cyclosporine blood concentrations. Since proliferation is known to be impacted by cyclosporine and other drugs given after transplantation, such as steroids, mycophenolate, sirolimus, and leflunomide, proliferation assays provide a more global assessment of immune function during multi-drug treatment.
Markers of Activation

Surface Antigens

Various antigens are expressed on the surface of T cells, and change once T cells receive an activation signal through the T cell receptor and costimulation through CD28 by an antigen presenting cell. Common markers of T cell activation include: CD25, the alpha subunit of the IL-2 receptor; CD95, the FAS receptor that signals apoptosis; CD134 (OX40), necessary for T cell survival after costimulation; CD154, the activating ligand for CD40 found on antigen presenting cells; CD11a, important for adhesion and diapedesis; CD69, important for cell activation, proliferation, and differentiation; and CD71, the transferrin receptor. Various combinations of surface antigens are commonly included in pharmacodynamic assays, and two studies by Barten showed lymphocyte proliferation and surface antigens were in general better correlated with cyclosporine blood concentrations than cytokines in human heart transplant recipients, and also showed lower surface antigen expression at peak versus trough blood concentrations. A study by Stalder and others also showed lower surface antigen expression (CD25, CD95, CD71, CD11a, and CD154) after oral cyclosporine therapy. Another study showed higher levels of CD4 positive, IL-2 receptor positive peripheral blood lymphocytes in heart transplant patients prior to a rejection episode, while IL-1 and IL-2 were unchanged. As with proliferation assays, surface antigens can be influenced by co-administered medications. A study validating human pharmacodynamic assays found concentration-dependent inhibition of CD25 and CD71 by cyclosporine, tacrolimus, sirolimus, mycophenolic acid, and methylprednisone when incubated in vitro, although sirolimus only had a small effect on CD25.
Cytokines

As far back as 1985, a study by Yoshimura and Kahan identified decreased IL-2 production from T cells of renal transplant patients treated with cyclosporine, and correlated higher IL-2 production (reflecting less effective cyclosporine-mediated suppression) with episodes of rejection.\textsuperscript{212} NFAT-regulated cytokines, especially IL-2, IFN-\(\gamma\), TNF-\(\alpha\), and granulocyte macrophage colony stimulating factor (GM-CSF) have since become popular pharmacodynamic measures of cyclosporine’s effects. Although glucocorticoids can also decrease transcription of cytokines, one immunologic biomarker validation study found that \textit{in vitro} inhibition of IL-2 was significant only for the calcineurin inhibitors cyclosporine and tacrolimus, and not for methylprednisolone, mycophenolic acid, or sirolimus.\textsuperscript{207} In this study, samples were activated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours, and cytokines were measured with flow cytometry.\textsuperscript{207} Freed and others showed methylprednisolone sodium succinate did inhibit IL-2 production after concanavalin A (ConA) stimulation for 24-32 hours,\textsuperscript{213} however, so the influence of concurrent medications is likely affected by the method and duration of stimulation. As another example, IL-2 messenger ribonucleic acid (mRNA) expression was suppressed in a concentration-dependent manner by cyclosporine, but was not affected by incubation with prednisolone or mycophenolic acid after a 2-hour incubation with the drug and PMA plus ionomycin.\textsuperscript{214} Though not as specific as calcineurin inhibition, cytokine monitoring most likely still offers a more targeted reflection of the effects of cyclosporine on the patient than surface antigens or lymphocyte proliferation.
Monitoring of IL-2, IFN-γ, and other cytokines has been frequently performed *in vitro* to evaluate immunosuppressive drug effects, and *ex vivo* after transplantation. Brunet and others showed peak but not trough IL-2 levels as measured by ELISA were significantly lower in transplant patients treated with cyclosporine than levels in healthy control patients. They also included a cyclosporine plus mycophenolate group where both peak and trough IL-2 were lower than healthy controls, and the peak cyclosporine plus mycophenolate also had significantly lower IL-2 than cyclosporine alone. These patients were also all treated with steroids. In another study by Brunet of patients on cyclosporine, mycophenolate, and steroids, IL-2 and IFN-γ expression 7 days after transplantation were significantly lower than healthy controls pre-dose and 2 hours after dosing. At 6 months after transplantation, IFN-γ pre-dose was no longer significantly different, while the other cytokine parameters remained significantly decreased. Barten and others used flow cytometry to show significantly lower expression of IL-2, IFN-γ, and TNF-α at peak relative to trough cyclosporine levels, while IL-4 was not significantly different, in heart transplant patients. In a similar study of cytokines by Barten and others, however, although all trended to decrease, only IL-2, IL-4, and IL-10 were significantly lower for the peak versus trough sample, and IFN-γ, TNF-α, and IL-8 were not significantly different. Hodge and others evaluated intracellular cytokines after lung transplantation and found reduced T cell IL-2 and TNF-α, but increased IL-4 relative to controls. They also specifically evaluated the CD4 and CD8 positive T cell subsets and found reduced IFN-γ expression by CD4 but not CD8 positive cells, and increased TGF-β expression by CD8 positive cells.
As described here, two approaches for cytokine monitoring are either to compare to a healthy control, or to compare the pre-dose and 2-hour sample. The general trend is for lower NFAT-regulated cytokine expression at peak cyclosporine blood concentrations relative to trough, and for lower cytokine levels after transplantation relative to healthy controls, though some cytokines like IL-4 are variable. A notable cytokine exception is TGF-β, which increases with cyclosporine therapy, and is higher at peak than trough cyclosporine levels. Cytokine levels do not always reflect clinical events, however. As mentioned earlier, Yoshimura and Kahan associated higher IL-2 with rejection, but other studies failed to show a correlation between IL-2 and IFN-γ and rejection, and even found lower IL-2 mRNA expression prior to rejection in some patients. Others have shown increased IFN-γ relative to pre-transplant levels in patients with acute kidney rejection.

Notable approaches to cytokine measurement include Härtel and others, who used a more physiologic activating stimulus (anti-CD3 and anti-CD28 monoclonal antibodies) and evaluated IL-2, IL-4 and TNF-α mRNA for different durations of incubation (0, 4, 8 and 24 hours). This group found a delayed increase in cytokine mRNA expression during costimulation in patients treated with cyclosporine, and proposed the “area of cytokine mRNA expression over time” as a potentially more sensitive way to assess immunosuppressive drugs. The work of Giese, Zeier, Meuer and others at the University of Heidelberg also suggests a promising method for cytokine monitoring. This group calculates the mean residual gene expression for IL-2, IFN-γ, and GM-CSF by dividing the peak adjusted number of gene transcripts by the trough gene transcripts, and averaging the values for the three cytokines evaluated. This method is suggested
to be more specific for cyclosporine because patients take other immunosuppressive medications separate from this two hour window, and their assay, which uses PMA and ionomycin stimulated cytokine expression, is reportedly insensitive to the effects of steroids and azathioprine in vitro. With this approach, a mean reduction in cytokine expression of 85% was found at two hours, and patient gene expression had recovered to trough levels by six hours after dosing. A lower residual gene expression (and thus greater immunosuppression) has been associated with a higher risk of both infection and neoplasia, and with a greater risk of non-melanoma skin cancer. These authors have successfully tapered cyclosporine therapy in stable renal transplant patients based on residual gene expression, which improved patient blood pressure and prevented the worsening of renal allograft function that was identified in the control (non-tapered) group. Based on this study and others, the authors suggested a target of 20-30% residual NFAT-regulated gene expression in long-term stable renal transplant patients, which is supported by an increased incidence of skin cancer in patients with gene expression below 15%, and an acute rejection episode documented in a patient with residual gene expression above 40%. 

**Calcineurin Inhibition**

Measuring calcineurin inhibition is the most specific measure of the direct effects of cyclosporine and tacrolimus, but is more technically challenging. In T cells, calcineurin activity is regulated by the presence of intracellular calcium, which is increased after T cell receptor binding. In vitro assays are performed with an excess of all reagents, and thus do not fully reflect actual intracellular calcineurin activity. Protocols involve measuring calcineurin’s ability to dephosphorylate a 19 amino acid
peptide that is part of the bovine cardiac cyclic AMP-dependent protein kinase regulatory subunit (RII). Other serine-threonine phosphatases (PP1, PP2A, and PP2C) can also dephosphorylate RII, so okadaic acid is included to inactivate PP1 and PP2A, and some protocols include either cyclosporine or ethyleneglycoltetraacetic acid (EGTA), the latter to chelate calcium, with both blocking calcineurin action and thus revealing PP2C effects.\textsuperscript{210} HPLC with ultraviolet detection can be used to identify the dephosphorylated peptide, a scintillation counter can be used to measure $^{32}$P if the peptide is radiolabeled, and a colorimetric assay with malachite green has also been used.\textsuperscript{161,210}

Calcineurin inhibition has been shown to parallel cyclosporine blood concentrations, and occurs rapidly after cyclosporine dosing.\textsuperscript{230} Halloran and others showed calcineurin levels to be significantly suppressed by 1 hour after cyclosporine administration and to remain suppressed through 4 hours after dosing, while 6, 8, and 12 hours were not significantly different than pre-dose calcineurin levels.\textsuperscript{230} Koefoed-Nielsen and others also examined the temporal profile of calcineurin inhibition after oral cyclosporine, and found a significant decrease by 1 hour, maximal suppression at 3 hours but, at the end of sampling at 6 hours, calcineurin levels did not return fully to baseline and were still significantly different than pre-dose.\textsuperscript{231} In both of these studies, an inverse correlation between cyclosporine concentration and calcineurin was identified. In another study of renal transplant patients, however, calcineurin inhibition was variable, and no correlation was identified between calcineurin inhibition and cyclosporine blood concentration at 0 or 2 hours, or between calcineurin inhibition and cyclosporine AUC\textsubscript{0-12}.\textsuperscript{232}
Calcineurin inhibition has not been well correlated with patient toxicity, but has been associated with transplant rejection in some transplant patients. Brunet and others showed lower trough calcineurin inhibition significantly correlated with biopsy-proven acute rejection in renal transplant patients on cyclosporine and mycophenolate.²⁰⁵ Fukudo and others identified increased calcineurin activity in patients with acute rejection treated with both cyclosporine and tacrolimus relative to patients that did not experience rejection.²³³ Higher calcineurin activity was identified in patients with graft versus host disease after allogeneic stem-cell transplantation in one study,²³⁴ while in contrast an older study actually found lower calcineurin activity in cyclosporine-treated patients experiencing graft versus host disease.²³⁵

**Other Assays**

Other pharmacodynamic measures have also been used to monitor cyclosporine treatment. The ImmuKnow® Immune Cell Function Assay developed by Cylex with the patent now held by Viracor-IBT Laboratories™ was cleared by the FDA in 2002 as a nonspecific measure of immunosuppression in transplant patients. The test measures adenosine triphosphate (ATP) production from CD4-positive T cells after a 15-18 hour incubation with PHA using luciferin and a luminometer.²³⁶ Lower ATP production reflects greater immunosuppression, and reportedly measures drug-associated immunosuppression regardless of which immunosuppressive drugs are used. Many studies of this assay have been performed, and while some do correlate lower ImmunoKnow® ATP levels with infection and higher ATP levels with rejection,²³⁷-²³⁹ other studies have not found clear associations with clinical outcomes.²⁴⁰,²⁴¹
In a recent study by Barten’s group, dendritic cell numbers were evaluated in heart transplant patients. The study revealed that higher numbers of plasmacytoid dendritic cells were associated with a lower risk of rejection, and that patients treated with tacrolimus had higher plasmacytoid dendritic cell numbers than cyclosporine-treated patients. Further study was recommended to define dendritic cell numbers that would predict rejection.242

**Sample Handling**

Similar to the discussions surrounding the best sample for pharmacokinetic analysis of cyclosporine use, different methods for pharmacodynamic sample evaluation have also been proposed. Older studies used PBMC or cell lines to have a purer cell population for evaluation. More recently, whole blood assays have been investigated to more closely mimic cell populations present *in vivo*, to avoid washing the drug out of the cytoplasm, and to maintain drug distribution among blood components. Whole blood assays showed similar responses as PBMC for proliferation assays in response to *in vitro* drug incubation,199 while cytokine responses, as measured by immunoassay, for PBMC versus diluted whole blood stimulated with PHA depended on the cytokine. Interleukin-6 and GM-CSF responses were similar, TNF-α and IFN-γ were higher in whole blood than PBMC, and IL-1 and IL-2 were higher in PBMC than whole blood.243 In a study assessing the IC$_{50}$ for calcineurin inhibition from *in vitro* cyclosporine, calcineurin inhibition was both significantly greater and more variable in PBMC versus whole blood.230 Another study showed greater calcineurin inhibition by cyclosporine in culture medium than whole blood, and this was attributed to cyclosporine partitioning to non-target areas in whole blood.244 Overall, whole blood assays are preferred where possible,
are generally technically less challenging and time-consuming, and seem to have less variability than assays performed with PBMC.

