INVESTIGATION INTO THE DISTRIBUTION, PHARMACOKINETICS AND TOXICITY OF THE IMMUNOSUPPRESSANT CYCLOSPORIN G

A Thesis Submitted to the
Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

By

Michal P. Lukowski, B.Sc.
University of Manitoba
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The undersigned certify that they have read a Master's thesis entitled: INVESTIGATION INTO THE DISTRIBUTION, PHARMACOKINETICS AND TOXICITY OF THE IMMUNOSUPPRESSAN CYCLOSPORIN G

submitted by MICHAL P. LUKOWSKI

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Approved ☒ Not Approved ☐

Advisor

Head

External Examiner

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To my parents

for their enthusiastic support, encouragement,

and love during my many years of studies.

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ABSTRACT

Cyclosporin G (CsG) is a unique endecapeptide, which is synthesized by the fungus Tolypocladium inflatum. It is an analogue of the better known immunosuppressant Cyclosporin A (CsA), which has been widely used in the prevention of allograft rejection in kidney, heart, and liver transplantation as well as graft-versus-host disease. However, nephrotoxicity along with other side-effects caused by CsA have prompted the search for so-called non-nephrotoxic derivatives that are equipotent to CsA. Preliminary work in animals suggests that CsG is as immunosuppressive as CsA but not as nephrotoxic, although conflicting reports exist. Further, CsG is extensively metabolized to yield a number of metabolites. However, it is presently not known whether any of the metabolites exhibit any toxic effects. Should metabolites prove to have significant toxic properties, it would be important to monitor these in blood to allow appropriate dosage adjustment to minimize toxicity.

We have therefore used the rabbit to study the toxicity, pharmacokinetics and tissue distribution of CsG. Using cultured renal cell lines we investigated the in vitro toxicity of CsG and its metabolites. The distribution of CsG and CsG metabolites between plasma and erythrocytes was also investigated.

Our studies demonstrate CsG to be less nephrotoxic than CsA in the rabbit. On average CsG appears to have a shorter half-life and faster clearance than CsA. There appears to be no difference in the volume of distribution between the two drugs. However, there are significant differences in the tissue distribution of CsG and CsA. CsG is less effective than CsA at inhibiting the growth of the renal

cell lines studied. Two of the hydroxylated CsG metabolites were able to significantly inhibit the DNA synthesis of a primary renal cell line. The distribution of CsG and metabolites in blood differs from that of CsA.

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LIST OF ABBREVIATIONS

ALT	alanine transaminase
AUC	area under the curve
AST	aspartate transaminase
C_{max}	maximum concentration
°C	degrees Celsius
CL	clearance
cm	centimeter
CsA	cyclosporin A
CsG	cyclosporin G
Da	Dalton
EDTA	ethylenediaminetetraacetate
FAB-MS	fast-atom bombardment mass spectroscopy
x g	relative centrifugal force
GFR	glomerular filtration rate
HPLC	high pressure liquid chromatography
hr	hour
IL-2	interleukin-2
i.m.	intramuscular
i.v.	intravenous
kg	kilogram
L	liter
LDL	low density lipoprotein
LLC-PK1	porcine proximal tubule renal epithelial cell line
m/z	mass over charge ratio
mg	milligram
mL	milliliter
mm	millimeter
mmol	millimole

uCi	microcurie
ug	microgram
uL	microliter
um	micrometer
nm	nanometer
PAS	Periodic Acid-Schiff
PGE_2	prostaglandin E_2
p.o.	oral administration
R	regression coefficient
RIA	radioimmunoasssay
RPF	renal plasma flow
s.c.	subcutaneous
$t_{½}$	half-life
TCA	trichloroacetic acid
TxB_2	thromboxane B_2
U	unit
v	volume
Vd(ss)	volume of distribution
VLDL	very low density lipoprotein
w	weight

I. INTRODUCTION

A. CLINICAL USE

Cyclosporin G (CsG) is a unique endecapeptide, which is synthesized by the fungus Tolypocladium inflatum (1). It is an analogue of the better known immunosuppressant Cyclosporin A (CsA), which has been widely used in the prevention of allograft rejection in kidney, heart, and liver transplantation as well as graft versus host disease (2-6). Most of the research to date has concentrated on the study of CsA. However, nephrotoxicity along with other side-effects caused by CsA has prompted the search for CsA analogues with enhanced or equipotent immunosuppressive properties and reduced or no toxic side-effects.

Since its first use in 1978 in renal transplantation, CsA has been shown to significantly improve graft survival (7,8). In patients undergoing kidney transplantation, one year graft survival increased 10 - 20% as compared to conventional steroid therapy (9). Patients older than 55 years of age and strong immune responders, benefit most from CsA therapy (9). Currently trials are also underway investigating the use of CsA in the treatment of autoimmune diseases such as uveitis, diabetes mellitus, multiple sclerosis, pure erythrocyte aplasia and psoriasis (10-13). The main advantage of CsA over other drugs is that it does not compromise the primary defense system against bacterial or viral infection. The reduction in the dose of steroids in patients given CsA has resulted in a decrease in the number of deaths due to infection. Furthermore, at pharmacological doses it is neither cytotoxic nor myelosuppressive unlike drugs conventionally used for transplantation (14).

B. CsA ANALOGUES

The fungus Tolypocladium inflatum produces a large number of CsA analogues of the same structural type. At least 25 of these natural cyclosporins have been isolated and their chemical composition characterized (1,15). The most common changes occur at position 2 of the molecule, where the alpha-aminobutyric acid residue of CsA can be replaced by alanine, threonine, valine or norvaline. There are 14 such analogues, seven of which contain an N-desmethylated amino acid residue as an additional alteration in the molecule. Five cyclosporin analogues contain one N-desmethylated amino acid residue (positions: 1,4,6,10,11) and one analogue contains two N-desmethylated amino acid residues (position 6 and 10). Substitutions of amino acids do not occur at position 3 (sarcosine) and position 8 (D-alanine) (1,15). Hence, natural analogues produced by the fungus Tolypocladium inflatum mainly differ by a change in position 2 and/or by introduction of a N-desmethylated amino acid residue.

In addition to these natural cyclosporins, about 750 semisynthetic or synthetic analogues were produced and tested in vitro, but only a few of them are available in sufficient quantity for in vivo characterization (1). Of the compounds tested, none of the natural cyclosporins or the synthetic analogues possess greater pharmacological potency than CsA in either in vitro tests or in in vivo models (1).

Recent studies have shown that amino acids 1, 2, 3, 10 and 11 are required for full immunosuppressive activity (16). However, CsA analogues with altered alkyl chains at amino acid 2 show lesser but significant immunosuppressive activity (16). One

such naturally occuring analogue is CsG. Some work in animals suggests that CsG is as immunosuppressive but not as nephrotoxic as CsA, although conflicting reports are found in the literature (17-22).

C. CHEMICAL STRUCTURE AND SYNTHESIS

In CsG, alpha-aminobutyric acid is replaced by norvaline at the amino acid 2 position found in CsA. It is a cyclic endecapeptide with a molecular weight of 1217. The chemical structure of CsG is shown in figure 1. All cyclosporins contain a previously unknown nine carbon atom amino acid in position 1. The absolute configuration of this amino acid was shown to be (4R)-4[(E)-2-butenyl]-4,N-dimethyl-L-threonine (MeBmt) and was established as the first amino acid of the sequence of cyclosporins. The proton spectra (Fast Atom Bombardment/Mass Spectroscopy, FAB/MS) of CsG are almost identical to CsA (23). The protonated molecular ion of CsG of m/z 1217 corresponds to an increase of 14 Da over CsA, which is consistent with the addition of a methyl group at amino acid position 2. There are four areas in the ^{13}C-NMR spectra where CsG and CsA differ significantly. All of these changes in the chemical shift correspond to the individual carbon atoms of the amino acid 2, the only structural difference between CsA and CsG (23). Analysis of CsA by x-ray, has shown it to be composed of two different components: a beta-pleated sheet conformation of residues 1-6 and an open loop of residues 7-11. The additional methyl group at amino acid 2 of CsG, allows this analogue to retain the three dimensional structure of CsA.

CsG can be produced using a cell free system, which employs the use of an enzyme fraction from Tolypocladium inflatum. However, it can be easily produced in vivo, where a

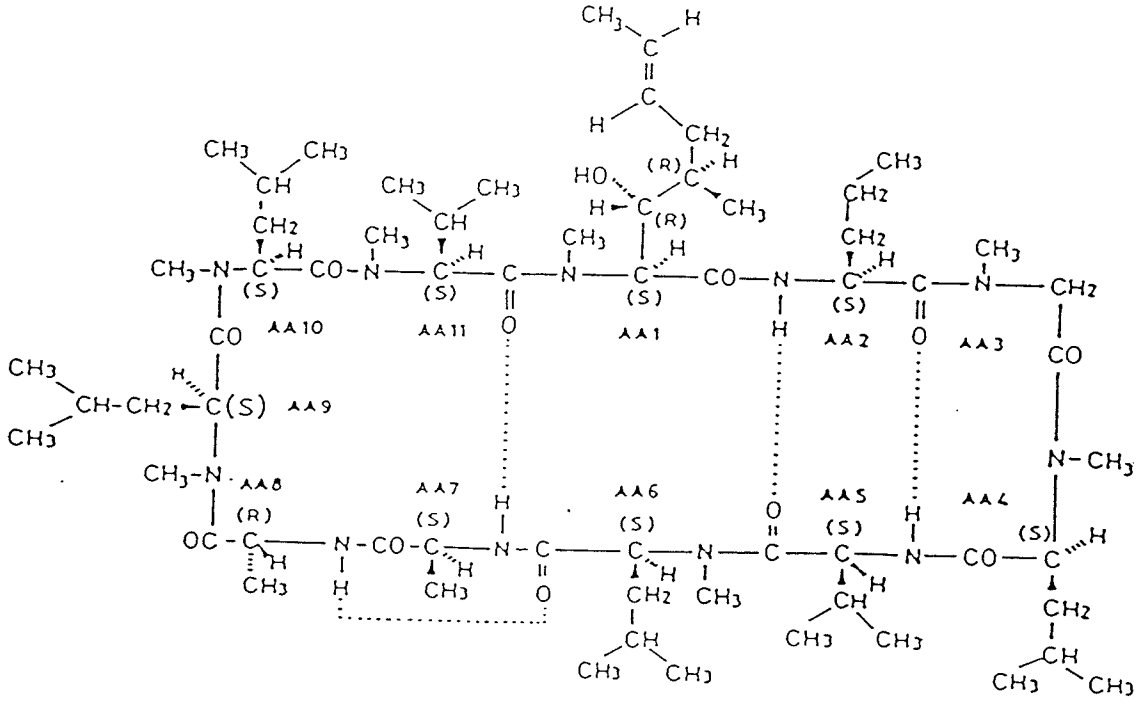


Figure 1. Chemical Structure of Cyclosporin G (CsG).

culture of this fungus can selectively produce CsG by adding norvaline to the fermantation media (15).