The activating protocol chosen also has the potential to influence test results. Most pharmacodynamic assays involve stimulating lymphocytes to induce proliferation and expression of activation-related surface antigens and cytokines. The most common T cell mitogens include ConA, PHA, mixed lymphocyte reactions, anti-CD3/anti-CD28 monoclonal antibodies, and PMA plus the calcium ionophore ionomycin. Fortunately, a study using microarrays found very similar gene expression patterns among T cells activated with anti-CD3 beads, PHA, costimulatory beads (anti-CD3/anti-CD28), and PMA plus ionomycin.245 Most studies use PMA and ionomycin for cytokine evaluation, and ConA for cell proliferation and surface antigen evaluation. A recent study attempting to validate techniques for biomarker evaluation in human whole blood suggested evaluating surface antigens after 72 hours incubation with 7.5 μg/mL ConA, and cytokines (IL-2 and TNF-α) after 4 hours with 15 ng/mL PMA and 0.75 μg/mL ionomycin.207

**Pharmacodynamics in Veterinary Medicine**

Very few studies have been performed investigating cyclosporine immune responses in veterinary medicine. A 1986 study showed PBMC from dogs treated with intravenous cyclosporine that were washed free of plasma actually had enhanced blastogenesis in the presence of ConA and PHA, while PBMC from untreated dogs incubated with 10% plasma from treated dogs showed suppression of proliferation that was greatest with plasma from 3 hours after cyclosporine dosing, and returned to baseline when incubated with plasma drawn at 72 hours. PBMC activated with PHA were more
sensitive to the effects of the cyclosporine-treated plasma than those activated with ConA. Results of this study suggested that cyclosporine’s effects on lymphocytes were reversible, and also confirmed that PBMC isolation affects results in canine samples. Recently, a study by Nafe and others evaluated IC$_{50}$ concentrations for canine PBMC incubated with different immunosuppressive agents. They reported an IC$_{50}$ to inhibit proliferation for cyclosporine of 15.8 +/- 2.3 ng/mL, which is significantly lower than whole blood IC$_{50}$ values reported for calcineurin inhibition ex vivo (253 ng/mL)$^{230}$ and IL-2 in vitro (173-340 ng/mL)$^{214}$ in humans, and may again be affected by the lack of non-target cyclosporine partitioning in PBMC in culture media. Strangely, this is also dramatically lower than cyclosporine IC$_{50}$ values for proliferation for both human whole blood (384 ng/mL) and PBMC (345 ng/mL) after PHA stimulation, though Nafe and others measured proliferation using flow cytometry, whereas the human study used tritiated thymidine incorporation.$^{199}$ A low proliferation IC$_{50}$ (55 ng/mL) was also documented for feline diluted whole blood when incubated with cyclosporine and ConA, possibly suggesting species variability.$^{248}$

Cyclosporine’s effects have also been evaluated on cytokine expression in vitro using qRT-PCR. Kobayashi and others showed concentration-dependent suppression of IL-2, IL-4, and IFN-γ in PBMC incubated with ConA and varying concentrations of cyclosporine for 5 hours, but an increase in TNF-α mRNA expression at 810 ng/mL of cyclosporine.$^{249}$ In a study of ConA-stimulated PBMC in cats (samples incubated for 10 hours), in vitro cyclosporine also showed a concentration-dependent inhibition of IL-2, IL-4, IFN-γ, and TNF-α. Cytokines were significantly decreased at cyclosporine concentrations of 150 ng/mL, and reached maximal suppression at 450 ng/mL. 1000
ng/mL of cyclosporine was also associated with a lower frequency of IL-2 secreting cells than untreated samples (measured with enzyme-linked immunospot assay).250

Studies of cyclosporine’s immunological effects in veterinary disease are even more scarce. A study of mRNA expression in tissue from canine anal furunculosis lesions showed significantly lower lesional IL-2 mRNA expression after cyclosporine treatment, and a trend towards lower IFN-γ.251 In Allenspach’s study of cyclosporine for the treatment of inflammatory bowel disease in dogs, reduced T lymphocyte numbers (but not total lymphocytes) were noted on intestinal biopsy samples of dogs performed after 10 weeks of oral cyclosporine therapy.252 Topical ophthalmic cyclosporine’s systemic effects have been evaluated in two studies. Gilger and others identified reduced PHA-stimulated PBMC proliferation after 1 months’ treatment with 2% cyclosporine, that became significantly reduced relative to healthy patients by 3 months.253 In contrast to this finding, Williams identified no effect of cyclosporine 0.2% ointment (Optimmune®) or 2% cyclosporine in corn oil on PHA or ConA driven PBMC proliferation after 1, 3, or 6 months of treatment. The highest blood concentration identified in this study was 15 ng/mL as measured using mass spectroscopy.254 The author is not aware of any studies attempting to correlate pharmacodynamic measures to cyclosporine response in dogs or cats.

**Monitoring Conclusions**

In their attempt to validate immunologic biomarkers, Böhler and others acknowledged that the primary limitation of pharmacodynamic assays is the lack of a target for immune suppression that is well correlated to patient outcome, and commented that this may not even be possible given that individual patients may require different
degrees of immunosuppression for clinical efficacy. Similarly, Barten and others acknowledged although pharmacodynamic assays clearly document a response to immunosuppressive agents, targets for inhibition are not known, and it is also uncertain whether proliferative responses need to be compared to pretreatment levels, or over time.

Thus, although pharmacodynamic assays offer the chance to assess immune responses, there are similar limitations to pharmacokinetic assays, with both assays presenting the greatest opportunity for intervention at the extremes of test responses. A very low blood cyclosporine concentration or very low inhibition of pharmacodynamic parameters increases the potential for treatment failure, while extremely high blood concentrations or complete immune suppression increase the potential for drug side effects, secondary infections, and cancer. Unfortunately, with appropriate blood concentrations and moderate suppression of immune function, it is still possible individual patients may respond unsatisfactorily. Pharmacokinetics and pharmacodynamics are thus likely both necessary to optimize immunosuppressive therapy, and provide complementary information. Prospective pharmacodynamic trials to develop and confirm target biomarker levels are also desperately needed to prove the benefits of immunologic monitoring in transplant medicine. In veterinary medicine, which has poorly defined blood cyclosporine targets and only preliminary investigations into pharmacodynamics, much research is needed to develop assays that can be used to monitor responses in patients treated with immunosuppressive medications, including cyclosporine.
Cyclosporine in Veterinary Clinical Use

The microemulsified formulation of cyclosporine, Atopica®, is approved for the treatment of allergic skin diseases in both dogs and cats at a dose of 5 mg/kg daily in dogs, and 7 mg/kg daily in cats. This includes atopic dermatitis in dogs, and eosinophilic plaques, miliary dermatitis, and self-induced alopecia in cats. Many studies have been performed evaluating cyclosporine for the treatment of atopy in dogs, and have confirmed similar clinical responses to glucocorticoids. Similar studies have confirmed cyclosporine’s efficacy for inflammatory skin conditions in cats. Cyclosporine concentrates in the skin, and this is suggested as a reason for the poor correlation between blood drug concentrations and clinical outcome found by Steffan and others in treating atopic dogs. The other approved formulation of cyclosporine is the ophthalmic preparation, Optimmune®, labeled for the treatment of keratoconjunctivitis sicca in dogs.

Cyclosporine has also been used for the extralabel treatment of other dermatologic conditions in dogs including sebaceous adenitis, pemphigus foliaceus, and anal furunculosis. Cyclosporine was effective in 12 dogs treated for sebaceous adenitis, but did not fully resolve signs of pemphigus foliaceus in any of the 5 dogs reported. Conversely, cyclosporine was found to be glucocorticoid sparing and effective in the treatment of 6 cats with pemphigus. Cyclosporine is well documented as an effective treatment for anal furunculosis, and is often combined with ketoconazole to impair CYP3A metabolism and allow higher blood cyclosporine concentrations to be reached with a lower oral cyclosporine dose. This is especially
important since anal furunculosis frequently affects large breed dogs such as German shepherds and Labrador retrievers.

In addition to its dermatologic uses, cyclosporine is used for other immune-mediated diseases in both dogs and cats. Allenspach and others showed improvement in 12 of 14 dogs with steroid-refractory inflammatory bowel disease treated with cyclosporine at a dose of 5 mg/kg daily. Though these authors measured blood concentrations, they felt that the limited number of samples precluded their ability to assess correlations between drug concentrations and clinical response. Cyclosporine has also been reported for the treatment of immune-mediated polyarthritis in dogs. A retrospective study reported poor success of cyclosporine at 5 mg/kg daily in 3 dogs, but a more recent prospective trial documented successful treatment in 7 of 10 dogs started at a dose of 5 mg/kg cyclosporine every 12 hours, with doses increased if necessary to reach a minimum trough blood cyclosporine concentration of 250 ng/mL if clinical signs were not improved within the first 7-14 days. In this study, some dogs experienced clinical improvement with trough blood cyclosporine concentrations as low as 70 ng/mL. Cyclosporine was also used in the successful treatment of chronic progressive polyarthritis in a female cat.

Less information is available regarding cyclosporine’s use for hematologic disorders, but it is used at higher doses (up to 10 mg/kg every 12-24 hours) for the treatment of immune-mediated hemolytic anemia (IMHA) and thrombocytopenia in dogs. In a study of 276 dogs with IMHA, 10.9% patients received cyclosporine as the primary immunosuppressive drug at a dose of 3-7 (median 5) mg/kg once daily, while 10.5% of dogs received it as a second-line drug at a dose of 2-7 (median 3.3) mg/kg every 12
A retrospective cross-sectional analysis of 88 IMHA dogs reported cyclosporine as an adjunctive treatment in 27% dogs, with a typical dose of 9.4 mg/kg once daily or 6.15 mg/kg twice daily. In this study, there was no significant effect on survival to discharge for cyclosporine coadministration. A small, 38 patient, prospective, double-masked randomized controlled trial showed no benefit of cyclosporine (3-5 mg/kg every 12 hours) plus prednisone (2-4 mg/kg every 24 hours) over prednisone alone on survival, though four of the prednisone only group relapsed, versus no dogs in the cyclosporine plus prednisone group. Limited evidence is available for the treatment of immune-mediated thrombocytopenia, but one abstract reports successful treatment in 3 of 4 dogs refractory to prednisone at a cyclosporine dose of 12-17 mg/kg/day increased to achieve a trough of 400-600 ng/mL, though one dog died of systemic aspergillosis. Cyclosporine has also been reported in combination with prednisolone for the treatment of pure red cell aplasia in cats and was successful for the treatment of 1 cat with immune-mediated thrombocytopenia.

Extralabel cyclosporine is also used for other inflammatory conditions. Cyclosporine at a dose of 2.5 mg/kg twice daily was successful in reducing stomatitis scores of cats that had persistent stomatitis after full or partial mouth extractions in a randomized, double-masked, placebo-controlled study. Interestingly, 72.3% of cats with a cyclosporine trough greater than 300 ng/mL had clinical improvement, versus 28.2% in cats with blood cyclosporine concentrations less than 300 ng/mL. A randomized, placebo-controlled trial of cyclosporine for the treatment of glomerulonephritis at a dose of 10 mg/kg every 24 hours adjusted to maintain a trough blood concentration of 250-400 ng/mL revealed median survival times of 16 months for placebo-treated dogs versus 11
months with cyclosporine, thus cyclosporine was not recommended for treatment of canine glomerulonephritis.\textsuperscript{280}

In neurology, cyclosporine reportedly improved clinical signs of myasthenia gravis in 2 dogs that were refractory to treatment with pyridostigmine and glucocorticoids.\textsuperscript{281} Cyclosporine has also been successfully used for the treatment of meningoencephalomyelitis of unknown etiology (MUE) in dogs. Two studies used a cyclosporine dose of 10 mg/kg once daily\textsuperscript{282} or 6 mg/kg twice daily,\textsuperscript{283} respectively, while a third reported a target drug trough concentration of 200-400 ng/mL when cyclosporine was used with prednisone and/or ketoconazole.\textsuperscript{284} Though cyclosporine does not normally cross the blood-brain barrier, Adamo and others suggested that the inflammation associated with MUE allows therapeutic cyclosporine concentrations to reach the central nervous system (CNS), or alternatively that cyclosporine’s effect on peripheral lymphoid organs recognized to initiate autoimmune disease is sufficient to reduce CNS inflammation without cyclosporine penetrating the blood-brain barrier.\textsuperscript{284}

Finally, cyclosporine is used for renal transplantation in both dogs and cats, although much more commonly in cats since renal transplantation in dogs has poor success rates.\textsuperscript{25-28} Whole blood trough cyclosporine concentrations between 300-700 ng/mL\textsuperscript{26,27,141} are recommended by different authors for renal transplantation in dogs, while feline post-transplant concentrations are lower at 250-500 ng/mL.\textsuperscript{25,162} Most transplant centers in veterinary medicine measure cyclosporine blood concentrations using HPLC.

There are many current and potential applications for cyclosporine within veterinary medicine. The wide range of dosing strategies offers an opportunity to
correlate biomarker levels, blood drug concentrations, and clinical response. The following chapters describe initial efforts to develop a panel of biomarkers to measure cyclosporine immune responses in the dog.
References


189. Marin JG, Levine M, Ensom MH. Is C2 monitoring or another limited sampling strategy superior to C0 monitoring in improving clinical outcomes in adult liver transplant recipients? Ther Drug Monit 2006;28:637-642.


203. Bohler T, Budde K, Schneider M, et al. Pharmacodynamic monitoring of lymphocyte proliferation and TGF-beta 1 expression at cyclosporine a (CyA) trough levels (C(0)) and 2 hours after intake (C(2)) of CyA in human renal allograft recipients. Transplant Proc 2001;33:3148-3150.


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CHAPTER III

CYCLOSPORINE A AFFECTS THE IN VITRO EXPRESSION OF T-CELL ACTIVATION-RELATED MOLECULES AND CYTOKINES IN DOGS


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Abstract

Cyclosporine is a powerful immunosuppressive drug that is being used with increasing frequency to treat a wide range of immune-mediated diseases in the dog. To date, ideal dosing protocols that will achieve immunosuppression with cyclosporine in dogs remain unclear, and standard methods that can measure effectiveness of immunosuppression have not been established. The aim of our study was to evaluate the effects of in vitro cyclosporine exposure on a panel of molecules expressed by activated T cells to ascertain their potential as biomarkers of immunosuppression in dogs. Blood was drawn from six healthy dogs, and peripheral blood mononuclear cells (PBMC) were
isolated and activated. Half of the cells were incubated with 200 ng/mL cyclosporine prior to activation, and the other half were not exposed to cyclosporine. Samples were analyzed using flow cytometry, and the expression of intracellular cytokines IL-2, IL-4, and IFN-γ was evaluated after 6, 12, and 24 h of drug exposure. Each cytokine exhibited a time-dependent suppression profile, and all but two samples activated in the presence of cyclosporine showed 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 h of drug exposure. Expression of these surface molecules decreased significantly when activated in the presence of cyclosporine. Our results suggest 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.

**Introduction**

Cyclosporine (CsA) is an important immunosuppressive drug with uses in both human and veterinary medicine. Veterinary applications include treatment of atopic dermatitis, anal furunculosis, inflammatory bowel disease, and a variety of other inflammatory and immune-mediated diseases.\(^1\)\(^-\)\(^3\) Derived from the fungus *Tolypocladium inflatum*, CsA is a hydrophobic cyclic endecapeptide. It specifically targets T cell function, potently inhibiting cell-mediated immunity with relatively less effect on humoral immunity.\(^4\) Inside T cells, CsA forms a complex with cyclophilin, which then binds to calcineurin preventing its activation. Calcineurin, a calcium-dependent serine/threonine phosphatase, is therefore unable to activate nuclear factor of activated T cells (NFAT) which regulates the production of several important cytokines including IL-2, IL-4, TNF-α and IFN-γ.\(^5\)\(^,\)\(^6\) Reduced production of IL-2, important for IL-2 dependent
growth and differentiation of T cells, is thought to be the main cause of the immunosuppressive effects of CsA.\textsuperscript{7}

Orally administered CsA is preferred for long term therapy and at home use in canine patients, but the bioavailability of available oral formulations is unpredictable. This necessitates the use of therapeutic drug monitoring to ensure that treatment is adequate, particularly when CsA is administered for potentially life-threatening conditions. Currently, pharmacokinetic assays measuring drug concentrations in the blood over time are the primary means to monitor CsA therapy in dogs. The pharmacokinetics of CsA in both people and dogs are well described,\textsuperscript{1,8-10} however, individual humans and dogs may not respond identically to the same blood level of CsA. Additionally, target CsA blood levels were originally established in dogs undergoing renal transplantation. These levels were extrapolated from human transplant literature and then empirically adjusted to achieve drug levels sufficient to prevent organ rejection in dogs while minimizing adverse effects.\textsuperscript{11} These target blood levels are those needed to prevent organ rejection, and may not be relevant for treatment of all naturally occurring canine inflammatory and immune-mediated diseases. Indeed, in pharmacokinetic studies of dogs with atopic dermatitis, no significant correlation was found between CsA blood level and response to therapy.\textsuperscript{8,10,12} Pharmacodynamic assays that study the relevant biological effects of a drug offer another option for CsA monitoring that may be more applicable to the veterinary patient.

There are numerous studies in the human literature pertaining to pharmacodynamic monitoring of CsA therapy,\textsuperscript{13-18} but few studies exist in the veterinary field. The effect of CsA on canine mRNA expression of various cytokines has been
investigated,\textsuperscript{19-21} and lymphocyte proliferation has been analyzed after topical CsA therapy for keratoconjunctivitis sicca\textsuperscript{22} and systemic CsA treatment.\textsuperscript{23} However, \textit{in vitro} flow cytometric analysis of the effects of CsA on the production of cytokines and surface molecules by circulating T lymphocytes has not been performed in the dog.

In our study, we used flow cytometry to measure the production of a panel of biomarkers, IL-2, IL-4, IFN-\(\gamma\), CD25 and CD95, that have been found to be down-regulated in humans treated with CsA.\textsuperscript{14} As mentioned previously, IL-2, IL-4, and IFN-\(\gamma\) are cytokines whose production is regulated by NFAT.\textsuperscript{5,6} CD25 (IL-2R\(\alpha\)) and CD95 (FasR) are T cell surface molecules with roles in activation and T cell development.\textsuperscript{24,25} The goals of our study were to examine the utility of these biomarkers in evaluating the \textit{in vitro} effects of CsA on canine lymphocytes and to determine the potential of these markers as pharmacodynamic measures of immunosuppression in dogs.

\textbf{Materials and Methods}

\textbf{Animals}

Six healthy adult Walker hounds, with no previous history of disease, were used as blood donors. Complete blood counts with differentials were performed periodically on each dog to ensure that cell counts were within the normal range. The dogs were housed in a university setting under standard conditions, and Institutional Animal Care and Use Committee (IACUC) approved protocols were followed.

\textbf{Reagents}

Complete media (CM) was prepared using RPMI 1640 supplemented with 10\% heat-inactivated FBS, 1\times GlutaMAX\textsuperscript{TM}, 1mM sodium pyruvate, 55\(\mu\)M 2-
mercaptoethanol, 75μg/mL gentamicin, 2mM HEPES, and 1μL/mL MEM amino acids solution without L-glutamine. For PBMC isolation, Histopaque®-1077 was purchased from Sigma–Aldrich (St. Louis, MO). Stock solutions of ConA (Sigma–Aldrich) were made with Ca²⁺-Mg²⁺-free PBS (PBS) at 1mg/mL. PMA and ionomycin were also purchased from Sigma–Aldrich, and stock solutions of 10mg/mL PMA and 1mM ionomycin were made in 100% ethanol. Brefeldin A and the BD Cytofix/Cytoperm Plus Kit were purchased from Becton Dickinson (San Jose, CA).

Antibodies used for intracellular staining were: FITC-conjugated monoclonal anti-dog CD3 (MCA1774F, AbD Serotec, Raleigh, NC), R-phycoerythrin (RPE)-conjugated monoclonal anti-bovine IL-4 (MCA1820PE, AbD Serotec), RPE-conjugated monoclonal anti-bovine IFN-γ (MCA1783PE, AbD Serotec), and biotinylated anti-canine IL-2 (BAF1815, R&D Systems, Minneapolis, MN). RPE-conjugated streptavidin (#60669, Anaspec, San Jose, CA) was used as a secondary label for IL-2. For surface molecule staining, RPE-conjugated anti-dog CD3 (MCA1774PE, AbD Serotec) was used with purified rabbit polyclonal antibodies for anti-CD25 (IL-2R, sc-664, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-CD95 (GTX13550, Genetex, Irvine, CA). FITC-conjugated goat anti-rabbit IgG antibody (GTX77059, Genetex) was used as a secondary antibody for the surface molecules. Isotype controls were purchased from Santa Cruz Biotechnology.

CsA was obtained from Bedford Laboratories (Bedford, OH). Injectable CsA at 50mg/mL was diluted, using 0.9% sodium chloride solution, to a concentration of 25μg/mL and was added to sample CM to reach a final concentration of 200ng/mL. This value was chosen because CsA concentrations higher than this resulted in negligible
additional suppression of mRNA expression during quantitative RT-PCR in dogs,\textsuperscript{19} and pilot studies in our laboratory showed it to be an effective drug concentration. Target trough cyclosporine blood levels in dogs vary widely depending on method and sample tested, and range from 100 to 600ng/mL.\textsuperscript{26-28} A concentration of 200ng/mL for PBMC in media and serum was therefore postulated to be comparable to therapeutic levels \textit{in vivo}.

**Blood collection and PBMC isolation**

Blood was drawn from the jugular vein of each dog and collected in heparinized tubes. PBMC were immediately isolated by density gradient centrifugation using Histopaque\textsuperscript{R}-1077 similarly to the previously described method.\textsuperscript{29} After isolation, cells were reconstituted in CM to a total volume equaling that of the original whole blood sample. This established a cell concentration approximately equivalent to that initially present in the healthy canine donors. All donors had white blood cell counts and differentials that were within the normal canine ranges.

**T cell cytokine assay**

200ng/mL CsA was added to half of the cells prior to activation, and the other cells were in CM only during this period. After 90min, PMA and ionomycin were added to the activated sample wells at a final concentration of 12.5ng/mL PMA and 0.8μM ionomycin, and all cells were incubated for 6, 12, or 24h at 37°C in a 5% CO\textsubscript{2} incubator. With 2h remaining in that incubation, brefeldin A was added at a final concentration of 1μg/mL to stop cytokine secretion from the T cells. After incubation, cells were collected from the wells (300μL per sample) and washed with PBS. Each sample was then incubated with FITC-conjugated anti-CD3 antibody for 30min at room temperature in the
dark to label the T cell population. Next, cells were fixed and permeabilized using the BD Cytofix/Cytoperm Plus Kit with slight modifications from the manufacturer’s instructions. Briefly, cells were fixed in the fixation/permeabilizing solution for 20min, centrifuged (400 × g for 7min), the supernatants aspirated, and incubated with 1x BD perm/wash for 10min, centrifuged again (400 × g for 7min), and the supernatants aspirated. Cells were then incubated with either RPE-conjugated anti-bovine IL-4, RPE-conjugated anti-bovine IFN-γ, or biotinylated anti-canine IL-2 for 30min at room temperature in the dark. Next, samples were washed with 1x BD perm/wash. The IL-2 samples had an additional 20min incubation with streptavidin at room temperature in the dark. These samples were washed again, and all cells were resuspended in PBS with 0.2% BSA.

**T cell surface molecule assay**

Half of the separated PBMC were untreated, and half were incubated with 200ng/mL CsA for 90min. Half of both groups were then activated with 0.5μg/mL ConA, and all cells were incubated for 36h at 37°C in a 5% CO₂ incubator. After incubation, cells were collected from the wells (300μL per sample) and washed with PBS. Each sample was incubated with RPE-conjugated anti-CD3 antibody, and either purified anti-CD25 antibody or purified antiCD95 antibody for 30min at room temperature in the dark. After washing with PBS, all samples were incubated with FITC-conjugated secondary antibody for a further 30min at room temperature in the dark. Cells were again washed and resuspended in PBS with 0.2% BSA.
Flow cytometric analysis

Forward scatter (FSC) and side scatter (SSC) were used to identify and gate the lymphocyte population, and a second gate was placed around CD3-positive cells to identify T cells and exclude B cells. Samples were assayed using a FACSCalibur Flow Cytometer (Becton Dickinson) and analyzed with CellQuest Pro software (Becton Dickinson). 10,000 events were collected in all experimental groups. Cell staining was evaluated by measuring mean fluorescence intensity (MFI) with single histogram statistics. Unactivated samples and isotype controls were used as negative controls in our study.

Statistical analysis

Differences in MFI and percent increase of MFI on activation between control and CsA-treated samples were analyzed for each biomarker at each time point using the Wilcoxon signed-rank test. Statistical analyses were performed using the UNIVARIATE procedure in SAS for Windows® version 9.2 (SAS Institute Inc., Cary, NC). \( P \) values ≤ 0.05 were considered significant. Percent increase of MFI on activation was calculated using Formula (3.1).

\[
\% \text{ Increase} = \frac{\text{Activated MFI} - \text{Unactivated MFI}}{\text{Unactivated MFI}} \times 100
\] (3.1)

Results

Intracellular cytokines

We analyzed the production of intracellular cytokines in activated cells incubated either alone or with 200ng/mL CsA for incubation periods of 6, 12, and 24h. Fig. 3.1 shows the decrease in expression of cytokines in activated CsA-treated cells relative to
activated untreated cells by evaluating both the percent increase in fluorescence upon activation and the MFI following activation. The increase on activation is calculated by comparing a sample’s unactivated fluorescence (unactivated MFI) to the fluorescence of a second aliquot of the same sample incubated with activating stimuli (activated MFI). This “percent increase on activation” value measures T cell function by assessing the cells’ capacity for increasing cytokine production in response to activation. Analyzing MFI in both the activated CsA-treated and untreated cells demonstrates the direct effect of CsA treatment on fluorescence intensity.

For IL-2, the percent increase in fluorescence intensity on activation was reduced in T cells exposed to CsA at 6, 12, and 24h, but this suppression was only statistically significant at 12h. The IL-2 overall MFI increased over time, but the results became more variable. A significant difference between the activated CsA-treated and untreated groups was found at 6 and 12h, but this difference was no longer significant at 24h (Fig. 3.1A’). IL-4 displayed a lower overall fluorescence, but differences were consistent so MFI and percent increase data were both significantly reduced in T cells exposed to CsA at 6 and 12h. At 24h, although fluorescence was greater, results were again less consistent and significance was lost (Fig. 3.1B and B’). IFN-γ samples showed a marked and consistent effect of CsA at 6, 12, and 24h, with statistically significant reductions at all time points for both percent increase on activation and MFI in CsA-treated cells compared to untreated cells (Fig. 3.1C and C’). Both IL-2 and IL-4 displayed greater untreated fluorescence at 24h than 6h, and IL-4 also demonstrated an enhanced ability to increase on activation over time. This is in contrast to IFN-γ, which had consistent MFI and activation levels across the testing interval.
Figure 3.1  Cyclosporine effects on activated T cell intracellular cytokines

Increase on activation and MFI of IL-2 (A, A’), IL-4 (B, B’), and IFN-γ (C, C’), for CsA-treated activated T cells relative to untreated controls. Canine PBMC were isolated, and half of the cells were incubated in the presence of CsA, and the other half were in CM only. Cytokine production was evaluated by flow cytometry after 6, 12, and 24h of activation with PMA and ionomycin. 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 CsA ($P \leq 0.05$).

Surface molecules

Surface molecule expression was evaluated for CsA-treated and untreated cells at 36h using 0.5μg/mL ConA as an activator. These data were analyzed using percent increase on activation (Fig. 3.2A) and MFI (Fig. 3.2B). Similarly to IL-4, CD25 and CD95 both had very low fluorescence. The differences in MFI and percent increase on
activation were greater for CD95 than CD25, although both markers consistently and significantly decreased with CsA treatment (Fig. 2A and B). Previous experiments in our laboratory indicated 36 h to be optimal for surface molecule assays (data not shown), so no further time points were assayed.

Figure 3.2  Cyclosporine effects on activated T cell surface molecules

Percent increase in expression on activation (A) and MFI (B) for T cell surface molecules CD25 and CD95 after 36h of incubation. Canine PBMC were isolated and either incubated with CsA or in CM only, and half of both groups were activated with ConA. Surface molecule expression was evaluated for unactivated and activated samples using flow cytometry. 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 CsA ($P \leq 0.05$).

Discussion

Optimal oral dosing protocols for CsA for dogs are not well established, despite its use as a major immunosuppressive drug in veterinary medicine. The majority of
investigations of the immunosuppressive effects of CsA are studied in dogs with inflammatory dermatologic or gastrointestinal disorders in which efficacy was primarily evaluated empirically by the practitioner and the owner through the assessment of clinical response. Additionally, while efficacy against several common inflammatory diseases has been established, there is little information regarding the relationship between CsA blood concentrations and the degree of immunosuppression in dogs.