D. METABOLISM

Recently, Copeland and Yatscoff reported on the isolation of CsG metabolites from urine of patients receiving the drug in a clinical trial (23,24). They successfully isolated 7 metabolites of CsG and structurally identified them using FAB/MS as well as ^{1}H-NMR and ^{13}C-NMR. Modifications were found to occur primarily on amino acid 1, 4 and 9. Metabolism mainly consists of oxidative processes resulting in the hydroxylation of amino acid 1 and 9, giving rise to metabolites GM1 and GM9, respectively. Metabolite GM4N, arises from the oxidative N-demethylation on amino acid 4 of CsG. The structures of these CsG metabolites are consistent to what has previously been identified for CsA (23,24). This is not surprising due to the similarities in structure and conformation between the two molecules.

To date over 14 metabolites resulting from the biotransformastion of CsA have been chemically characterized. The cyclic oligopeptide structure of CsA is preserved in all identified metabolites. Metabolism of CsA involves the hydroxylation of amino acid 1, 4, 6 and 9 and oxidative N-demethylation of amino acid 4, giving rise to metabolites AM1, AM4, AM6, AM9 and AM4N, respectively (25). The major metabolites of CsA are AM1, AM9 and AM4N, which can then be further metabolized via hydroxylation, demethylation, cyclization or acidification of the appropriate amino acid. Likewise, the corresponding metabolites of CsG, namely GM1, GM9, and GM4N have been hypothesized to be primary metabolites of CsG and hence it is assumed that they will be further metabolized in a similar

fashion as described above for CsA. A dihydroxylated (GM19), hydroxylated and demethylated (GM4N9) and two cyclyzed metabolites (GM1c and GM1c9) have been already identified (24). These findings suggest that both CsG and CsA are metabolized by the same cytochrome P450 III A system, which is responsible for the metabolism of many hydrophobic compounds (26).

E. IMMUNOSUPPRESSION

In initial studies, CsA was found to markedly suppress haemagglutinin formation against sheep erythrocytes in mice (27). Its action is mainly restricted to T and B cells of the lymphoid system (28). CsA acts primarily by blocking the activation of lymphocytes at an early stage. It inhibits the production of IL-2, a T cell derived lymphokine which promotes the expansion of clones of effector lymphocytes which can be activated activated by transplantation antigens (27-29). CsA also indirectly inhibits monocyte function by suppressing production of T cell lymphokines, gamma-interferon, macrophage inhibitory factor, and macrophage chemotactic factor (14,30,31). At the cellular level, the inhibition of T lymphocyte proliferation is possibly mediated through binding to the intracellular protein cyclophillin (32). This protein has been found to catalyze the cis-trans isomerization of peptide bonds involving prolyl residue, the rate determining step for possible crucial protein folding processes in the immune response (32).

None of the natural cyclosporins or synthetic analogues possess greater pharmacological potency than CsA in either <u>in vitro</u> or <u>in vivo</u> models (1). A number of <u>in vitro</u> and <u>in vivo</u> studies have shown CsG to be as immunosuppressive as CsA (18,20,21,33). CsG was found to have a immunosuppressive profile similar to CsA, through its ability to

inhibit mitogen and alloantigen-induced production of gamma interferon, lymphotoxin and tumor necrosis factor, when peripheral blood mononuclear cells from normal individuals were subjected to CsG treatment (33). However, CsG was found to be 2-3 fold less immunosuppressive than CsA when using a human mixed lymphocyte culture system (23).

The immunosuppressive efficacy of either CsG or CsA metabolites have been studied by a number of investigators using a variety of in vitro systems. These include primary and secondary mixed lymphocyte reactions as well as mitogen stimulated systems (24,34,35). The immunosuppressive activity of CsA metabolites is affected by the site of modification, with the primary metabolites, AM1, AM9, and AM4N being most active (36). Metabolite AM1 retains 10-20% of the activity of CsA (36). The same appears to be true for CsG metabolites. The primary CsG metabolites GM1, GM9 and GM4N are the most active metabolites identified to date (24). Their immunosuppressive activity is 5-10% that of CsG (24). The role of metabolites in the overall immunosuppression remains uncertain, until in vivo animal studies can confirm these results.

CsG immunosuppressive efficacy in vivo has been compared with that of CsA in experimental animal models. In dogs with renal allografts, CsG was found to be immunosuppressively equipotent to CsA (20,21). The same findings were made in rat renal and heart allograft models where the animals received 10 mg/kg CsA or CsG, although at lower doses (5 and 7.5 mg/kg) CsA proved to be a superior immunosuppressant (17,18). Other studies that investigated experimental animal allograft models were able to show CsG to be equipotent to CsA, although a lesser potency has also been reported (22,37). The reason for the conflicting data

could possibly reside in the variation of dosage and route of administration of the drug as well as species and organ transplantation differences (17,18,20-22,37,38).

F. PHARMACOKINETICS

1. Man

In a recent study, the pharmacokinetics of CsG were investigated in 6 patients with terminal renal failure after oral administration (600 mg) of the drug (39). Based on HPLC (high pressure liquid chromatography) determination of whole blood concentrations, the resulting pharmacokinetic parameters of CsG were similar to those described for CsA. Maximum CsG concentration in blood was reached between 2.5 and 3 hours and the bioavailability was in the range of 24 to 55%. After a 4-hour intravenous infusion (3.5 mg/kg), the terminal elimination half-life ($t_{½}$) for CsG was 18.9 hours , which is in good agreement with 15.8 hours found for CsA (39). The total body clearance (CL) was 0.55 L/hr/kg, and the volume of distribution at steady state ($V_{d(ss)}$) was reported as 5.97 L/kg. CL and $V_{d(ss)}$ are both less for CsA, 0.34 L/hr/kg and 4.5 L/kg, respectively (40,41). In another study two renal transplant patients to whom cyclosporin G was administered orally, the drug exhibited on average, an increased $V_{d(ss)}$ of 5.36 L/kg and CL of 1.758 L/hr/kg, with decreased $t_{½}$ of 2.37 hr as compared with CsA (42).

CsA can be classified as a low to intermediate clearance drug. Both its clearance and elimination half-life are highly variable among patients and seem to be influenced by the type of

transplant, age, disease state and concurrent therapy (43). CsA is poorly absorbed after intramuscular (i.m.) administration and is therefore administered either orally (p.o.) or intravenously (i.v.) (44). Subcutaneous (s.c.) administration gives the most consistent pattern of absorption in the rat, but has not been used in man (45). After oral administration the absorption of cyclosporin from the intestine is slow and incomplete (40,43). The mean time from ingestion to peak cyclosporin blood level is approximately 4 hours. The inter-patient variation is striking, ranging in renal transplant patients between 1 and 8 hours (43). The bioavailability, which represents the percentage of a dose of cyclosporin that reaches the systemic circulation and is calculated from paired oral and intravenous administration , is approximately 30% and can range from 1% to 89% (40,43,46,47).

2. Animals

A few studies have compared CsG and CsA in animals. In 1987 Grant et.al. demonstrated in dogs that the area under the curve (AUC), the time to maximum concentration (C_{max}) and $t_{½}$ were generally higher for CsG in serum when compared to CsA after oral ingestion, although the difference was not statistically significant (48). After intravenous administration, the CL of CsG in dogs was significantly less compared to CL of CsA, with no difference in $t_{½}$ or $V_{d(ss)}$ (49). In rats the plasma CsG levels after a three week oral treatment with 10 mg/kg/day were higher than plasma levels of rats receiving the same dose of CsA (1).

The pharmacokinetics of CsG have also been studied in the rabbit (50). Here the CL of CsG was found to be significantly higher than that of CsA after a 15 mg/kg intravenous infusion. The $t_{½}$ of

CsG was significantly lower and there was no difference in $V_{d(ss)}$.

G. DISTRIBUTION IN BLOOD AND TISSUE

To date little is known about the distribution of CsG and CsG metabolites in blood. However, since CsG is structurally similar to CsA, one can assume some similarities.

The distribution of CsA between red blood cells and plasma is dependent upon a patient's hematocrit (40,51). Transplant recipients often have a low hematocrit due to chronic disease or intraoperative blood loss, resulting in altered drug distribution in these patients. In blood with a low hematocrit, a greater proportion of the drug resides in the plasma. This is due to the fact that the percentage of CsA associated with cells decreases as the hematocrit decreases, thus changing the overall redistribution of CsA. If CsA is added to drug-free whole blood, the relative concentration of CsA in plasma increase, when the respective concentration in whole blood exceeds between 500 to 1000 ug/L at 37°C (52). This is also true of metabolites AM1, AM9 and AM4N69 (52).