Flow cytometry is a frequently used technique to study CsA effects in human research, but has been of limited use in veterinary medicine, possibly due to the limited availability of antibodies. Using flow cytometry, Gilger et al. described suppressed lymphocyte proliferation after 1 to 3 months of topical 2% CsA administration in keratoconjunctivitis sicca patients. In another study, CsA’s ability to suppress green fluorescent protein (GFP)-specific immune responses against GFP-transduced hematopoietic stem cells was evaluated. The use of flow cytometry to monitor systemic CsA therapy in dogs is a novel approach, and is the goal of our research group.

In vitro, activated canine T cells showed consistent suppression of IL-2, IL-4, and IFN-γ production when incubated in the presence of CsA. Each cytokine had an individual suppression profile that varied over time from 6 to 24h, which, except for the 24h IL-2 time point, was consistent for the six dogs sampled. In previous work in our laboratory (data not shown), IL-2 results were more variable at longer incubations than at those less than 24h, and this held true in this experiment. Overall, the most consistent data for cytokine protein expression were found with a 12h incubation, at which time all three markers showed a significant difference in percent activation and MFI between untreated and CsA-treated groups.
Other pharmacodynamic assays in dogs examining cytokine mRNA expression in cells treated with CsA have found similar results. In one study, the mRNA expression of IL-2, IL-4, and IFN-γ was found to be inhibited in PBMC treated with CsA, but TNF-α was found to increase. The suppression we identified with IL-2, IL-4, and IFN-γ corroborates those mRNA results, and is similar to what has been found in humans. Kobayashi’s TNF-α finding is contrary to work with human cells, however, in which TNF-α was found to have decreased expression in the presence of in vitro CsA. Anti-canine TNF-α antibodies for flow cytometry were unavailable at the time of publication, so this cytokine could not be studied in our project. Another group studied IL-2 and IFN-γ mRNA expression in anal furunculosis lesions in dogs, and also found decreased cytokine levels after CsA therapy. Since mRNA expression does not always correlate with the production of biologically active proteins, confirmation of changes in T cell cytokine production by flow cytometry is valuable. A study in cynomolgus monkeys found comparable results when analyzing CsA effects using flow cytometry and quantitative RT-PCR. A similar study in dogs is warranted to identify whether quantitative RT-PCR or flow cytometry is more sensitive at detecting changes in T cell cytokine production, and could direct future pharmacodynamic work.

The expression of surface molecules CD25 and CD95 also decreased in the presence of CsA. The response of T cell surface molecules to immunosuppressive therapy has been evaluated in human whole blood, where expression of both CD25 and CD95 was found to decrease in the presence of CsA. These samples were incubated for three days, but in our laboratory, 36h provided a more optimal incubation period. Our
assay provides the first determination of the response of canine T cell surface molecules to CsA in vitro, and confirms a comparable effect to that found in humans.

This study is the first to use flow cytometry to measure T cell cytokine and surface molecule expression as pharmacodynamic measures of CsA immunosuppression in dogs. With our identification of a panel of biomarkers that both increase upon activation and are suppressed by CsA, further investigation is warranted to determine if expression of the markers is suppressed in dogs treated with oral CsA using an ex vivo assay. In order for the assays to be clinically valuable, suppression will need to be dose-dependent. Further work could also involve correlating our pharmacodynamic assays with traditional pharmacokinetic blood CsA levels. It is not known whether dogs will respond identically and predictably to a certain blood drug level, or if suppression of biomarkers of immunosuppression from a baseline established for each dog will be required in order to optimize immunosuppressive therapy. Böhler et al. conducted a rigorous analysis of several immunosuppressive markers in humans, and examined both intra-assay and inter-individual variation. An analogous investigation into the reproducibility of results in dogs would help identify a pharmacodynamic strategy for veterinary CsA monitoring.35

In conclusion, there is a clear need for pharmacodynamic studies that establish target CsA levels that are relevant to immune-mediated diseases commonly encountered in canine practice, but to date such studies have been very limited in number and scope. Assays that reflect actual T cell function hold promise as objective measures of the level of immunosuppression present in the body, and could help identify a better way to utilize CsA in clinical patients. This report describes one such assay that used flow cytometry to
evaluate the ability of T cells to express activation molecules and produce cytokines. We demonstrated substantial suppression of five canine immunological markers when exposed to *in vitro* CsA. These results suggest that CsA has a similar mechanism in dogs to that found in humans, and that flow cytometric assays may be a valuable tool for measuring CsA effects in dogs.

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References


CHAPTER IV
EFFECTS OF CYCLOSPORINE AND DEXAMETHASONE ON CANINE T-CELL EXPRESSION OF IL-2 AND IFN-GAMMA AS MEASURED BY FLOW CYTOMETRY AND QUANTATIVE RT-PCR

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Abstract

Cyclosporine and glucocorticoids are powerful immunosuppressive agents used to treat many inflammatory diseases in dogs. Cyclosporine inhibits calcineurin-dependent pathways of T cell activation and resultant T cell cytokine production, and glucocorticoids directly inhibit genes coding for cytokines. Little work has been done comparing the effects of these agents on T cell cytokine production in dogs. Our study measured T cell interleukin-2 (IL-2) and interferon-gamma (IFN-\(\gamma\)) production using flow cytometry and T cell IL-2 and IFN-\(\gamma\) gene expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in activated canine T cells incubated...
with cyclosporine and dexamethasone \textit{in vitro}. For flow cytometric assays, diluted whole blood was cultured for 7 hours in the presence of cyclosporine (10, 100, 500, and 1000 ng/mL) or dexamethasone (10 ng/mL, 100 ng/mL, 1 μg/mL, and 10 μg/mL). For qRT-PCR, whole blood was cultured for 5 hours with the same drugs at the same concentrations, and RNA was then extracted from leukocytes. Flow cytometry and qRT-PCR both demonstrated inhibition of IL-2 and IFN-γ that was concentration-dependent in response to cyclosporine, and was more variable for dexamethasone. Quantitative RT-PCR but not flow cytometry documented significant reduction of IL-2 expression after dexamethasone treatment, while both methods showed concentration-dependent suppression of IFN-γ. Quantitative RT-PCR also revealed additional cytokine suppression at higher cyclosporine concentrations, an effect not found using flow cytometry, and may therefore be the preferred method for cytokine determination in dogs. Suppression of IL-2 and IFN-γ in activated T cells may have potential as an indicator of the efficacy of cyclosporine and glucocorticoids in suppressing canine T cell function \textit{in vivo}, and may therefore be of value for characterizing the immunosuppression induced by these drugs in clinical patients.

\textbf{Introduction}

Glucocorticoids and cyclosporine are commonly used as immunosuppressive agents in both veterinary and human medicine. Glucocorticoids act through binding the glucocorticoid receptor in cell cytoplasm, and then translocating to the nucleus to modulate the activity of glucocorticoid response elements. Glucocorticoids alter the expression of various cytokines and cell processes in lymphocytes and other immune cells, and affect both innate and adaptive immune responses.\textsuperscript{1} Glucocorticoids also
inhibit the transcription factors nuclear factor of kappa B (NF-κB) and activator protein 1 (AP-1), independent of their effects on glucocorticoid response elements. The primary mechanism of action of cyclosporine is by inhibiting calcineurin, which in turn blocks activation of the transcription factor nuclear factor of activated T cells (NFAT) and decreases production of pro-inflammatory cytokines like IL-2, IL-4, IFN-γ, and tumor necrosis factor-alpha (TNF-α). A combination of glucocorticoids and cyclosporine leads to combined inhibition of NF-κB, AP-1, and NFAT, thereby blocking the three transcription factors triggered after T cell receptor binding and causing an additive or synergistic impact on T cell activity.

Pharmacodynamic monitoring of levels of the cytokines produced by T cells has been explored in human transplantation medicine as a means of identifying the biological effects of a given immunosuppressive regimen on a patient. Pharmacodynamic monitoring allows for individualized therapy, and such monitoring has been able to associate very low cytokine expression with the potential for infection and malignancy, and high residual T cell cytokine expression with inadequate immunosuppression and the potential for transplant rejection. Immunologic assays are also beginning to be explored in veterinary medicine to assess the effects of cyclosporine and other immunosuppressant medications on lymphocyte responses in dogs and cats. Most of this work has been done in in vitro drug incubation studies, but our laboratory has also evaluated T cell cytokine production ex vivo in dogs taking oral cyclosporine.

In human medicine, measurement of T cell production of NFAT-regulated cytokines is considered useful for monitoring calcineurin inhibitor therapy after transplantation, even in the presence of other co-administered immunosuppressive drugs.
However, some of these other agents (especially glucocorticoids) are also known to affect T cell cytokine expression, and could influence the results of pharmacodynamic assays. In fact, glucocorticoid-mediated T cell suppression has been shown to be associated with reduced cytokine levels, including reduced levels of IL-2.\textsuperscript{17} Since cyclosporine is frequently used concurrently with glucocorticoids, it is important to determine what effects glucocorticoids may have on any proposed biomarker assay used for pharmacodynamic monitoring.

Different methods have been used for T cell cytokine evaluation in both animals and humans, but few studies compare the results found with techniques reporting different measures of cytokine expression, such as protein expression with flow cytometry, and relative gene expression with quantitative RT-PCR. The authors are only aware of one study comparing different measures of expression in the context of immunosuppressive pharmacodynamics, which found the two methods of measurement to provide comparable results in cynomolgus monkeys.\textsuperscript{18}

The present study investigates the \textit{in vitro} effects of exposure to cyclosporine and dexamethasone on T cell cytokines measured using both flow cytometry and qRT-PCR in a whole blood assay. Goals of this study were to explore the possible effects of concurrent glucocorticoid administration on assays intended for cyclosporine monitoring in dogs given cyclosporine, and to compare the responses of the two different measures of cytokine expression across a range of drug concentrations. Samples from three dogs were also used to determine the time of maximal cytokine expression after activation to provide technique development information for subsequent drug effect studies.
Materials and Methods

Animals

Six adult Walker hounds, determined to be healthy based on normal complete blood count, serum biochemistry, urinalysis, and negative heartworm status, were used as blood donors. They were housed in a university setting, and protocols approved by the Institutional Animal Care and Use Committee (IACUC) were followed.

Reagents

Complete media (CM) was prepared as previously described. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO), and stock solutions of 1 mg/mL PMA and 1 mM ionomycin were made in 100% ethanol. Working solutions of cyclosporine (Bedford Laboratories, Bedford, OH) and dexamethasone (Bimeda, Irwindale, CA) were prepared daily and diluted with sodium chloride. Brefeldin A, the BD Cytofix/Cytoperm™ Plus Kit, and BD Pharm Lyse™ were purchased from Becton Dickinson (San Jose, CA).

Antibodies used for flow cytometry were: Fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-dog CD3 (MCA1774F, AbD Serotec, Raleigh, NC), R-phycoerythrin (R-PE)-conjugated monoclonal anti-bovine IFN-γ (MCA1783PE, AbD Serotec), and biotinylated anti-canine IL-2 (BAF1815, R&D Systems, Minneapolis, MN). RPE-conjugated streptavidin (#60669, Anaspec, San Jose, CA) was used as a secondary label for IL-2. Isotype controls were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

For quantitative reverse transcription PCR (qRT-PCR), RNA was extracted using a QIAamp® RNA Blood Mini Kit, and on-column DNase treatment was performed with
the RNase-Free DNase Set, both from Qiagen (Valencia, CA). Assays were performed using a SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit with Rox used as a reference dye (Invitrogen, Carlsbad, CA).

**Blood collection and stimulation**

Blood was collected from the jugular vein into heparinized blood tubes. After plating, samples were activated with 0.8 μM ionomycin and 12.5 ng/mL phorbol 12-myristate 13-acetate (PMA). For flow cytometry, samples were diluted 1:9 with complete media, and brefeldin A was added one hour after activation at a final concentration of 1 μg/mL to stop cytokine secretion from T cells. For qRT-PCR analysis, heparinized whole blood was used.

**In vitro assays**

**Incubation study**

Blood from 3 dogs was activated with PMA and ionomycin, and incubated at 37°C and 5% CO₂ for 2, 4, 6, 8, 10 and 12 hours to determine the optimal time for cytokine assessment. As described above, whole blood was used for qRT-PCR analysis, and diluted whole blood samples for flow cytometry had brefeldin A added 1 hour after starting the incubation.

**Exposure to cyclosporine and dexamethasone**

Prior to activation for the in vitro drug effect assessment, blood samples were incubated with various concentrations of cyclosporine (10, 100, 500, and 1000 ng/mL) and dexamethasone (10 ng/mL, 100 ng/mL, 1 μg/mL, and 10 μg/mL) for 1 hour. Cyclosporine concentrations were based on previously established achievable blood
concentrations after oral cyclosporine dosing, and concentrations found to suppress T cell indices \textit{in vitro}.\textsuperscript{10,15} Dexamethasone concentrations encompassed established typical blood concentrations after oral dosing, and approached the higher concentrations tested by Nafe and others.\textsuperscript{13,19,20} Based on results from the prior incubation study, samples were then activated and incubated for five hours for qRT-PCR analysis, and seven hours for flow cytometry. Brefeldin A was added one hour after activation for flow cytometry samples. An untreated activated sample was also included, and unactivated samples were run for each treatment condition as a control.

**Flow cytometry**

Flow cytometry was performed similarly to the previously published method,\textsuperscript{14} with modifications for a whole blood assay, as briefly described below. After incubation, cells (350 uL per sample) were collected from the wells and immediately incubated with FITC-conjugated anti-CD3 antibody for 25 minutes at room temperature (RT) in the dark. Red blood cells were lysed using BD Pharm Lyse\textsuperscript{TM} according to the manufacturer’s instructions. Following lysis, cells were washed and then fixed and permeabilized using the BD Cytofix/Cytoperm\textsuperscript{TM} Plus Kit as described previously.\textsuperscript{14-16}

Samples were assayed using the FACSCalibur\textsuperscript{TM} Flow Cytometer (Becton Dickinson) and analyzed with CellQuest\textsuperscript{TM} Pro software (Becton Dickinson). Lymphocytes were identified using forward scatter and side scatter, and 10,000 events were collected per sample. An additional gate was placed around CD3+ cells to include T cells and exclude B cells. Cytokine staining was evaluated for CD3+ lymphocytes by measuring mean fluorescence intensity (MFI) with single histogram statistics. Negative controls included unactivated samples and isotype controls.
RNA isolation and qRT-PCR analysis

After activation, total RNA was isolated from 1 mL heparinized whole blood as previously described. Cytokine gene expression analysis was performed similarly to the previously described method, with the modifications described below. Briefly, a 20 µL reaction volume was used with 30 ng template and 200 nM of each primer. Samples were run on a Stratagene™ Mx3005P Real-Time PCR system, and Stratagene™ MxPro QPCR software, version 4.10 (Agilent Technologies, Santa Clara, CA) was used for analysis. Experimental samples were run in triplicate, with non-template controls run in duplicate. Relative gene expression was calculated with mean Ct values using the $2^{-\Delta\Delta Ct}$ method, where: $\Delta\Delta Ct = (Ct_{GOI} - Ct_{norm})_{treated} - (Ct_{GOI} - Ct_{norm})_{untreated}$, and GOI is the gene of interest (IL-2 and IFN-γ) and norm is the normalizer gene (GAPDH).

Statistical analysis

Mean fluorescence intensity data were analyzed for flow cytometry, and the percent reduction in cytokine gene expression relative to untreated sample expression was evaluated for qRT-PCR. Following visual assessment of quantile-quantile plots of the data (PROC UNIVARIATE, SAS for Windows 9.3, SAS Institute, Inc., Cary, NC, USA), the data were judged to not be normally distributed. Consequently, nonparametric methods of analysis that accounted for the hierarchical structure of the data were utilized. For each outcome (flow cytometry: IL-2, IFN-γ; qRT-PCR: IL-2, IFN-γ), the data were ranked and then analysis of variance type statistics were obtained through PROC MIXED (SAS for Windows 9.3, SAS Institute, Inc., Cary, NC, USA) by using the ANOVAF option and the MIVQUE0 estimation method for the covariance parameters and a REPEATED statement specifying dog identity as the subject and an unstructured...
covariance structure. Either cyclosporine or dexamethasone concentration was included in the models as a fixed effect. Differences in least square means with the SIMULATE adjustment of $P$ values were used for multiple comparisons of each of the four concentration levels to the untreated sample. To allow method comparison using the same outcome, percent reduction in cytokine expression relative to untreated levels was calculated for flow cytometry to parallel the results found with qRT-PCR. The effect of method (flow cytometry vs qRT-PCR), drug concentration, and their interaction on percent reduction in cytokine expression was then assessed. A $P$ value of less than 0.05 was considered to be significant for all analyses.

**Results**

**Incubation study**

Results of the blood sample incubation study are shown in Figure 4.1. For both methods, cytokine production after PMA and ionomycin activation was similar for the three dogs tested. For flow cytometry (Fig. 4.1 A and B), both IL-2 and IFN-$\gamma$ MFI increased to reach a maximum at either 6 or 8 hours, and then decreased with longer testing intervals, except for 1 dog that had its highest IFN-$\gamma$ level at 12 hours. Quantitative RT-PCR results (Fig. 4.1 C and D) are shown as the $\Delta\Delta$Ct value between the 2, 4, 6, 8, 10, and 12-hour incubation value and unstimulated cytokine production. Cytokine production increased substantially from 2 hours to 4 hours for both IL-2 and IFN-$\gamma$, and mildly more at 6 hours, but remained at a similar expression level thereafter. To allow the greatest potential to find a difference in expression and the most stable expression level, the time cytokines reached an approximate maximum was selected.
Seven hours of activation is recommended for flow cytometry, and five hours for qRT-PCR. These incubation times were used in the following experiments.

Figure 4.1  Cytokine expression at various durations of activation with PMA plus ionomycin

Cytokine expression measured using flow cytometry (A and B) in diluted whole blood, and qRT-PCR (C and D) in whole blood after 2, 4, 6, 8, 10, and 12 hours of incubation with 12.5 ng/mL PMA, and 0.8 μM ionomycin. Brefeldin A was added 1 hour after activation for flow cytometry samples. Flow cytometry results are shown as the mean fluorescence intensity for CD3-positive lymphocytes, and qRT-PCR results are shown as the ΔΔCt value of stimulated-unstimulated cytokine values.

Cyclosporine and dexamethasone treatment

Cyclosporine caused a concentration-dependent decrease in IL-2 and IFN-γ expression as measured with both flow cytometry and qRT-PCR (Figure 4.2). At 10 ng/mL cyclosporine, no significant difference was identified for either cytokine with
either method relative to untreated control samples \((P \geq 0.21)\). By 100 ng/mL cyclosporine, however, both IL-2 and IFN-\(\gamma\) expression were significantly reduced for both methods \((P \leq 0.01)\). No additional suppression of cytokine production was noted at higher concentrations of cyclosporine using flow cytometry (Fig. 4.2 A, \(P \geq 0.16\)), while significantly greater suppression of both cytokines was noted at 500 ng/mL than 100 ng/mL with qRT-PCR (Fig. 4.2 C, \(P \leq 0.025\)). No additional suppression, however, occurred with an increase to 1000 ng/mL \((P \geq 0.81)\).

For dexamethasone, results from flow cytometry and qRT-PCR were not as closely related as they were for qRT-PCR. IL-2 was not significantly suppressed with any dexamethasone concentration as measured by flow cytometry (Fig 4.2 B, \(P \geq 0.095\)), while qRT-PCR showed low but uniform significant reduction in gene expression at all concentrations tested (Fig. 4.2 D, \(P \leq 0.037\)). Suppression of IFN-\(\gamma\) was concentration-dependent after treatment with dexamethasone, and reached maximum levels at 100 ng/mL for both methods (Fig. 4.2 B and D). Dexamethasone caused more suppression than cyclosporine at lower concentrations, but at higher concentrations, cyclosporine caused a greater degree of cytokine suppression.

When considering the interaction between method and drug concentration on the percent reduction in cytokine expression, there was no difference between flow cytometry and qRT-PCR for cyclosporine at 10 or 100 ng/mL \((P \geq 0.29)\), while at 500 and 1000 ng/mL, qRT-PCR showed a significantly greater degree of suppression of both IL-2 and IFN-\(\gamma\) than flow cytometry \((P \leq 0.01)\). For dexamethasone, 10 ng/mL and 10 \(\mu\)g/mL showed significantly greater suppression for IL-2 with qRT-PCR relative to flow cytometry \((P \leq 0.028)\), and all drug concentrations were significantly different between
the two methods for IFN-γ, reflecting the greater degree of suppression noted for qRT-PCR versus flow cytometry ($P \leq 0.001$).

**Figure 4.2**  Cytokine expression after *in vitro* exposure to varying concentrations of cyclosporine and dexamethasone

Cytokine expression measured using flow cytometry (A and B) in diluted whole blood, and qRT-PCR (C and D) in whole blood. Samples were activated with PMA and ionomycin and incubated with cyclosporine (10, 100, 500, 1000 ng/mL) and dexamethasone (10 ng/mL, 100 ng/mL, 1 μg/mL, 10 μg/mL). Flow cytometry results for CD3-positive lymphocytes and qRT-PCR gene expression results are shown as the percent expression relative to untreated samples. Letters are used to indicate significant differences for IL-2, and numbers for IFN-γ. The presence of a letter/number indicates a significant difference from untreated values, and treatments that do not share a letter/number are significantly different ($P < 0.05$).
Discussion

This study documents \textit{in vitro} responses to exposure to two commonly used immunosuppressive medications, cyclosporine and dexamethasone, using canine whole blood assays. Both drugs are known to reduce the expression of pro-inflammatory cytokines,\textsuperscript{25-28} and this effect was reflected in the present study. Both cyclosporine and dexamethasone reduced the gene expression of IL-2 and IFN-\textgamma. Cyclosporine caused similar suppression of both cytokines, and caused a greater inhibition at high drug concentrations than did dexamethasone. Dexamethasone, in contrast, caused greater suppression of IFN-\textgamma than IL-2. Flow cytometry was used to confirm effects on protein production, and documented marked suppression of both IL-2 and IFN-\textgamma for cyclosporine, but only IFN-\textgamma was significantly decreased after dexamethasone treatment.

While flow cytometry cytokine suppression plateaued at cyclosporine concentrations greater than 100 ng/mL, qRT-PCR was notable in reflecting significantly greater suppression at 500 ng/mL than 100 ng/mL. This is important because 100 ng/mL is at the very low end of published target blood cyclosporine concentrations in dogs, and concentrations higher than this are frequently achieved with oral drug dosing.\textsuperscript{29} Although being sensitive to the effects of low drug concentrations is important for a biomarker, the ability to discriminate greater degrees of immunosuppression at higher blood concentrations is likely to further increase the utility of the assay, since the intent of the assay is not only to document the effects of cyclosporine on T cells, but to also identify patients with excessive immunosuppression that may be at risk of secondary infections.

In this study, a statistical comparison of the degree of reduction in marker expression between flow cytometry and qRT-PCR was performed. Although the clinical
consequences of decreased protein production relative to decreased gene expression cannot be established, and an argument can be made that decreased protein expression is ultimately the more relevant endpoint, the greater magnitude of reduction at higher drug concentrations identified for cytokine gene expression compared to protein production is potentially valuable from an analytical standpoint. Reductions of lesser magnitude are at greater risk of being confounded by biological and analytical variability. In this sense, the 90% decrease in gene expression identified at high cyclosporine concentrations is appealing. Quantitative RT-PCR also carries the advantage of enabling storage of RNA after extraction, thereby allowing samples collected on different days to be run on the same plate to further reduce analytical variability.

One strength of the described techniques is the use of whole blood and diluted whole blood assays. Cyclosporine is known to distribute into the different whole blood components and to bind to non-target sites, and thus effective inhibitory drug concentrations for pharmacodynamic measures are generally lower when tested in PBMC than in whole blood, because in whole blood not as much free cyclosporine is available to affect T cells. This effect can sometimes lead to marked differences in effective inhibitory drug concentrations, as in the study by Batiuk and others that identified the concentration of cyclosporine needed to cause 50% reduction in calcineurin activity (IC$_{50}$) as 2 ng/mL in peripheral blood leukocytes in culture medium versus 102 ng/mL for peripheral blood leukocytes in whole blood, and an IC$_{50}$ for IFN-γ production of 18 ng/mL in peripheral blood leukocytes in culture medium versus 690 ng/mL for peripheral blood leukocytes in whole blood.$^{30}$ Stein and others recognized this difference between IC$_{50}$ values for cyclosporine in isolated lymphocytes versus whole blood, and
championed whole blood values as being more clinically relevant and reflective of trough blood concentration targets in human transplant medicine, which are commonly 100-400 ng/mL. Other studies, however, have documented smaller differences based on sample preparation, such as a cyclosporine IC$_{50}$ for phytohemagglutinin-stimulated proliferation of 345 ng/mL for PBMC and 384 ng/mL for whole blood. Separation of PBMC also removes any drug present in plasma or red blood cells, which can impact assays that require incubation. Because of these factors, results of in vitro incubation studies with cyclosporine, especially when using isolated blood components rather than whole blood, are sometimes of questionable biological relevance.

Most previous investigations of immunosuppressive drug effects in dogs and cats have used peripheral blood mononuclear cells (PBMC). Density gradient isolation of PBMC has been associated with variable effects on cytokine production in humans. Using immunoassays, DeGroote and others reported increased IFN-γ and TNF-α in whole blood versus PBMC, while IL-1 and IL-2 were higher in PBMC. Härtel and others reported increased IL-2, IL-4, and TNF-α mRNA expression in PBMC versus whole blood, while IFN-γ levels were unchanged. Stordeur and others identified increased IFN-γ mRNA in whole blood relative to PBMC, supporting the findings of DeGroote, however, and suggested that rapid IFN-γ mRNA degradation at room temperature may have influenced the findings of Härtel.

Although an insufficient number of drug concentrations were tested to precisely identify the inhibitory concentration causing 50% reduction in cytokines (IC$_{50}$) in this study, cytokine suppression was already approximately 50% suppressed at 100 ng/mL cyclosporine when measured using flow cytometry, and gene expression was
approximately 55% of untreated levels at this concentration. Nafe and others reported an IC$_{50}$ for proliferation for cyclosporine of 15.8 ± 2.3 ng/mL in canine PBMC stimulated with concanavalin A. Although an IC$_{50}$ was not calculated, visual inspection of cytokine mRNA expression plots by Kobayashi and others also suggest approximately 50% inhibition of IL-2, IL-4, and IFN-γ mRNA expression in canine PBMC treated with 10 ng/mL of cyclosporine. These data agree with the previously reported tendency for IC$_{50}$ values to be lower in PBMC than whole blood, but interestingly, the values are much lower than most IC$_{50}$ values for lymphocyte proliferation or cytokine expression in whole blood or PBMC in humans. A study by Batiuk and others found IC$_{50}$ values of less than 20 ng/mL for human leukocyte calcineurin inhibition and IFN-γ production in culture medium, and studies by Hirano have documented very low IC$_{50}$ values (< 10 ng/mL) for blastogenesis by human PBMC, but most studies report cyclosporine IC$_{50}$ values of 200-500 ng/mL depending on the parameter studied. In cats, one study of lymphocyte proliferation in whole blood documented an IC$_{50}$ for cyclosporine of 55 ng/mL, while mRNA concentrations of IL-2, IL-4, and IFN-γ in feline PBMC were not significantly inhibited by 50 ng/mL cyclosporine, but were more than 50% inhibited by 150 ng/mL cyclosporine.

It is unclear whether the apparently greater lymphocyte responsiveness to cyclosporine in veterinary species is due to differences in assay performance, or to differences in species susceptibility to immunosuppressive effects. Genuine differences in species T cell susceptibility to cyclosporine may have important clinical ramifications, and could result from factors such as differences in cellular cyclophilin A levels, calcineurin activity, or P-glycoprotein function. Published target blood cyclosporine
concentrations are similar in dogs, cats, and humans, despite the fact that little work has been done to correlate cyclosporine blood concentration with clinical response in veterinary medicine. Several studies in dogs measured blood concentrations when cyclosporine was used for dermatologic disease, and found poor correlations between cyclosporine blood concentrations and clinical improvement in atopic dermatitis and anal furunculosis.\textsuperscript{40,41} Cyclosporine is recognized to concentrate in the skin of dogs, however, which may have affected these findings.\textsuperscript{42,43} Differences in T cell susceptibility could, potentially, indicate a need for different target concentrations in dogs and cats than in humans.