CsA also rapidly alters its distribution in blood with changes in temperature. The concentration of CsA measured in plasma can easily be affected by sample preparation. If whole blood is stored at either 4°C or room temperature, results for CsA in the plasma are lower than in whole blood stored at 37°C (53). Re-equilibration of the former to 37°C before the cells are removed increases the analytical recovery of CsA in plasma (53). The optimal equilibration interval is 30 minutes.

It has been shown that the blood-plasma ratio of CsG of 1.2 is smaller than that observed for CsA (1.5), when plasma is

separated at room temperature (39). This suggests that at equivalent whole blood concentration of the two drugs, more CsG would be found in the plasma.

CsA is a highly lipophilic drug and in a sample of whole blood at room temperature (21°C) with a CsA concentration of 500 ug/L, 60% of the drug is bound to erythrocytes (54). The fraction not bound to erythrocytes is mostly bound to lipoproteins, with less than 5% of CsA present as free drug in plasma (55). CsA is predominantly bound to cholesterol-containing lipoproteins, that is very low density lipoprotein (VLDL) and low density lipoprotein (LDL) (55).

No data has yet been reported on the tissue distribution of CsG. However, the distribution of CsA in human and animal tissue has been previously investigated (25,43,54). In keeping with the lipophilic nature of CsA, the drug accumulates in body fat. Maurer et al (25) showed the greatest accumulation of ^{3}H-CsA in the liver and, in descending order, fat, kidney, reticuloendothelial and endocrine systems, and blood. ^{3}H-CsA was not detected in the central nervous system. The absence of CsA from the nervous system is due to its inability to cross the blood-brain barrier (56).

H. THERAPEUTIC DRUG MONITORING

During the last decade, clinicians have increasingly used drug concentrations measured in blood as a tool to monitor and regulate dosage of drugs with narrow therapeutic ranges. Information on the therapeutic monitoring of CsG is limited. However, its clinical potential is currently under investigation. The toxic effects of CsG have not been well established and hence

it is difficult to speculate about a therapeutic range for this analogue. However, one study has reported the pharmacokinetics of CsG in patients with renal failure to be similar to CsA, and as a consequence the therapeutic range for CsG may not be significantly different from CsA (39). Secondly, the effect of metabolites on the overall toxicity are unknown, since it is only recently that CsG metabolites have been isolated and characterized (23). Should CsG metabolites prove to have significant toxic properties it would be important to monitor their levels in the blood.

Substantially more information is available on the monitoring of CsA. The optimal range of concentrations of CsA in the blood - those required for immunosuppression but which produce the least degree of toxicity - is narrow. It is therefore important that concentrations of CsA in blood be monitored accurately and regularly and the results used in conjunction with other laboratory and clinical data to guide dosage adjustment. However, the scientific literature on CsA monitoring has been confusing due to the use of different non-specific and specific methods for measurement of CsA, the use of different sample matrices in which to measure the drug and the varied criteria used for defining renal toxicity or rejection (57).

Recently, a Canadian Consensus Panel (57) has made several recomendations in the hope to improve consistency in the practice of CsA therapeutic drug monitoring. Due to the need for consistency, which would make the comparison of inter-laboratory results more meaningful the panel suggested the use of whole blood as the sample matrix. The use of whole blood was also suggested because of the temperature dependance that governs the distribution of CsA in plasma, making sample preparation much

more difficult to control. Further, it was recommended to measure the parent drug only, since to date all data suggests that metabolites are both less immunosuppressive and less toxic than CsA itself. High pressure liquid chromatography (HPLC) and immunoassays in which a selective monoclonal antibody is used allow for the specific quantitation of CsA only.

I. SIDE-EFFECTS

The use of CsA is also associated with side-effects. These include nephrotoxicity, hepatotoxicity, vascular complications, hypertension, seizures and tremors, gastrointestinal problems, hypertrichosis, gingival hyperplasia, and lymphoma (58). However, the impairment of kidney function is the major complication in transplant patients, occuring in almost all patients (59).

The extensive toxicologic evaluation of CsA, necessary for the initiation of clinical trials in the 1970's, showed that it was well tolerated by various animal species and was without systemic side-effects at therapeutic doses (9,59). However, the first clinical trials of CsA in 1978 in patients undergoing renal transplants showed nephrotoxicity to be the major complication (7).

CsA nephrotoxicity can be divided into two types: tubular and vascular, which can be further subdivided into acute and chronic categories (9,60). The clinical findings of acute tubular and acute vascular toxicity are characterized by a number of functional changes, which are dose-dependent and reversible by a reduction in the dose of CsA. Tubular toxicity is manifested by alterations in the reabsorption and excretion of magnesium,

potassium and uric acid (61). These derangements lead to decreased serum magnesium and a mild increase in potassium and uric acid levels. Injury to the renal vasculature results from a increased vasoconstriction of the afferent arteriole and decreased glomerular filtration rate (GFR) (61). This decrease in renal function is most easily detected by an increase in serum creatinine and serum urea. A reduction in the dose of CsA will correct the acute tubular and vascular functional changes.

Chronic CsA induced damage to the renal tubules and vasculature result in morphological changes (60,62). These include isometric vacuoles and ballooning within the proximal tubule cells, cellular inclusion bodies that are mostly giant mitochondria as well as single cell necrosis and some microcalcification within the tubular cells. Damage to the vasculature develops only in the afferent arteriole, where damage to the endothelium and smooth muscle layers can lead to vessel occlusion and a localized loss of blood supply (9,60,62). Such changes to the vascular system are permanent and are therefore of most concern. The observed occlusion of renal arterioles may eventually lead to glomerular collapse and obsolescence, tubular degeneration and atrophy as well as striped fibrosis (9,60,62). The above mentioned arteriolopathy and tubular atrophy, together with presence of a very characteristic striped interstitial fibrosis, usually occurs after a minimum of 1 month of CsA treatment and is irreversible (9).

The mechanism leading to this CsA induced renal failure is poorly understood. The most important pathogenic factor is probably a direct toxic effect on renal vessels. In kidney transplant patients with CsA-associated arteriolopathy a decrease in the production of vasoactive prostaglandins and an increased

excretion of the vasoconstrictor thromboxane B_2 (TxB_2) into the urine has been observed (9). A decreased synthesis of prostaglandins with vasodilatory properties coupled with the increased excretion of those with vasoconstrictive effects may result in an increase in renal vascular resistance. Endothelial cell damage occuring in the presence of impaired vasodilatory prostaglandin synthesis could predispose to platelet aggregation and thrombosis. This would result in renal parenchymal ischemia with the characteristic degenerative changes in the nephron and the interstitium.

To facilitate the understanding of the mechanism by which CsA injures the kidney, the effect of the drug on renal cell lines has been studied (63-69). The porcine proximal renal epithelial cell line ($LLC-PK_1$) has been extensively used to study CsA toxicity in vitro (63,64,67,69). This cell line retained most of the morphological and biochemical characteristics of epithelial tubule cells. Walker et al demonstrated that CsA concentrations of 10000 ug/L significantly inhibited DNA and protein synthesis of these cells (63). Toxicity of CsA directed at the sodium-glucose transporter has also been demonstrated in this cell line (64). It is thought that this particular inhibition of glucose uptake may account for CsA-associated glycosuria observed in human subjects (64). In addition, Copeland et al demonstrated that CsA metabolites generally exhibit activity less than 10% of that of CsA when DNA, RNA and protein synthesis were tested in this cell line (65). However, metabolite AM4N exhibited higher potency of 17-50% of CsA for the various metabolic parameters measured (65).

The effects of CsA have also been investigated in a primary cell line. Mesangial cells can be isolated and maintained

under selective conditions for a prolonged period of time, while still exhibiting biological and biochemical activities of freshly isolated cells (70). These cells occupy a central position in the renal glomerulus and their response to CsA could possibly explain some of the observed toxicity. These cells have the ability of contracting in response to vasoactive substances released by platelets and endothelial cells (71). In addition mesangial cells also release prostagladins and others mediators of inflammation themselves and hence they can act on each other in an autocrine fashion.

The proliferation of a primary rat renal mesangial cell culture was significantly suppressed at 2000 ug/L of CsA as measured by DNA synthesis (66). Stahl et.al. also used a rat mesangial culture to study the effect of CsA on the production of vasodilatory prostaglandin E_2 (PGE_2) (72). They demonstrated that CsA reduces PGE_2 formation by rat mesangial cells in culture, an effect that may contribute to lower circulating levels of this vasodilatory compound and help explain the reduction in GFR that is observed in patients treated with CsA. Due to the structural similarities between CsG and CsA it can be assumed that CsG would exhibit a similar effect on this primary cell line. However, comparible studies investigating the effects of CsG have not been reported.

The toxic side effects of CsG have also been studied in animals. However, attempts to develop an animal model of the human renal injury, particularly in its chronic form, have been unsuccessful. Because of the lack of a specific animal species and strain to study nephrotoxicity, investigations into the toxicity of CsG as well as CsA have yielded quite confusing results. CsG was found to be less nephrotoxic than CsA in several

studies using mice and rats (17,37,38). It was shown to be significantly less toxic than CsA in mice (37). In rats treated with either CsA or CsG (25mg/kg for 21 days), there was no change in RPF (renal plasma flow) and GFR for the CsG treated rats as compared to controls (73). However, RPF and GFR were significantly reduced in the CsA treated animals. There was no difference between CsG, CsA or control group in blood pressure and serum creatinine (73). Further, CsG treated animals showed lack of tubular atrophy upon examination of the kidney. In comparison, 40% of CsA treated animals showed this abberation (73). Rats treated with 50 mg/kg of either CsG or CsA for 50 days showed some degree of renal fibrosis as did rats treated with 10 mg/kg of CsA for 130 days (17). However, no significant damage was seen in rats treated with 10 mg/kg of CsG for 130 days (17). Other studies have reported both CsG and CsA to be devoid of nephrotoxicity in animals. Grant et.al. compared CsG and CsA in a rat renal allograft model and found that neither drug was nephrotoxic at doses of 5, 7.5 and 10 mg/kg for 28 days (18). In mongrel dogs with renal allografts, there was no significant difference in the renal function between CsG and CsA treated animals.