The immune-modulating effects of dexamethasone and other steroids have also been investigated extensively \textit{in vitro}. One study comparing the effects of cyclosporine and methylprednisolone on human T cells documented that both cyclosporine and methylprednisolone inhibited IL-2 expression to a greater extent than lymphocyte proliferation, and reported an additive, but not synergistic, effect of the drugs in combination.\textsuperscript{5} Other studies have documented cyclosporine to be less effective than prednisolone at inhibiting whole blood lymphocyte proliferation, but noted a synergistic effect when the drugs were used in combination.\textsuperscript{6,44} In contrast to cyclosporine, where whole blood assays decrease the effective amount of cyclosporine at the target site, higher proliferation IC\textsubscript{50} values are noted for dexamethasone in PBMC relative to whole blood,\textsuperscript{32} suggesting that assay effects are drug-dependent. Dexamethasone IC\textsubscript{50} is reported as 4.5 ng/mL in PBMC, and 3.5 ng/mL in whole blood. Lower IC\textsubscript{50} values in whole blood than PBMC are also reported for methylprednisolone and prednisolone.\textsuperscript{32}

Another study reported dexamethasone at 39 ng/mL significantly inhibited PBMC
proliferation in humans.\textsuperscript{45} In a study of lymphocyte proliferation by Nafe and others, however, a much higher dexamethasone IC\textsubscript{50} value of 1.36 ± 0.75 mg/mL (3460 ± 1900 μM) was reported for canine PBMC.\textsuperscript{13}

In the present study, 10 ng/mL, 100 ng/mL, 1 μg/mL and 10 μg/mL dexamethasone were tested. Uniform but fairly minimal suppression of IL-2 was identified with qRT-PCR, while no effect on IL-2 production was identified with flow cytometry. Dexamethasone induced greater suppression of IFN-γ compared to IL-2, and suppression was concentration dependent with both methods of measuring cytokine expression, but flow cytometry did not attain a 50% reduction in cytokine expression, while gene expression results were less than 50% at all concentrations tested. Maximum blood dexamethasone concentrations in dogs after 0.1 mg/kg of the drug is administered intravenously ranged from 43 ng/mL to 297 ng/mL with an average of 118 ng/mL,\textsuperscript{20} and averaged 571 ng/mL for dexamethasone in alcohol at 1 mg/kg intravenously.\textsuperscript{19} Typical dexamethasone doses used clinically range from 0.1-0.3 mg/kg, so the concentrations evaluated in this study include and exceed typical blood dexamethasone concentrations.

Although decreased expression of cytokines including IL-2 and IFN-γ is recognized to be a primary and well-documented effect of glucocorticoids,\textsuperscript{25,28,46,47} recent studies by two different groups documented a lack of effect of \textit{in vitro} exposure to methylprednisolone on human whole blood IL-2 mRNA expression,\textsuperscript{38} and of methylprednisone on human whole blood IL-2 cytokine production by flow cytometry.\textsuperscript{48} Our study shows that cyclosporine causes a greater degree of T cell cytokine suppression at high concentrations than dexamethasone, which is expected given the specific mechanism of action of cyclosporine involves inhibition of NFAT-regulated cytokines.
The minimal suppression of IL-2 following incubation with dexamethasone deserves further study, however, and suggests that IL-2 has the potential to be a fairly specific biomarker for cyclosporine in dogs receiving concurrent glucocorticoids.

In conclusion, this study provides an *in vitro* comparison of the effects of cyclosporine and dexamethasone on whole blood T cell cytokine assays in dogs. Cyclosporine caused concentration-dependent suppression of IL-2 and IFN-γ, with qRT-PCR better able to detect additional suppression at higher drug concentrations. Dexamethasone caused mild suppression of IL-2 as measured with qRT-PCR, but IL-2 levels were not significantly affected when measured by flow cytometry. In contrast, dexamethasone did cause concentration-dependent suppression of IFN-γ, and produced a significantly greater degree of suppression when measured by qRT-PCR compared to flow cytometry. This study determined that optimal incubation times for cytokine analysis in dogs are five hours with PMA and ionomycin for qRT-PCR, and seven hours for flow cytometry. Further work is needed to determine the analytical method with the greatest utility for reflecting the immunosuppression achieved by cyclosporine and glucocorticoids *in vivo*, but our results suggest that cytokine monitoring may be a viable method for monitoring the effects of immunosuppressive drugs in dogs.

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References


47. Reem GH, Yeh NH. Interleukin 2 regulates expression of its receptor and synthesis of gamma interferon by human T lymphocytes. Science 1984;225:429-430.

CHAPTER V

EFFECTS OF ORAL CYCLOSPORINE ON CANINE T-CELL EXPRESSION OF IL-2 AND IFN-GAMMA ACROSS A 12-H DOSING INTERVAL


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Abstract

The duration of immunosuppressive effects following oral cyclosporine in dogs is unknown. This study used flow cytometry and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) to evaluate the effects of high-dose oral cyclosporine across a 12-h dosing interval. Expression of interleukin-2 (IL-2) and interferon-gamma (IFN-\(\gamma\)) was compared before and after 8 days of cyclosporine at 10 mg/kg every 12 h in six healthy dogs. Samples were collected at 0, 2, 4, and 8 h
postdosing for analysis of unactivated and activated T-cell and whole blood cytokine expression using flow cytometry and qRT-PCR, respectively, and at 0, 2, 4, 6, 8, and 10 h postdosing for measurement of cyclosporine concentrations. Flow cytometry and qRT-PCR both demonstrated significant marked reductions in IL-2 and IFN-γ levels at 0, 2, 4, and 8 h after dosing compared to pretreatment levels ($P < 0.05$) for activated samples, with less consistent effects observed for unactivated samples. Both flow cytometry and qRT-PCR are viable techniques for measuring cyclosporine pharmacodynamics in dogs, yielding comparable results with activated samples. Two hours postdrug administration is the preferred time for concurrent assessment of peak drug concentration and cytokine expression, and T-cell activation is needed for optimal results.

**Introduction**

Cyclosporine, a calcineurin inhibitor, is an important immunosuppressive agent in both dogs and humans. A potent inhibitor of T-cell activation, cyclosporine decreases the expression of nuclear factor of activated T-cell (NFAT) -regulated cytokines, including interleukin-2 (IL-2), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α).\(^1\) Cyclosporine is commonly used in the treatment of inflammatory and immune-mediated diseases in dogs, but there is substantial controversy regarding appropriate dosage regimens and how to best assess response to therapy. Pharmacokinetic monitoring is available, but there is limited information regarding appropriate therapeutic cyclosporine blood concentrations for various disease states in dogs.\(^2\) Veterinarians generally evaluate trough cyclosporine concentrations while, in human transplant medicine, peak concentrations collected 2 h postdosing are better correlated with both transplant rejection and the development of cyclosporine toxicity.\(^3\)-\(^7\)
Blood cyclosporine concentrations in people do not predict patient response in all situations, and much work in human medicine has therefore been directed at pharmacodynamic monitoring of cyclosporine therapy.\textsuperscript{8-13} Pharmacodynamic studies of cyclosporine typically involve assays of either calcineurin activity or lymphocyte function. Lymphocyte proliferation, surface antigen expression, and cytokine production have all been evaluated after cyclosporine therapy, with most work in veterinary medicine focusing on reductions in peripheral blood and affected tissue cytokine levels.\textsuperscript{14-17} Flow cytometry and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) are two commonly used techniques to measure T-cell cytokine responses, but assess different outcomes. Flow cytometry measures actual protein levels, while qRT-PCR looks only at messenger ribonucleic acid (mRNA) expression, which does not necessarily correlate with protein production.

Previous work in our laboratory has both evaluated and validated techniques to measure cytokine levels in dogs treated with cyclosporine using flow cytometry and qRT-PCR.\textsuperscript{18-20} Our initial work demonstrated cyclosporine-mediated suppression of cytokines and activation-related surface antigens for T cells incubated with cyclosporine,\textsuperscript{19} and subsequent work confirmed that IL-2 and IFN-\(\gamma\) are suppressed after oral cyclosporine administration in dogs.\textsuperscript{18} However, it has not been established if cyclosporine-mediated suppression of NFAT-regulated cytokines is consistent across the dosing interval, or if there is T cell recovery as the next dose is approached. This study evaluated the levels of the cytokines IL-2 and IFN-\(\gamma\) measured using both flow cytometry and qRT-PCR across a 12-h oral cyclosporine dosing interval. Blood cyclosporine concentrations were also measured. The goals of this study were to compare results obtained using both flow
cytometry and qRT-PCR, and to determine the optimal time and method for pharmacodynamic measurement of cyclosporine’s effects on T cells.

Material and Methods

Dogs

This project involved six healthy, purpose-bred, adult female Walker hounds. Prior to the study, each dog received a physical examination, complete blood count, serum biochemistry profile, urinalysis, fecal flotation, and heartworm testing, with no significant abnormalities noted. Study protocols and animal care regimens were approved by the Mississippi State University Institutional Animal Care and Use Committee. Mississippi State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Cyclosporine administration

A repeated-measures design was used. Before drug administration, blood was collected from all dogs for pretreatment evaluation of IL-2 and IFN-\(\gamma\) using both flow cytometry and qRT-PCR. The dogs were divided into two groups of three dogs to simplify sample processing. The first three dogs were then given oral microemulsified cyclosporine (Atopica®, Elanco, Greenfield, IL, USA), at a dose of 10 mg/kg every 12 h for 8 days. On day 8, blood was collected immediately prior to the next dose (0 h or ‘trough’ sample), and at two-hour intervals (2, 4, 6, 8, and 10 h) after drug administration, for cyclosporine blood concentration determination. Additional blood was collected predosing (0 h), and at 2, 4, and 8 h after dosing for cytokine analysis using flow cytometry and qRT-PCR. Six days after the first three dogs began drug
administration, the other three dogs began oral cyclosporine at the same dose and had blood collected at the previously described time points after 8 days of treatment. All blood was collected using jugular venipuncture with a 20-gauge needle and syringe, with collected blood immediately transferred to heparinized vacutainers for cytokine evaluation, and ethylenediaminetetraacetic acid (EDTA) tubes for cyclosporine blood concentration determination.

**Cyclosporine blood concentrations**

Blood was collected into EDTA anticoagulant tubes, and shipped to the Auburn University Veterinary Clinical Pharmacology Laboratory on ice for analysis within 48 h of collection. Samples were thawed to room temperature and then mixed by inversion to assure homogeneity. Cyclosporine was detected in canine EDTA whole blood using the Siemens (New York, NY, USA) Cyclosporine Immunoassay® (CSA) and the Siemens Cyclosporine Extended Range Immunoassay® (CSAE) on a Siemens (New York, NY, USA) Dimension Xpand Plus® general chemistry analyzer. For CSA, the upper limit of quantitation was 500 ng/mL, and the lower limit of quantitation was 25 ng/mL. CSA was calibrated using the Siemens CSA Calibrator®, and quality control was performed using More Diagnostics (Los Osos, CA, USA) RAP/TAC/CSA Controls®. For CSAE, the upper limit of quantitation was 2000 ng/mL, and the lower limit of quantitation was 350 ng/mL, with the ability to dilute up to 6000 ng/mL. CSAE was calibrated with the Siemens CSAE Calibrator®, and quality control was performed using More Diagnostics Cyclosporine C2 Controls®.
**Cytokine analysis**

**Flow cytometry**

Flow cytometric analysis was performed as previously described by our laboratory, with modifications as described below. Antibodies used were as follows: FITC-conjugated monoclonal anti-dog cluster of differentiation 3 (CD3) (MCA1774F, AbD Serotec, Raleigh, NC, USA), RPE-conjugated monoclonal anti-bovine IFN-γ (MCA1783PE, AbD Serotec), and biotinylated anti-canine IL-2 (BAF1815, R&D Systems, Minneapolis, MN, USA). The secondary label for IL-2 was RPE-conjugated streptavidin (#60669, Anaspec, San Jose, CA, USA). Heparinized blood was diluted with complete media at a ratio of one part blood, nine parts media. Half of the diluted whole blood samples were not activated (‘unactivated’), and the other half were activated with 12.5 ng/mL phorbol 12-myristate 13-acetate (PMA, cat. P8139) and 0.8 μM ionomycin (cat. I0634), both purchased from Sigma-Aldrich (St. Louis, MO, USA). All samples were then incubated for 7 h at 37°C in a 5% CO₂ incubator. Brefeldin A (cat. 555029, BD Biosciences, San Jose, CA, USA) was added 1 h after activation at a final concentration of 1 μg/mL to stop cytokine secretion from T cells. After incubation, 350 μL of cell suspension was collected per sample, and anti-CD3 monoclonal antibody was added directly to the whole blood mixture and incubated for 25 min at room temperature in the dark. Red blood cells were lysed with BD Pharm Lyse™ (cat. 555899, BD Biosciences, San Jose, CA, USA) as recommended by the manufacturer. Cells were then fixed and permeabilized using the Becton Dickinson (BD) Cytofix/Cytoperm™ Plus Kit (cat. 554714, BD Biosciences, San Jose, CA, USA), stained for IL-2 and IFN-γ, and prepared for flow cytometry as previously described.
A BD FACSCalibur™ flow cytometer was used for staining evaluation, with data analyzed using BD CellQuest™ Pro software (BD Biosciences, San Jose, CA, USA). Lymphocytes were identified using forward scatter and side scatter, and 5000 lymphocytes were collected per sample. An additional gate identified CD3+ cells, and cytokine expression was measured from cells within the lymphocyte and CD3+ gates. Cell staining was measured using mean fluorescence intensity (MFI) values with single histogram statistics, and isotype controls and unstained samples were used as negative controls.