Concern has also been raised about the possible hepatotoxic side effect of CsG. Bilirubin levels were found to be elevated in rats receiving 10 mg/kg CsG as compared to CsA treated animals (19). Further, bilirubin was consistently elevated in dogs with renal allografts receiving CsG as compared to those receiving CsA as their imunosuppressive regimen (20). This has also been found true in primates, where at doses of 16 mg/kg i.m. for 14 months followed by 20 mg/kg i.m. for a total of 2 years, an increased serum bilirubin was measured as compared to CsA (74).

All of the animals discussed above failed to exhibit the chronic CsA-associated nephrotoxicity at therapeutic doses of the drug in man, which consists of vascular thrombosis, tubular atrophy, leukocyte infiltration and interstitial fibrosis. Ryffel et.al. reported the occurence of arteriolopathy in spontaneously hypertensive rats given CsA (75). Such lesions however, are identical to the spontaneously occuring lesions in this strain, and hence the significance of this model remains unclear. In another study, rats were administered CsA at 25 mg/kg for 28 days, resulting in renal lesions consistent with chronic CsA nephrotoxicity (76). A variety of strains of rabbits have been used to investigate CsA-associated nephrotoxicity; however, none developed the typical renal lesions consistent with chronic CsA nephrotoxicity (77-79). Gratwohl et al observed an increased in serum creatinine in New Zealand White rabbits treated with CsA for 60 days (77). However, no histologic changes were reported in the kidney.

II. RATIONALE AND OBJECTIVES

CsA has been shown to be an effective immunosuppressive agent for the prevention of graft rejection after organ transplantation. However, the major problem with the use of this drug is the high incidence of nephrotoxicity. This has lead to the search for CsA analogues which retain the immunossuppressive properties of CsA but are less toxic. The CsA analogue CsG has been reported in preliminary studies to be as immunosuppressive as CsA and possess reduced renal side-effects. However, the results are presently inconclusive.

Little is known regarding the therapeutic range for CsG. Prior to establishment of such a range the distribution of the drug in blood must be determined to establish what medium, plasma or whole blood, would be more suitable for monitoring. The development of a therapeutic range would reduce the frequency of side effects.

In this study we proposed to:

1. Investigate the <u>in vivo</u> toxicity of CsG in New Zealand White rabbits.

2. Study the <u>in vitro</u> toxicity of CsG and metabolites in a transformed and a primary renal cell line.

3. Investigate the influence of CsG and metabolite concentration as well as temperature on their distribution among plasma and cells of whole blood. This will help to determine which medium, blood or plasma, may be the most suitable for monitoring of the drug.

III. METHODS

A. QUANTITATION OF CsG AND CsA

1. HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

Cyclosporin in blood, plasma and tissue was measured using a HPLC procedure similar to that originally described by Carruthers et.al. (80) and modified by our laboratory (81). 25 uL of 20,000 ug/L internal standard was added into 1.0 mL of sample, followed by addition of 2.0 mL of 0.2 N NaOH and 6.0 mL of diethyl ether. When CsG was being measured a 20,000 ug/L CsA was used as the internal standard, and a 20,000 ug/L CsG internal standard was employed for the measurement of CsA. Subsequent to shaking for 10 minutes, the diethyl ether layer was transferred to another extraction vial containing 2.0 mL of 0.2 N HCl. Specimens were shaken for another 10 minutes and the diethyl ether layer was then transferred into 12 x 75 mm test tubes, where the ether was evaporated to dryness under nitrogen at room temperature. The dried extract was reconstituted in 300 uL of mobile phase (acetonitrile/ methanol/ H_2O, 50/20/30), followed by addition of 300 uL of hexane. The specimen was vortexed for 30 seconds and the mobile phase layer was transferred to an injection vial. Quantitation of samples occured on a Varian (Varian Inc., U.S.A.) HPLC, which consisted of the following components: 9090 autosampler, 2080 column heater, 2510 pump, 2050 variable wavelength detector, and a 4400 integrator. Chromatographic separation was performed isocratically on a spherasorb C-8 columnn (5 um, 25 x 0.46 cm) (Chromatography Sciences Inc., Canada) preceded by a pellicular and silica precolumnn (30-40 um)

(Upchurch Scientific, U.S.A.). Mobile phase employed was as described above. The solvent flow rate was 1.0 mL/min with the precolumn and column heated to 70°C. Peaks eluted from the column were monitored at 214 nm. Cyclosporin was calculated from the area under the curve in relation to that of the internal standard.

2. RADIOIMMUNOASSAY (RIA)

Quantification of CsG and CsA was performed using a RIA kit supplied by Sandoz Inc.(Sandoz Ltd., Switzerland). It involves the use of a [^{3}H]cyclosporine as tracer and a selective monoclonal antibody that is specific for CsA, or a nonselective antibody that recognizes both the parent drug and metabolites. The selective monoclonal antibody detects only CsA and exhibits <2% cross-reactivity with CsA metabolites (81). The non-selective monoclonal antibody recognizes CsA and exhibits cross-reactivities of > 50% with CsA metabolites (24).

All CsA metabolites that have been characterized present modifications on residues 1,4,6, and 9 which are located on one side of the CsA molecule (82). The selective monoclonal antibody recognizes these residues and is therefore very sensitive to modifications at these positions. CsG is modified on amino acid 2 and we have shown in our laboratory that both the selective and non-selective monoclonal antibody show 100% cross reactivity with CsG. The majority of CsG metabolites examined exhibit < 3% cross-reactivity with the selective antibody. Metabolite GM9 exhibits 10% cross reactivity with this antibody (24). Metabolites exhibited cross-reactivity to varying degrees with the non-selective antibody. Cross-reactivity ranges from 6% for GM4N9 to 70% for GM1 and GM9. GM1c

is the most cross-reactive metabolite with 120% cross reactivity (24).

Standards were prepared in drug-free plasma or whole blood with a range 25 ug/L to 800 ug/L. Prior to assay, all samples were pretreated to remove protein, by addition of 100 uL of sample into 1.0 mL of methanol. Specimens were centrifuged for 5 minutes at 1,600 x g and the methanolic supernatant was used for the assay. The antibody (100 uL) was added to sample and incubated at 4°C for 1 hour. [^{3}H]CsA and unlabelled cyclosporine present in the sample compete for the monoclonal antibody. Separation of the bound and free fraction was achieved by charcoal chromatography and the radioactivity of the antibody bound fraction in each specimen was measured with a liquid scintillation counter using a quench corrected counting program. A standard curve was constructed by plotting %B/Bo vs CsA concentration in the standards and the unkown samples read off this curve.

2. PHARMACOKINETICS

CsG or CsA was administered via a single intravenous injection to New Zealand White rabbits (2.5-3.5 kg) at a dose of 1.0, 2.5, 5.0 mg/kg. The drug was weighed out and dissolved using a solvent system of 60:40 ethyl alcohol and sterile saline. CsG or CsA was administered (total volume 1.0 ml) over 2 minutes into the right marginal ear vein. The rabbits were housed in individual cages with food being restricted during pharmacokinetic analysis. Immidiately before infusion of the drug, an in-line catheter (with a 20-gauge needle) and heparin lock were placed in the left marginal ear vein to facilitate blood sampling. Heparinized whole blood (500-1000 uL) was obtained prior to infusion and at the following times after

administration of the drug : 3,6,10,20, 30,45,60,90 minutes and 2,3,4,6,8,10,12,18,21,24 hours. All samples were stored at 4°C. Model independent parameters were calculated with the respective blood concentration time data by Dr. N. Honcharik, Pharmacy Department, Health Sciences Centre. These included total body clearance (Cl) and the steady state volume of distribution ($V_{d(ss)}$). Clearance is an index of drug elimination from the central compartment by hepatic biotransformation and excretion by the kidney or in the feces. It can be expressed as follows:

$$\text{Clearance} = \frac{\text{Dose}}{\text{Area under the the blood concentration curve (AUC)}}$$

The volume of distribution of a drug provides an estimate of the extent of its distribution through body-fluid compartments and of its uptake by tissues. A large volume of distribution implies wide distribution and/or extensive tissue uptake. Volume of distribution is expressed as follows:

$$\text{Volume of distribution} = \frac{\text{Dose}}{\text{AUC} * t_{½}}$$

The terminal elimination half-life ($t_{½}$) is obtained from the slope of the terminal portion of the blood concentration curve. Model independent parameters were calculated using the computer program PKCALC (83).

C. DISTRIBUTION IN BLOOD AND TISSUE

Whole blood from normal individuals was supplemented with CsG to 100 and 500 ug/L. Aliquots of whole blood were incubated at room temperature (about 22°C) and at 4°C for a minimum of 4 hours and then equilibrated in a 37°C water bath for 0, 10, 15, 30, 60,120 and 180 minutes. All specimens were centrifuged and the plasma was decanted with the CsG concentration being analyzed by HPLC.

Whole blood from normal individuals was supplemented with CsG or metabolites to the following concentrations: 100, 500, 1000, and 5000 ug/L. Samples were then equilibrated in a 37°C water bath for 30 minutes followed by centrifugation. Plasma and cell fractions were separated and CsG or metabolites quantitated by HPLC.

To investigate the distribution of CsG in vivo, venous blood was collected from New Zealand white rabbits receiving CsG, 12 hours after their last dose. Samples were equilibrated in a 37°C water bath for 30 minutes, followed by centrifugation and prompt removal of plasma. CsG in plasma and whole blood was measured by HPLC and RIA using both the selective and non-selective monoclonal antibodies.

New Zealand white rabbits that had been receiving CsG for 30 days were sacrificed with a lethal dose of sodium pentobarbital and the body cavities exposed. Samples of kidney, liver, spleen, heart, fat, pancreas and brain were obtained and frozen at -40°C. To determine tissue CsG concentration, samples diluted in saline (1:3, weight:volume) were homogenized using a Brinkmann homogenizer (Kinematica GmbH, Switzerland). CsG was extracted using diethylether and quantitated on HPLC.

D. IN VIVO STUDIES

Age-matched New Zealand white rabbits (2.5 to 3.5 kg) were housed in individual cages and randomly assigned to 5 groups of 5 animals each. Rabbits received either CsG or CsA intravenously via the marginal ear vein daily for 30 days at doses of 2.5 mg/kg and 5.0 mg/kg. The vehicle cremophor-EL (poly[oxyethylene]-40-ricinoleic acid) was administered to a control group in a volume equivalent to that of animals receiving either drug.

Animals were placed into metabolic cages in the last week of study to obtain 24 hour urine collections. Serum and whole blood were collected to establish creatinine clearance and CsG or CsA concentration. Creatinine concentrations in both serum and urine were determined by the alkaline picrate method on the Beckman Astra Analyzer (Beckman Instruments, USA). Cyclosporine concentrations were determined by HPLC and RIA.

Samples were analyzed for potassium and sodium, as well as for liver enzymes: aspartate transaminase (AST) and alanine transaminase (ALT). Potassium and sodium concentrations as well as enzyme activities were measured using standard laboratory procedures in the Clinical Chemistry laboratory at the Health Sciences Centre.

Morphological investigations were carried out by Dr. J.A. Thliveris from the Department of Anatomy, University of Manitoba. Briefly, at the end of the study the animals were sacrificed with a lethal dose of sodium pentabarbital. Tissue samples were obtained from four different regions of each kidney and processed for light microscopic and ultrastructural evaluation. For light

microscopy, sections were stained with hematoxylin and eosin, Mallory-Azan and PAS, and scored semi-quantitatively on a scale of 0 to 4+ for the presence of 1) leucocyte infiltrates, 2) tubular atrophy, 3) interstitial fibrosis, and 4) arteriolopathy. Quantification of the glomerular tuft area and volume density and arteriole wall thickness (area) was assessed at a magnification of 120x for glomerular tuft area and volume density and 530x for arteriole thickness using a ZIDAS (Zeiss Interactive Digital Analysis System, Carl Zeiss, Germany) image analysis system. Electron microscopy was used to examine qualitatively 1)tubular vacuolization, 2) changes in cytoplasmic organelles, and 3) integrity of the cell membrane at the adluminal surface. Specimens for both light and electron microscopy were examined without foreknowledge of their source.

E. IN VITRO STUDIES

A pig kidney epithelial cell line LLC-PK_1 (American Type Culture Collection, USA) which expresses many proximal tubular characteristics, and a primary rabbit mesangial cell line were used to investigate CsG toxicity *in vitro*. Mesangial cells were isolated in our laboratory from outgrowths of collagenase-treated isolated rabbit renal glomeruli. Morphologic examination of the isolated cells revealed ultrastructural features consistent with those of mesangial cells *in vivo* (24). Cells were grown in 25-cm^2 culture flasks in medium 199 with 10% fetal calf serum in a 5% CO_2 incubator. The cells were subcultured when confluent using 0.02% EDTA and 0.05% trypsin.

The experiments were performed in 6 well plates. Cells were plated at 2.5×10^4 cells/well in 2.5 mL of media containing

the following CsG concentrations: 0, 500, 1000, 2500, 5000, 10000, and 20000 ug/L. Cells were grown for 5 days or until confluency. The cells in the resulting suspension were counted with a hemacytometer, with a fraction being analyzed for protein content by the method of Lowry et.al. (84). The incorporation of [^{3}H]-thymidine for the measurement of DNA synthesis was quantified by adding 2uCi [^{3}H]-thymidine (70-85 uCi/mmol) (Amersham Canada Ltd., Canada) to the cell cultures 18 hours prior to trypsinization. Just prior to trypsinization, the cells were washed twice with saline and recovered by treatment with trypsin and EDTA as above. The cells were lysed by freezethawing and cellular material precipitated using 10% (w/v) TCA. The precipitate was resuspended in saline with a portion of the suspension being taken for protein determination. The remaining amount was transferred to a sampling manifold fitted with Whatmann GF/C glass fibre filters. The filters were thoroughly washed with 10 mL of 50% ethanol. The radioactivity retained on the filters was counted in 10 mL scintillation fluid using a quench corrected counting program on a beta liquid scintillation - spectrophotometer (LKB, Finland).

F. STATISTICS

Statistical assessment of the data obtained was carried out by using analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant. The statistical analysis for the morphological studies additionally used Duncan's multiple-range test and the Kruskal-Wallis rank-sum test where appropriate (85).

IV. RESULTS

A. BLOOD DISTRIBUTION STUDIES

The effect of time of re-equilibration at 37°C of whole blood supplemented with CsG was investigated. The results are shown in figures 2 and 3. It can be seen, that for either samples stored at 4°C (figure 2) or room temperature (figure 3) complete equilibration occurs within 10 minutes of incubation.

Further, we investigated the influence of analyte concentration on the distribution of CsG and two of the CsG metabolites, GM1 and GM9, among plasma and cells. The comparison of concentrations in whole blood and plasma are shown in figure 4. The relative proportion of CsG in plasma as compared to whole blood remained unchanged from 100 to 1000 ug/L, with an average plasma to whole blood ratio of 1.0. At 5000 ug/L this ratio increased to 1.5. Figure 5, depicts the comparison of concentrations in plasma and red blood cells (RBC). The relative proportion of CsG in plasma remained unchanged at concentrations 100 to 1000 ug/L, with a plasma to RBC ratio of 1.4. That ratio increased to 2.3 when the CsG concentration in whole blood was 5000 ug/L.

The distribution of CsG metabolites GM1 and GM9 between the plasma and cells of whole blood at 37°C is demonstrated in figure 4. The plasma to whole blood ratio for GM1 at concentrations 100 to 1000 ug/L was about 0.5 and its plasma to RBC ratio at this concentration range was 0.3. At 5000 ug/L of GM1 in whole blood both plasma to whole blood and the plasma to RBC

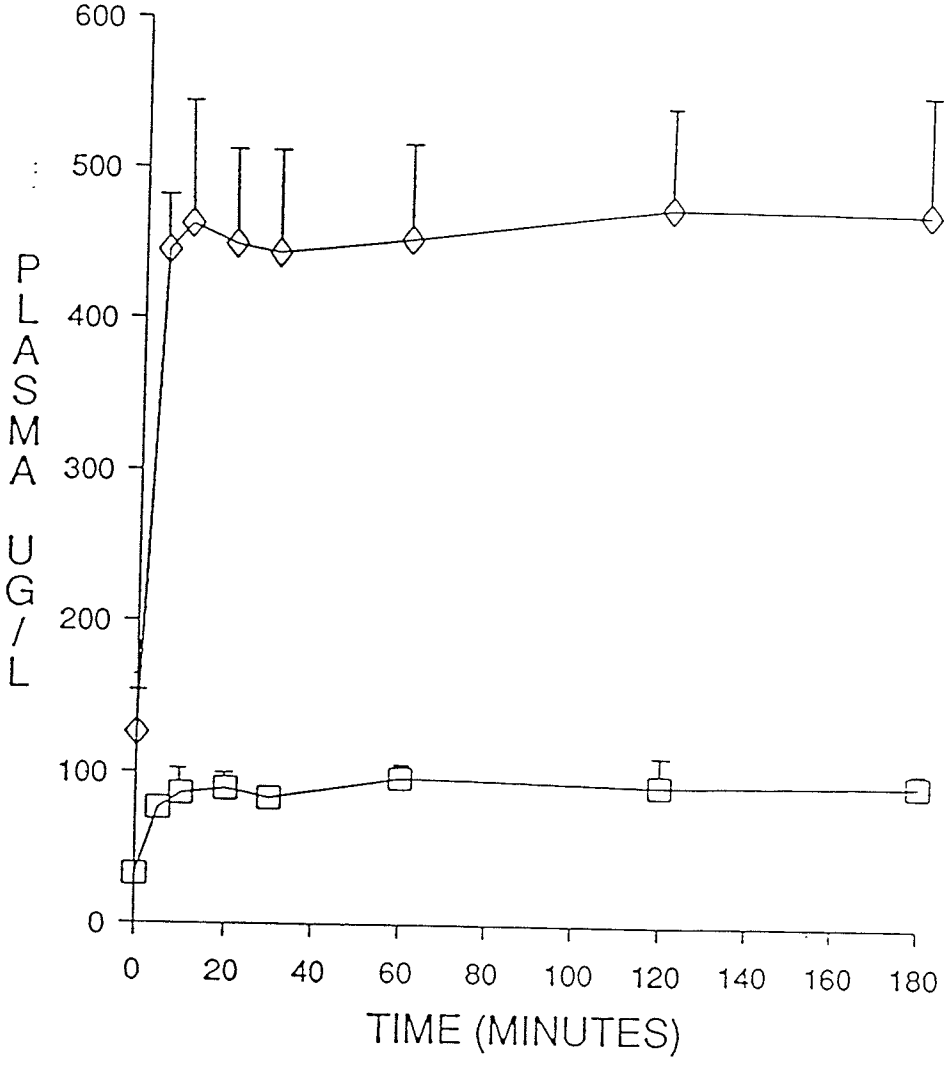


Figure 2. Effect of time of re-equilibration at 37°C on concentrations of CsG in plasma. Whole blood was supplemented with CsG to 100 ug/L (⊟) and 500 ug/L (◇), stored at 4°C and plasma separated by centrifugation after upto 3 hours of re-equilibration at 37°C. Results are expressed as mean ± SD (n = 3).