**Quantitative RT-PCR**

Heparinized whole blood was activated using 12.5 ng/mL PMA and 0.8 μM ionomycin. Another set of blood samples was left unactivated, and all samples were incubated for 5 h at 37°C and 5% CO₂. Total RNA was extracted and qRT-PCR analysis of IL-2 and IFN-γ expression performed as previously described by our laboratory, with the minor modification of using 1.5 ng/μL RNA template in a 20 μL final reaction volume. RNA was frozen at 80 °C until analysis, and samples from each dog were analyzed on a single qPCR plate. Reactions were analyzed on a Stratagene™ Mx3005P using Stratagene™ MxPro QPCR software v4.10 for analysis (Agilent Technologies, Santa Clara, CA, USA).

Relative gene expression was assessed for threshold cycle (Ct) using the 2-ΔΔCt method where ΔΔCt = (CtGOI - Ctnorm)_{treated} - (CtGOI - Ctnorm)_{untreated}, GOI is the gene of interest, and norm is the reference gene (GAPDH).
Statistical analysis

A repeated-measures design was utilized in this study. Visual assessment of the data using histograms with UNIVARIATE procedure of SAS® for Windows® 9.3 (SAS Institute, Inc., Cary, NC, USA) indicated that the data were not normally distributed. Consequently, the data were transformed by taking the reciprocal square root of each value. Histograms indicated the transformed data were approximately normally distributed. A separate mixed-effects model for each outcome was used to test for a time effect using the MIXED procedure of SAS® for Windows® 9.3. A first-order autoregressive covariance structure was specified in the repeated statement to accommodate the repeated measures. For outcomes in which time had a significant effect ($P \leq 0.05$), comparisons were made between each pair of time points using differences in least square means. Tukey Kramer adjustment was used for adjustment of $P$ values for the multiple comparisons. A $P$ value of less than or equal to 0.05 was considered to be significant for all analyses.

Results

Cyclosporine blood concentrations

Blood cyclosporine concentrations are presented in Fig. 5.1. After 8 days of cyclosporine at 10 mg/kg every 12 h, peak concentrations were found at two hours postdosing. At two hours postdosing, the range was 1944–5148 ng/mL, and the median was 3040 ng/mL. Trough concentrations (0 h) ranged from 375 to 1021 ng/mL, with a median of 701 ng/mL.
Figure 5.1  Cyclosporine blood concentrations after 10 mg/kg oral dosing

Blood cyclosporine concentrations in six dogs measured on Day 8 after 7 days of oral cyclosporine dosed at 10 mg/kg every 12 h. Each box represents the interquartile range (IQR) from the 25th to 75th percentile. The point inside each box represents the median, and whiskers extend to maximum and minimum values. Cyclosporine concentrations were measured using an immunoassay.

Cytokine analysis

Flow cytometry

Flow cytometry results are shown in Figs 5.2 and 5.3. Activated sample expression of IL-2 and IFN-γ was significantly reduced relative to pretreatment values at 0, 2, 4, and 8 h after dosing for both cytokines ($P < 0.05$). There was also significantly reduced expression for the 2, 4, and 8 h samples relative to the 0 h (trough cyclosporine blood concentration) sample for IFN-γ, and for the 8 h sample relative to the 0 h sample for IL-2. All activated cyclosporine samples in all dogs at all post-treatment time points showed markedly lower protein expression than pretreatment samples (Fig. 5.2).
Figure 5.2  Flow cytometry: Activated T-cell expression of IL-2 and IFN-γ

Activated T-cell expression of IL-2 (a) and IFN-γ (b) measured using flow cytometry in 6 dogs on Day 8 after oral cyclosporine dosing at 10 mg/kg every 12 h. Each box represents the IQR. The point inside each box represents the median, and whiskers extend to maximum and minimum values. Times that do not share a letter are significantly different ($P < 0.05$) based on mixed model analysis of data transformed by taking the reciprocal square root.

Unactivated sample analysis (Fig. 5.3) revealed significant reduction in IL-2 cytokine levels for hours 0, 2, 4, and 8 relative to pretreatment. IL-2 cytokine levels at hour 8 were also significantly lower than at hours 0, 2, and 4. Only hours 2 and 8 were significantly lower than pretreatment for IFN-γ levels, and hour 8 was also significantly lower than hour 0. There was an overall smaller difference in MFI across the sampling
times for unactivated samples than for activated samples, largely due to unactivated pretreatment samples having much lower fluorescence than activated pretreatment samples.

Figure 5.3  Flow cytometry: Unactivated T-cell expression of IL-2 and IFN-γ

Unactivated T-cell expression of IL-2 (a) and IFN-γ (b) measured using flow cytometry in 6 dogs on Day 8 after oral cyclosporine dosing at 10 mg/kg every 12 h. Each box represents the IQR. The point inside each box represents the median, and whiskers extend to maximum and minimum values. Times that do not share a letter are significantly different ($P < 0.05$) based on mixed model analysis of data transformed by taking the reciprocal square root.
Quantitative RT-PCR

Quantitative RT-PCR results are shown in Figs 5.4 and 5.5. Activated expression of IL-2 and IFN-γ mRNA was significantly reduced relative to pretreatment values at 0, 2, 4, and 8 h after dosing for both cytokines ($P < 0.05$). In contrast to flow cytometry, there was no statistically significant variation in the degree of suppression across the dosing interval. All activated cyclosporine samples in all dogs at all post-treatment time points showed markedly lower gene expression than pretreatment samples (Fig. 5.4). There were no significant differences in mRNA expression for unactivated samples (Fig. 5.5).
Figure 5.4  Quantitative RT-PCR: Activated T-cell expression of IL-2 and IFN-γ

Activated whole blood mRNA expression of IL-2 (a) and IFN-γ (b) measured using qRT-PCR in six dogs on Day 8 after oral cyclosporine dosing at 10 mg/kg every 12 h. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method where $Ct = (Ct_{GOI} - Ct_{norm})_{treated} - (Ct_{GOI} - Ct_{norm})_{untreated}$, GOI is the gene of interest, and norm is the reference gene GAPDH. Data are expressed as a percentage of cytokine expression in pretreatment samples, which are given a value of 100%. Each box represents the IQR. The point inside each box represents the median, and whiskers extend to maximum and minimum values. Times that do not share a letter are significantly different ($P < 0.05$) based on mixed model analysis of data transformed by taking the reciprocal square root.
Figure 5.5  Quantitative RT-PCR: Unactivated T-cell expression of IL-2 and IFN-γ

Unactivated whole blood mRNA expression of IL-2 (a) and IFN-γ (b) measured using qRT-PCR in six dogs on Day 8 after oral cyclosporine dosing at 10 mg/kg every 12 h. Relative quantification was performed using the 2^-ΔΔCt method where Ct = (CtGOI - Ct(norm))treated - (CtGOI - Ct(norm))untreated, GOI is the gene of interest, and norm is the reference gene GAPDH. Data are expressed as a percentage of cytokine expression in pretreatment samples, which are given a value of 100%. Each box represents the IQR. The point inside each box represents the median, and whiskers extend to maximum and minimum values. No significant differences identified.

Discussion

To the authors’ knowledge, this study provides the first paired evaluation of flow cytometry and qRT-PCR for measurement of cyclosporine pharmacodynamics in dogs.
Our results show that high-dose oral cyclosporine markedly and consistently suppresses cytokine expression in healthy dogs in activated blood samples when evaluated using either technique. Flow cytometry did show an extra degree of suppression of protein expression at 2, 4, and 8 h relative to 0 h activated samples for IFN-γ, and a significant difference between hour 0 and hour 8 for activated IL-2. Overall, however, there was minimal variability seen in suppression across the oral dosing interval, and even at trough drug concentrations 12 h after the previous oral cyclosporine dose (hour 0), both IL-2 and IFN-γ were significantly reduced from pretreatment levels for activated samples using both methods.

Unactivated cytokine expression showed similar findings as with activated samples for flow cytometry, although differences in fluorescence were of smaller magnitude. Activated samples had total separation of MFI values for pre- relative to posttreatment samples, while unactivated fluorescence was lower and had overlap in pre- and post-treatment MFI values. The most prominent suppression was seen for 8-h unactivated samples, which demonstrated significantly lower protein expression than all other times for IL-2, and were lower than pretreatment and 0-h samples for IFN-γ. Unactivated qRT-PCR samples, on the other hand, showed no significant differences between pretreatment and post-treatment expression levels. Activation of lymphocytes increases the production of cytokines, so lower expression and smaller differences among treatment times were expected for unactivated samples. Testing unactivated samples would decrease processing time, but because of the poorer separation in pre- and post-treatment cytokine levels, PMA and ionomycin activation of blood samples is
recommended for pharmacodynamic evaluation of cyclosporine’s effects. The remainder of this discussion will focus on activated cell expression results.

Previous work in our laboratory has evaluated both flow cytometry and qRT-PCR for assessment of cyclosporine pharmacodynamics.\textsuperscript{18-20} Our earlier results suggested that both techniques provide comparable results, and this study confirms that both methods document similar marked suppression of T-cell function for an extended period after each oral dose of cyclosporine. A previous study by Flores and others evaluated the ability of flow cytometry and qRT-PCR to assess suppression of cytokine expression caused by \textit{in vitro} exposure to cyclosporine and tacrolimus in blood from cynomolgus monkeys and concluded that both techniques could be used interchangeably when evaluating cyclosporine pharmacodynamics in monkeys.\textsuperscript{22} Our study confirms that both methods appear to be similarly interchangeable in dogs, at least at high drug doses.

The additional degree of cytokine protein suppression shown using flow cytometry between trough drug concentrations and later time points in the dosing interval suggests that this method may be more discriminating and able to reflect subtle differences in suppression of T-cell function at high cyclosporine concentrations. As suppression of cytokine mRNA expression in our study was slightly more rapid, sustained and complete than protein expression, our results suggest that the qRT-PCR assay may be more sensitive to the effects of cyclosporine and may therefore be able to identify suppression of T-cell function at lower drug concentrations, but further studies at lower cyclosporine concentrations would be needed to evaluate this possibility.

Blood cyclosporine concentrations following oral cyclosporine dosing in the current study were similar to those reported in previous studies, with a peak at two hours
and a gradual reduction in blood concentrations over subsequent hours. Previously, trough cyclosporine concentrations of 500 ng/mL have been suggested as a target for attainment of adequate immunosuppression in dogs. However, although only four of the six dogs in this study reached a 500 ng/mL trough concentration, with one dog attaining a trough of only 375 ng/mL, all dogs showed marked suppression of cytokine expression. Although ideal peak cyclosporine concentrations for dogs have not been published, a target peak drug concentration of 800–1400 ng/mL is recommended by the Auburn University Veterinary Clinical Pharmacology Laboratory for dogs, and all of the dogs in the current study exceeded this level. In fact, the lowest peak in this study was 1944 ng/mL, and the median was 3040 ng/mL. Given the pronounced suppression of cytokines in our study, our results suggest that, in dogs as in people, it is likely that peak cyclosporine concentrations may correlate better with immunosuppressive effects compared to trough concentrations. Although our study only looked at two cytokines and therefore may not reflect all of the immune effects of cyclosporine, these two cytokines are known to reflect the drug’s main mechanism of action.

In human pharmacokinetic studies, cyclosporine area under the curve (AUC) has been shown to have the best correlation with clinical outcome. Numerous studies have confirmed the lack of correlation of trough cyclosporine concentrations with AUC and clinical outcome and have identified improved outcomes when measuring peak drug concentrations as a surrogate measure for AUC. In humans, cyclosporine absorption occurs primarily in the first 4 h after oral administration, and drug absorption exhibits high inter- and intra-individual variability that is not adequately reflected by trough measurements. Temporally, calcineurin inhibition has been shown to closely
follow cyclosporine blood concentrations in humans and mice, with little residual inhibition of calcineurin enzyme activity once blood drug concentrations drop below peak levels. Interestingly, however, residual suppression of T-cell expression of NFAT-regulated cytokines can persist long beyond peak cyclosporine levels and the expected parallel transient inhibition of calcineurin, as demonstrated by our study. The results of our study, along with consideration of past studies, suggest that peak cyclosporine blood concentrations determine the degree of calcineurin inhibition but that, even when blood drug concentrations then drop markedly, the residual effects of transient calcineurin inhibition on NFAT-regulated cytokine expression persist for a sustained period of time. Pharmacodynamic monitoring of cytokine expression in dogs may therefore be the best means of determining both the extent and duration of suppression of T-cell function. Further evaluation of cyclosporine pharmacokinetics and pharmacodynamics in dogs, and correlation of results with clinical outcome in canine patients treated with cyclosporine, is warranted.

One goal of the present study was to determine the ability of both flow cytometry and qRT-PCR to detect differences in T-cell cytokine expression across the cyclosporine dosing interval. Previous studies in human medicine used changes in cytokine expression from trough to peak cyclosporine blood concentrations as indicators of the degree of immunosuppression. Although flow cytometry did show mildly increased suppression from trough to peak levels, overall the cytokine levels in our study were markedly suppressed at all time points after cyclosporine administration, and there was minimal change across the dosing interval. The high oral cyclosporine dose used in this study most likely caused maximal immunosuppression without allowing time for immune
recovery between doses. We chose a 10 mg/kg twice daily dose of oral cyclosporine for this study because, based on our previous work, this dose tended to place trough drug levels at or around the previously published target immunosuppressive concentration of 500 ng/mL. Lesser cyclosporine doses would be expected to cause more variation in cytokine levels over time and, in fact, previous work in our laboratory confirmed that when the much lower approved oral cyclosporine dose for canine atopic dermatitis (5 mg/kg once daily) was administered to healthy dogs, suppression of activated T-cell cytokine expression 8 h after drug dosing in individual dogs varied from minimal to marked. Based on studies in people, it is possible that marked suppression of T-cell function at the time of peak cyclosporine blood concentrations with subsequent partial recovery of suppression at the time of trough levels may be useful as an indicator of adequate but not excessive immunosuppression and that the dose of cyclosporine used in our study caused a greater degree of immunosuppression than might be needed clinically. Further studies with various cyclosporine doses across the entire dosing interval will be needed to determine whether the degree of suppression of T-cell function varies postdosing in individual dogs at lower drug doses. For dogs on high doses of cyclosporine, however, the results of our present study suggest that, using qRT-PCR, cytokine analysis performed at any time point during therapy will likely reflect maximal immunosuppression. For flow cytometry, however, samples 2–8 h postdosing may be more representative.