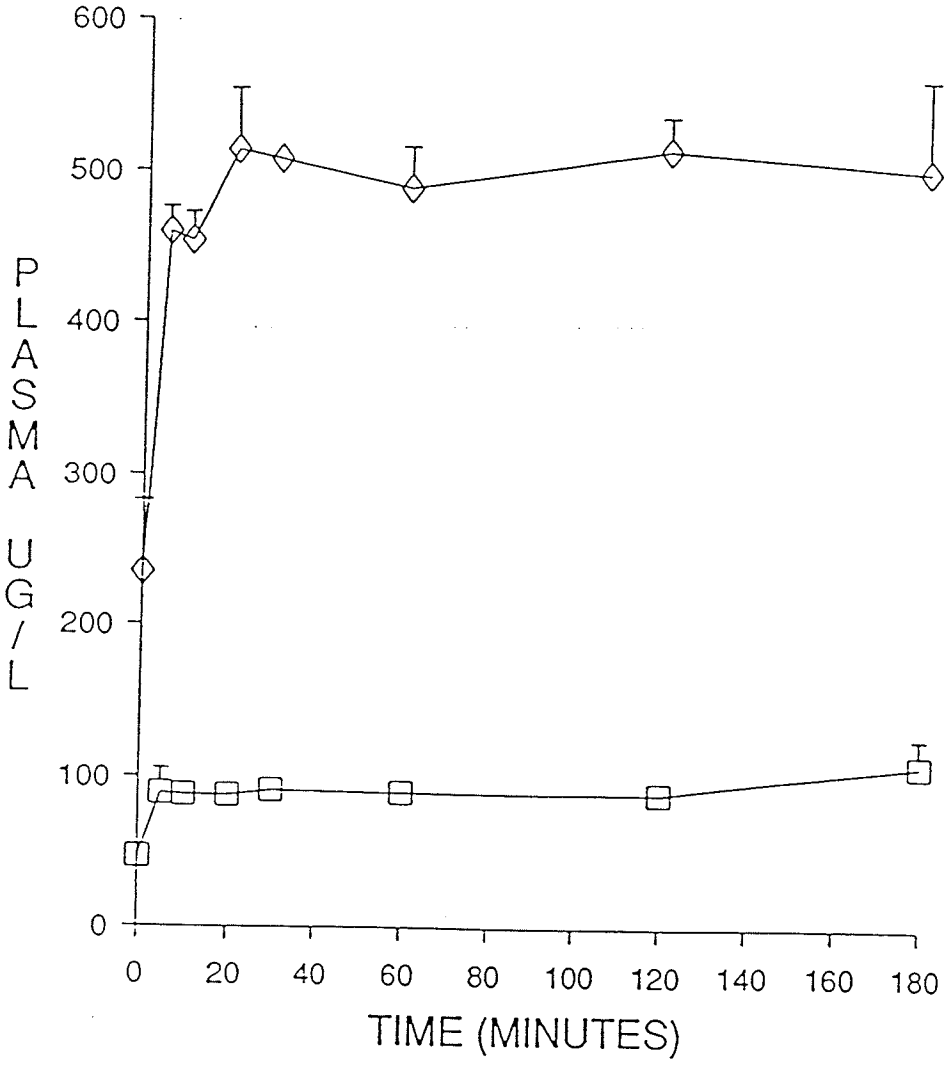


Figure 3. Effect of time of re-equilibration at 37°C on concentrations of CsG in plasma. Whole blood was supplemented with CsG to 100 ug/L (⊟) and 500 ug/L (◇), stored at room temperature and plasma separated by centrifugation after upto 3 hours of re-equilibration at 37°C. Results are expressed as mean ± SD (n = 3).

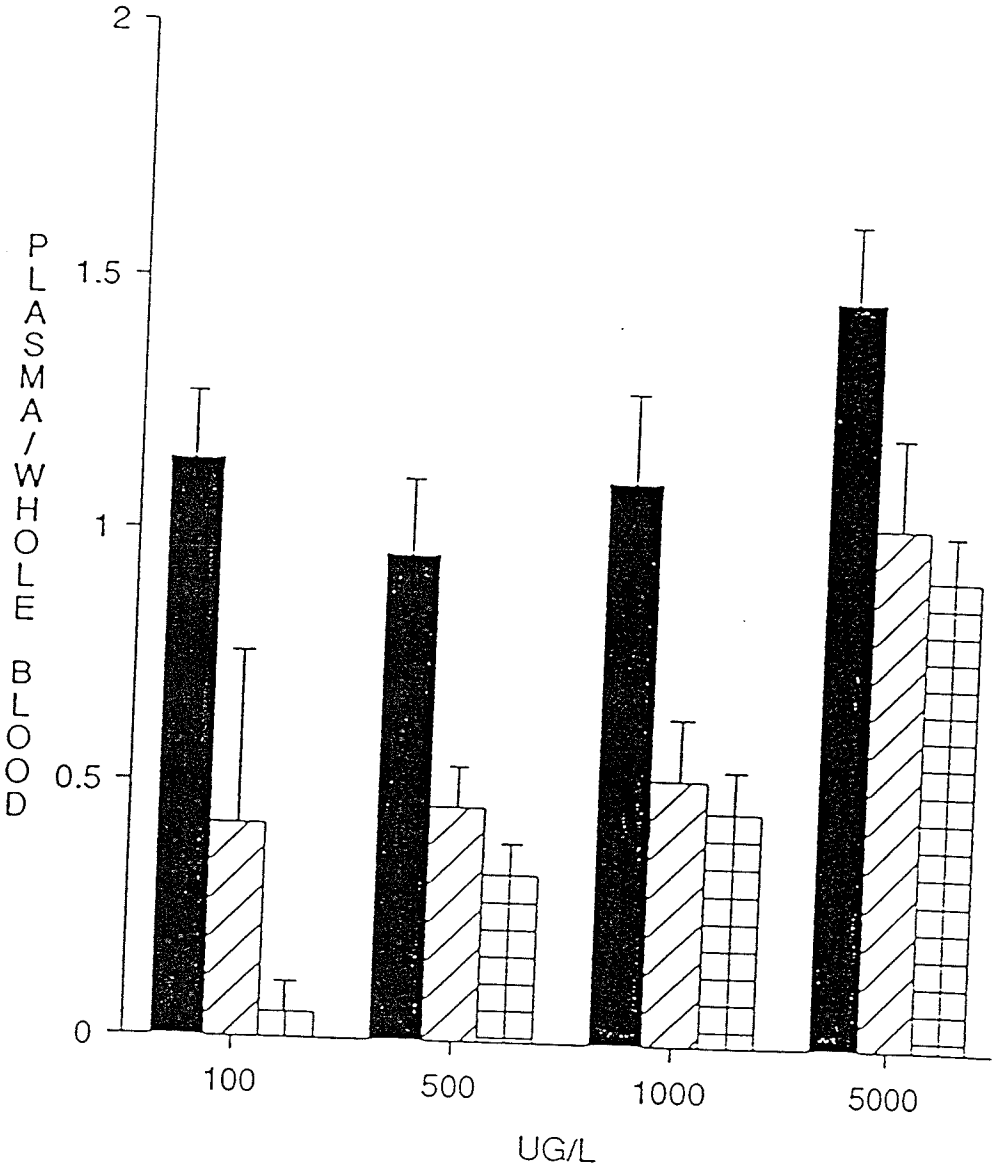


Figure 4. Comparison of plasma CsG and CsG metabolites concentrations with that in whole blood. Whole blood was supplemented with either CsG (■), GM1 (▨), or GM9 (▦) to 100, 500, 1000, and 5000 ug/L. All samples were re-equilibrated at 37°C for 30 minutes and plasma separated by centrifugation. Results are expressed as mean ± SD (n ≥ 3).