To allow direct comparison with our previous work with cyclosporine, in which effects were assessed after 1 week of drug therapy, we chose to monitor the effects of cyclosporine on T-cell function after a full week of drug dosing, and our study confirmed
that by this time cytokine expression was markedly suppressed. Undoubtedly, the immunosuppressive effects of cyclosporine will begin to manifest before completion of a full week of therapy, and further studies will be needed to determine how rapidly suppression of T-cell cytokine expression occurs after drug therapy is commenced.

Our study has confirmed that at high drug doses expression of the cytokines IL-2 and IFN-γ is consistently reduced by cyclosporine, and suggests that, as in humans, NFAT-regulated cytokine assays show great promise as biomarkers of drug-induced immunosuppression. In human medicine, the information provided by pharmacokinetic and pharmacodynamic monitoring is considered to be complementary, and the authors suggest that utilization of both techniques will be needed to develop optimal immunosuppressive regimens in canine patients.

Limitations of this study include the relatively small number of animals, the lack of an untreated control group, the single dog breed (Walker hound) used, and the use of only healthy animals. The sample size is typical for a standard pharmacokinetic study, and results were consistent among all dogs, with marked differences noted between pretreatment and post-treatment results. A formal untreated control group was not included, and provision of a control group would have confirmed a lack of diurnal effect. However, a single untreated dog was included in the flow cytometry groups to ensure appropriate sample activation at all times (data not shown). The Walker hound is not known to have issues with cyclosporine metabolism or variations in activity of the efflux pumps that handle drug metabolism and is expected to serve as an acceptable model for all dog breeds. Cyclosporine is commonly used for inflammatory and immune-mediated diseases, and it is possible that the presence of these conditions will affect cytokine
responses to cyclosporine. As results from this study reflect healthy dogs only, further work will be needed to confirm the relevance of these assays in diseased clinic patients.

In conclusion, our study describes the use of flow cytometry and qRT-PCR to measure expression of the cytokines IL-2 and IFN-γ after oral cyclosporine administration. Both techniques demonstrated marked reduction in activated cytokine levels at 2, 4, 8 and 12 h after administration of high doses of cyclosporine, and the degree of suppression of T-cell function varied only slightly across the 12-h dosing interval. Unactivated sample results were more variable, especially for qRT-PCR. Based on these results, pharmacodynamic monitoring of T-cell function could be performed using PMA and ionomycin activation at any time point in the 12-h interval between drug doses in dogs receiving chronic high-dose cyclosporine. Concurrent cyclosporine blood concentration measurement revealed 12-h trough concentrations that, based on previous studies, would be considered acceptable in only four of six dogs, but high (above reference laboratory ‘target’ range) 2 h peak concentrations in all dogs. As peak drug concentrations were attained in all dogs at 2 h postdosing, and cytokine expression was also markedly suppressed in all dogs at 2 h postdosing, 2 h after administration of high doses of cyclosporine is likely the most promising single time point for concurrent pharmacokinetic and pharmacodynamic assessment, although further study is needed to clarify the value of cytokine expression changes from trough to peak drug concentrations at lower drug doses. Both flow cytometry and qRT-PCR provided similar information, and our study suggests that both assays can be used interchangeably when monitoring cyclosporine therapy, at least at high drug doses. Further studies at different cyclosporine doses, and at earlier intervals after commencement of therapy, will be necessary to
evaluate the full range of effects of the drug on T-cell function and to fully determine the best technique for cytokine assessment.

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References


This dissertation describes initial investigations into pharmacodynamic assays of the effects of cyclosporine in dogs. We showed that incubation with cyclosporine inhibited the production of activation-related cytokines and surface antigens from canine T cells \textit{in vitro} using flow cytometry, and then modified this assay for whole blood. We then investigated whole blood responses to \textit{in vitro} incubation with cyclosporine and dexamethasone across a range of concentrations, and identified concentration-dependent suppression of IL-2 and IFN-\(\gamma\) after cyclosporine treatment with both flow cytometry and qRT-PCR. Cytokine production was maximally suppressed at 100 ng/mL cyclosporine by flow cytometry, and at 500 ng/mL cyclosporine by qRT-PCR. Dexamethasone caused greater inhibition of IFN-\(\gamma\) than IL-2, and this effect was more pronounced when measured with qRT-PCR than flow cytometry. Our final study investigated cytokine expression by flow cytometry and qRT-PCR in dogs after high dose oral cyclosporine, and documented uniform marked suppression of both IL-2 and IFN-\(\gamma\) gene expression measured using qRT-PCR across a 12-hour dosing interval. For flow cytometry, samples at the time of trough blood drug concentrations were significantly less suppressed than at the time of peak concentrations and subsequent sampling times for IFN-\(\gamma\), and the 8-hour sample was significantly more suppressed than samples at trough blood concentrations for IL-2. These results suggest that flow cytometry was able to reflect immune recovery...
as the next cyclosporine dose was reached. Unactivated samples were also evaluated in this study, but no significant differences in gene expression following administration of cyclosporine were found using qRT-PCR, and flow cytometry effects were less pronounced and more variable compared to the effects observed when T cells were activated. This confirms the need for activation of lymphocyte samples to enhance cytokine production when evaluating cyclosporine pharmacodynamics.

The transition from PBMC to whole blood assays reflects an important adjustment that preserves the effects of cyclosporine present in the plasma, and also more closely mimics the environment *in vivo*. Chapters IV and V describe two comparisons of qRT-PCR and flow cytometry with whole blood assays. Confirmation that gene expression measured using qRT-PCR to a large extent parallels protein production measured using flow cytometry is valuable. Our studies found similar results for both methods, with the most notable difference being that IL-2 gene expression, but not protein production, was significantly lower after *in vitro* incubation with dexamethasone. It is interesting that while qRT-PCR seemed to be the more discriminating test, and more able to reflect the effects of higher cyclosporine concentrations *in vitro*, in the *ex vivo* study reported in Chapter V, flow cytometry but not qRT-PCR detected subtle differences in cytokine levels across the oral dosing interval. This finding underscores the need for confirmation of *in vitro* findings with subsequent oral dosing studies in dogs.

One limitation of our oral dosing study is that it was performed only in healthy dogs. Concurrent disease has the potential to make cytokine levels more variable and to influence patient drug disposition, and the influence of disease on the effects of
cyclosporine deserve further investigation through pharmacokinetic/pharmacodynamic correlation in clinic patients.

The original overall hypothesis of our research laboratory was that a comprehensive panel of biomarkers of immunosuppression could be used to objectively and accurately establish target doses and drug concentrations of cyclosporine in the dog. Some biomarker panels for cyclosporine therapy have included lymphocyte proliferation, surface antigen expression, and cytokine evaluation. While a larger panel does offer the opportunity for more detailed and sensitive evaluation of the effects of an immunosuppressive drug, the potential biomarkers evaluated in our studies were all suppressed after exposure to cyclosporine, and all seemed to provide similar information. For this reason, we elected to pursue the biomarkers that showed the most significant suppression in response to cyclosporine, which, not surprisingly, were gene transcription and protein production of NFAT-regulated cytokines.

In another study by our laboratory not described in this dissertation, both high dose cyclosporine and the atopy cyclosporine dose of 5 mg/kg once daily were investigated after oral dosing in dogs. This study showed that IL-2 and IFN-γ, but not IL-4, were significantly decreased by high dose oral cyclosporine when measured using flow cytometric evaluation of PBMC. Interferon-γ production was also significantly decreased by the lower atopy dose of cyclosporine. These results, along with the studies in this dissertation, suggested that IL-2 and IFN-γ were the best biomarkers for monitoring the effects of cyclosporine in dogs, so we persisted with these markers in subsequent studies. Although not as comprehensive as a full panel of biomarkers,
limiting the number of biomarkers evaluated also makes application to clinic patients more practical and cost-effective.

Our overall hypothesis also proposed that our biomarker assays could be used to establish target drug concentrations and drug doses in dogs. This is an area where substantial investigation is still needed. Chapter V describes the effects only of a single high dose of cyclosporine and, as expected, biomarkers were dramatically suppressed. The blood concentrations obtained in this study supported the comments of Dr. Dawn Boothe, who suggested that achieving reliable peak cyclosporine concentrations is more readily accomplished than attaining target trough concentrations.² All of the dogs in this study far exceeded Dr. Boothe’s target peak drug concentrations of 800-1400 ng/mL (actual range 1944-5148 ng/mL), while only 5 of 6 dogs achieved minimum target trough concentrations of 400-600 ng/mL, and only 3 dogs’ trough values exceeded this range. Additional work at lower oral cyclosporine doses is needed to determine if cytokine expression correlates better with peak or trough blood concentrations. Target blood concentrations will also need to be critically evaluated based on cytokine expression and clinical responses to best determine ideal dosing protocols for dogs. Based on our study, the marked immunosuppression seen in all dogs suggests that reevaluation of at least target blood trough concentrations is needed.

Another important consideration when suggesting adjusting dosing recommendations for cyclosporine based on biomarker assays is the effect of concurrent medications. Co-administered medications have the potential to influence cytokine assays, and we did notice an effect, especially for IFN-γ, caused by exposure to dexamethasone in vitro using our assays. Two other studies have reported no significant
effect from *in vitro* exposure to either methylprednisone/methylprednisolone or mycophenolic acid on T cell IL-2 production,\(^3,4\) and it may have been the short 2-4 hour incubations in these studies that limited this interference. In clinic patients, determining the individual effects of each drug on IL-2 or IFN-γ production may arguably be less important, since the overall goal of combination drug therapy is greater reduction in T lymphocyte and other immune cell responsiveness. Presently, however, it is important to be able to determine the relative contribution of other drugs to biomarker suppression, because the best optimization of cyclosporine therapy will include correlation with clinical outcome in patients where additional immunosuppressive drugs are frequently co-administered. Therefore, further investigation of our assays in dogs receiving multiple immunosuppressant medications is needed.

The described work shows that our proposed biomarkers are successfully able to reflect the effects of *in vitro* and orally administered cyclosporine and that, as expected, cyclosporine causes reduction of T cell activation-related surface antigens and NFAT-regulated cytokines. Pharmacodynamic measures are intended to compliment the information obtained from pharmacokinetic assays. Blood drug concentrations are still needed to identify differences in drug absorption, and to determine the effects of drugs that alter cyclosporine’s metabolism, such as ketoconazole. Although achieving a target level of T cell suppression is encouraging, it is not yet known what level of suppression is required for different diseases in veterinary medicine, or if a single target level will lead to a consistent outcome among dogs. Therefore, for life-threatening immune-mediated diseases and transplantation, attainment of target peak or trough blood concentrations along with concurrent evaluation of cytokine function is suggested to provide as much
information as possible to optimally individualize therapy. Once more studies are performed correlating both pharmacokinetic and pharmacodynamic responses to clinical outcomes, this recommendation may be able to be revisited for certain diseases. Based on experience in human medicine, however, it is likely that dog pharmacodynamic responses will be highly variable even with similar blood drug concentrations, thus necessitating the continued performance of both types of assays.

Pharmacodynamic monitoring carries the advantage of avoiding several issues associated with pharmacokinetic monitoring. By measuring effects on the final immune target cell, the T cell, pharmacodynamic assays circumvent pharmacokinetic issues like the relevance of free drug concentration in plasma versus total drug concentration, and the influence of any biologically active metabolites. Pharmacodynamic assays also have the potential to allow further investigation of the effects of the ABCB1 genotype and variations in P-glycoprotein function on patient cyclosporine response. Mealey and others showed that blood concentrations of cyclosporine and other P-glycoprotein substrates were not affected by decreased P-glycoprotein expression. However, Mealey and others also found that, at comparable blood concentrations of loperamide, P-glycoprotein deficient dogs were sedated while wild-type dogs were not, suggesting that a deficiency of P-glycoprotein at the blood-brain barrier can lead to significant differences in local drug effects at the level of the central nervous system. A similar effect may exist for lymphocytes, which also utilize P-glycoprotein as an efflux pump to expel cyclosporine from the cell cytoplasm. P-glycoprotein deficiency could lead to decreased drug efflux from affected lymphocytes and greater immune suppression in response to the same blood concentration of cyclosporine. Pharmacodynamic assays
have the ability to identify these target cell responses, and thereby provide important clinical information with the potential to help explain individual patient variability to cyclosporine.

Less certain is whether measuring circulating T cell responses is relevant for local rather than systemic diseases, or for conditions affecting tissues where cyclosporine concentrates, such as the skin. Cyclosporine may cause a local effect within the gastrointestinal tract, for example and, as such, the effects of the drug on circulating T cell responses may not be directly relevant to gastrointestinal effects. In contrast, the opposite argument has been made for the use of cyclosporine in dogs with meningoencephalomyelitis of unknown origin (MUE). Cyclosporine poorly crosses the blood-brain barrier, but has still been found to be clinically effective for MUE. Adamo and others suggest that inflammation may allow cyclosporine to cross the blood-brain barrier or that, since autoimmune responses originate in peripheral lymphoid organs, suppressing circulating T cell function may be sufficient to create a successful clinical response. Further study is needed to correlate pharmacodynamic responses with clinical outcome for different disease states.

The described cytokine assays are ready for immediate investigation in clinic patients being treated with cyclosporine. Future investigations could also evaluate cytokine responses at different drug doses to confirm the best assay for assessing cyclosporine pharmacodynamics in dogs, and could also evaluate the effects of co-administered medications. These assays would also likely be useful to evaluate other calcineurin inhibitors, such as tacrolimus. If it turns out, as is suggested in human medicine, that suppression of NFAT-regulated cytokines is more reflective of the effects
of cyclosporine than other immunosuppressive drugs, this will be helpful in the continued clinical evaluation of cyclosporine in dogs. However, this would also raise the question of what assays can be performed to evaluate the effects of other medications, and to assess the overall immune status of the patient. Last, it will be important to understand the effects of different disease states on baseline cytokine expression to be able to effectively interpret patient responses to cyclosporine.

This dissertation describes preliminary work investigating cyclosporine pharmacodynamics in dogs. Assay results are encouraging, and with further investigation, cyclosporine pharmacodynamics may become an accepted method of cyclosporine monitoring, with the ability to enhance the understanding of individual patient immune responses to a potentially life-saving drug.
References