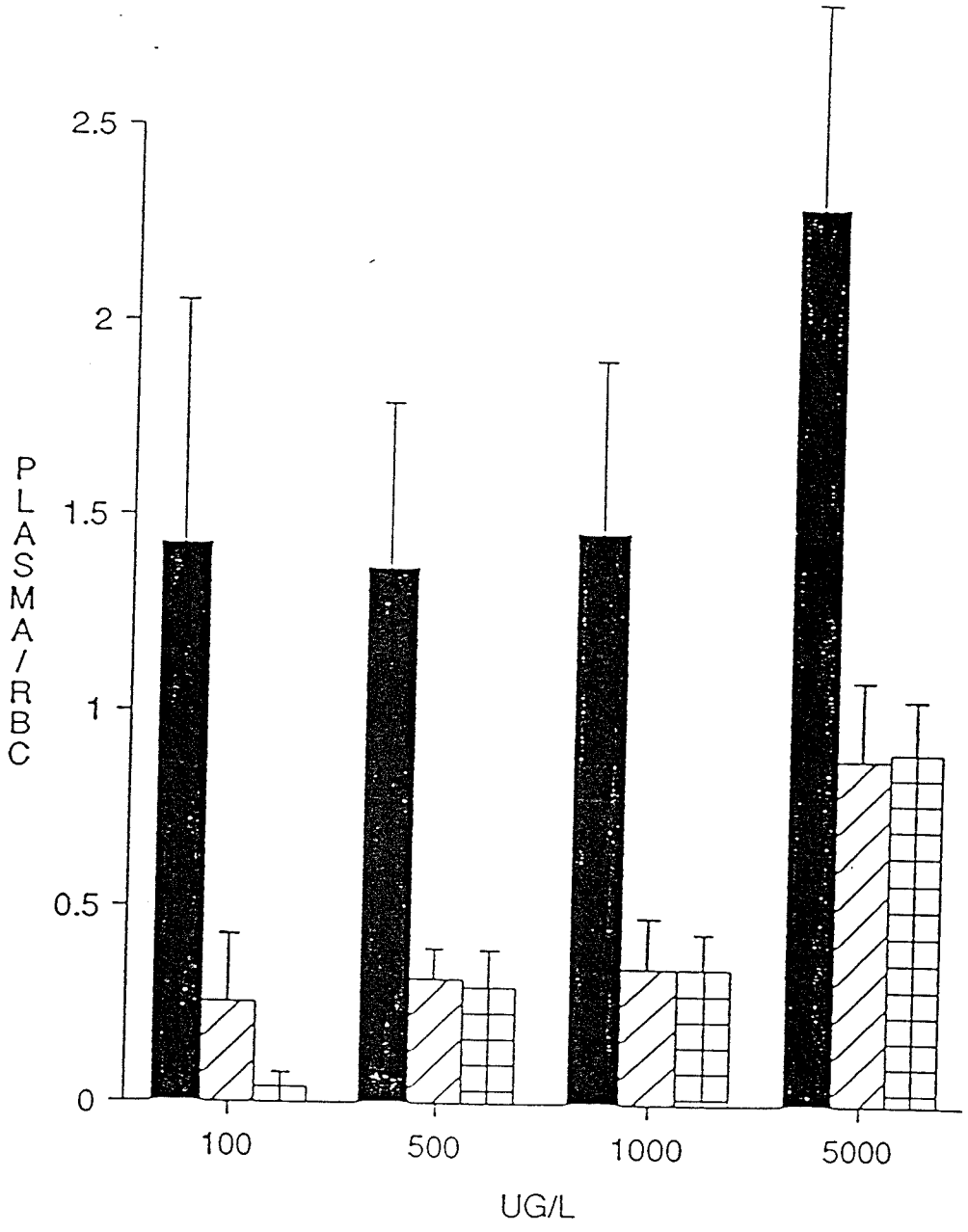


Figure 5. Comparison of plasma CsG and CsG metabolites concentrations with that in red blood cells (RBC). Whole blood was supplemented with either CsG (■), GM1 (▨), or GM9 (▦) to 100, 500, 1000, and 5000 ug/L. All samples were re-equilibrated at 37°C for 30 minutes and RBC separated by centrifugation. Results are expressed as mean ± SD ($n \geq 3$).

ratio increased to 1.0 and 0.9 respectively. The metabolite GM9 showed a concentration dependent distribution at the concentratrations examined. Increasing the concentrations of this metabolite from 100 to 1000 ug/L changed the plasma to whole blood ratio from 0.05 to 0.5, and increased to 0.9 at 5000 ug/L. The same trend was observed as we measured more closely the distribution of GM9 between plasma and RBC. The plasma to RBC ratio increased from 0.04 to 0.4 at concentrations between 100 and 1000 ug/L and was 0.9 at 5000 ug/L .

B. BLOOD LEVEL MONITORING

The use of a selective (RIA-S) and non-selective (RIA-NS) monoclonal antibody, as well as HPLC for the monitoring of CsG and CsA in plasma and whole blood of rabbits receiving the drug was investigated.

Regression analysis and correlation of CsG and CsA concentrations in whole blood and plasma are depicted in table 1. The mean CsA concentrations in whole blood as measured by HPLC when compared to those measured by the RIA-S gave a ratio of 0.9. The same comparison of whole blood samples from rabbits receiving CsG gave a ratio of 1.1. When whole blood samples from rabbits treated with CsA were measured by RIA-S or RIA-NS, the respective mean CsA concentrations obtained were 72 and 233 ug/L. The resulting ratio between whole blood concentrations obtained by RIA-NS and RIA-S was 3.2. Comparable analysis of whole blood from rabbits receiving CsG resulted in a RIA-NS to RIA-S ratio of 1.4, with mean CsG values obtained by RIA-NS and RIA-S of 113 and 82 ug/L, respectively. The blood to plasma ratio as measured by RIA-S was 0.3 and 0.7 for CsA and CsG treated rabbits, respectively.

Table 1. Comparison of CsG and CsA concentrations in whole blood and plasma from rabbits treated with either drug*.

CsG							
N	X	Y	$\bar{X}$	$\bar{Y}$	Y/X	R	REGRESSION EQUATION
18	WB (RIA-S)	WB (HPLC)	70	77	1.1	0.97	y = 2.8 + 1.0x
24	WB (RIA-S)	WB (RIA-NS)	82	113	1.4	0.96	y = -9.8 + 1.5x
14	WB (RIA-S)	PL (RIA-S)	97	70	0.7	0.50	y = 23 + 0.5x

CsA							
N	X	Y	$\bar{X}$	$\bar{Y}$	Y/X	R	REGRESSION EQUATION
14	WB (RIA-S)	WB (HPLC)	132	122	0.9	0.92	y = -1.6 + 0.9x
20	WB (RIA-S)	WB (RIA-NS)	72	233	3.2	0.91	y = 22 + 2.9x
25	WB (HPLC)	PL (HPLC)	224	76	0.3	0.50	y = 34 + 0.2x

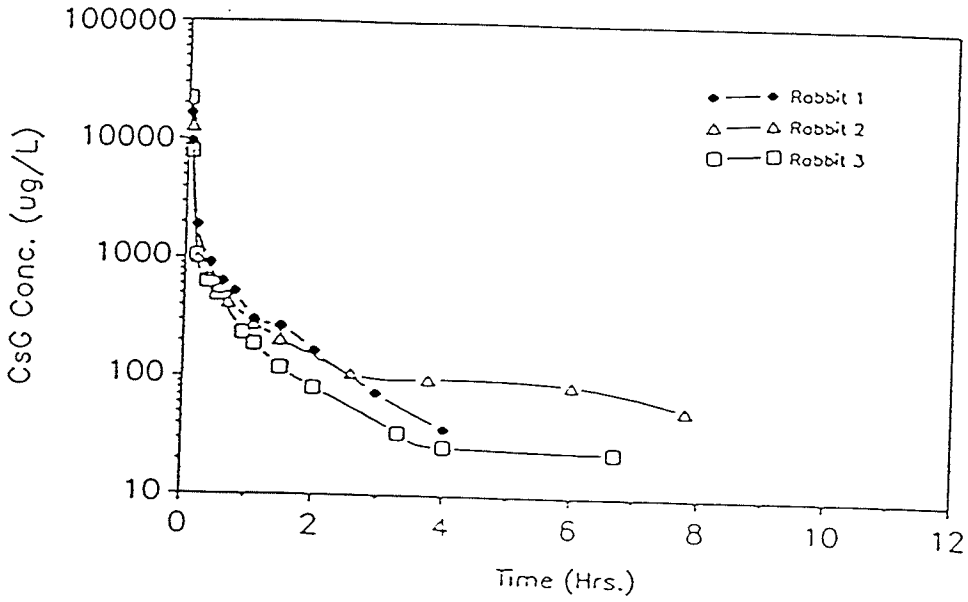
* = CsG and CsA concentrations in whole blood (WB) and plasma (PL) were determined by a monoclonal RIA with selective antibody (RIA-S), a monoclonal RIA with a non-selective antibody (RIA-NS), and by HPLC.

C. PHARMACOKINETICS

We investigated the pharmacokinetics of CsG and CsA in the rabbit after a single dose given intravenously as a bolus. The pharmacokinetic profiles of CsG and CsA in three rabbits are shown in figures 6 and 7. The drug was prepared in a solution of 60% ethyl alcohol and a total volume of 0.9 mL was administered at doses of 2.5 and 5.0 mg/kg. The detailed calculations of the various pharmacokinetic parameters are shown in table 2. There were no statistically significant differences ($p < 0.05$) between any of the parameters. However, on average CsG showed a larger clearance rate and a shorter half-life than CsA at both doses.

D. IN VIVO STUDIES

Rabbits received vehicle (cremophor-EL), CsG or CsA intravenously at doses of 2.5 or 5.0 mg/kg for 30 days. We investigated renal and hepatic function as well as histological changes in the kidney and liver. The functional and chemical parameters at time of sacrifice studied in rabbits receiving either of the analogues as well as controls are presented in figure 8 and table 3. It was noted that serum aspartate transaminase (AST) was elevated in rabbits treated with 2.5 and 5.0 mg/kg/day of CsA, when compared to rabbits treated with vehicle (figure 8). There was no observable trend among all five groups of rabbits with respect to serum alanine transaminase



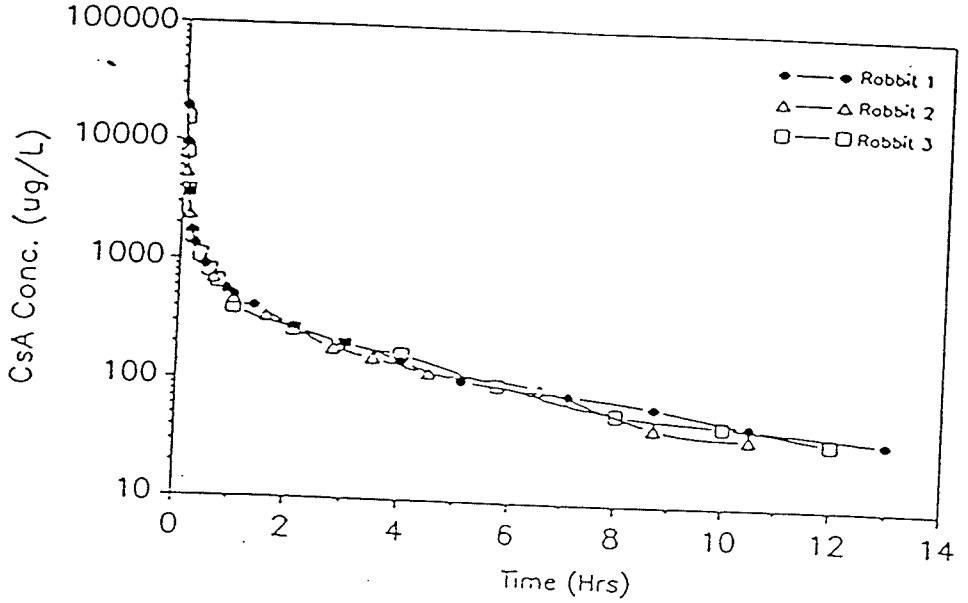


Figure 6. Pharmacokinetic profile for 2.5 mg/kg of CsG (Top) and CsA (Bottom) administered i.v. to rabbits.

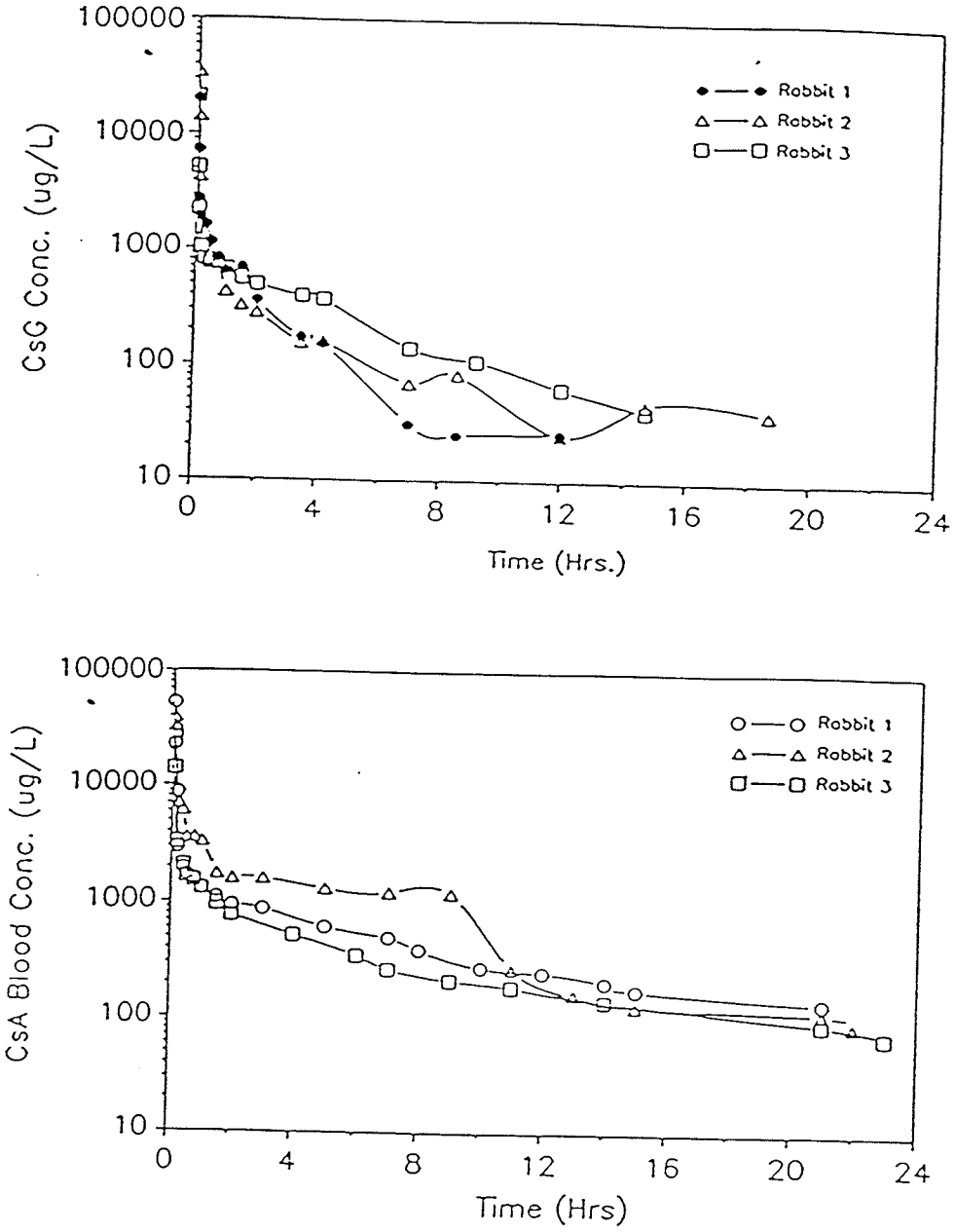


Figure 7. Pharmacokinetic profile for 10.0 mg/kg of CsG (Top) and CsA (Bottom) administered i.v. to rabbits.

Table 2. Pharmacokinetic parameters* of CsG and CsA in rabbits.

CYCLOSPORIN G (CsG)			
DOSE (mg/kg)	CL (mL/min/kg)	$V_{d(ss)}$ (L/kg)	$t_{½}$ (hrs)
2.5	16.2 (12.8 - 18.8)	1.2 (0.6 - 1.8)	2.8 (1.0 - 4.2)
10.0	38.2 (33.1 - 47.8)	8.0 (4.1 - 11.9)	5.2 (1.7 - 9.7)

CYCLOSPORIN A (CsA)			
DOSE (mg/kg)	CL (mL/min/kg)	$V_{d(ss)}$ (L/kg)	$t_{½}$ (hrs)
2.5	11.9 (10.7 - 12.9)	2.2 (1.9 - 2.5)	4.5 (3.3 - 5.3)
10.0	11.7 (7.3 - 16.3)	5.1 (2.9 - 6.9)	10.0 (8.2 - 12.8)

* = values expressed as mean (n = 3) and range in brackets.

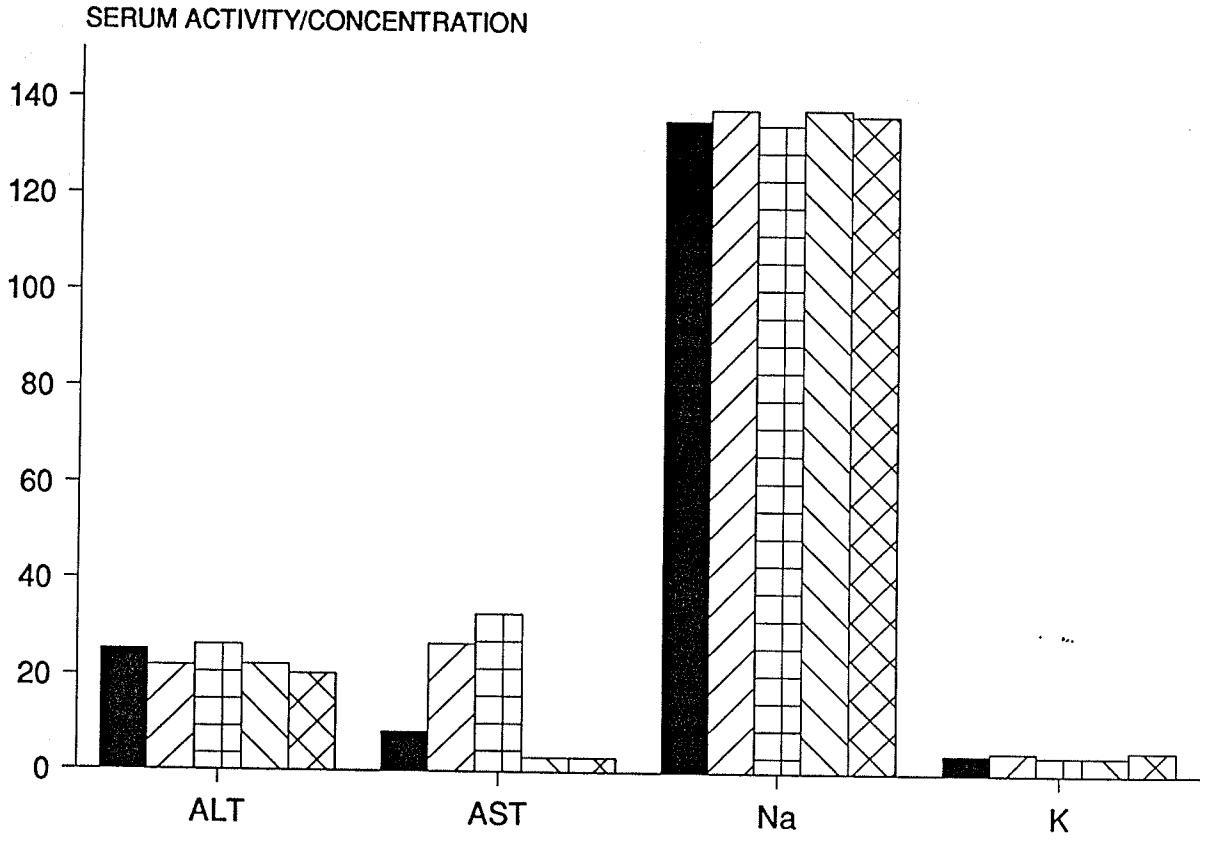


Figure 8. Comparison of chemical parameters in serum of rabbits treated intravenously daily for 30 days with CsG or CsA. ALT = alanine transaminase (U/L), AST = aspartate transaminase (U/L), Na = sodium (mM), K = potassium (mM). Control ( ), 2.5 mg/kg CsA ( ), 5.0 mg/kg CsA ( ), 2.5 mg/kg CsG ( ), 5.0 mg/kg CsG ( ). Values expressed as mean ± SD ($n \geq 4$).

Table 3. Comparison of Chemical and Functional Parameters* in Rabbits Treated Intravenously Daily for 30 Days with CsG or CsA.

Group (n = 5)	Urine Creatinine (mmol/L)	Urine Volume (mL/24 hrs)	Serum Creatinine (umol/L)	Creatinine Clearance (mL/min)	CsG# (ug/L)	CsA (ug/L)
CsG (5.0 mg/kg)	6.36 ± 4.07	194 ± 97	102 ± 11	7.02 ± 3.39	175 ± 162	
CsA (5.0 mg/kg)	6.56 ± 2.40	271 ± 84	86 ± 11	7.53 ± 1.59		87 ± 18
CsG (2.5 mg/kg)	7.98 ± 5.12	173 ± 86	94 ± 7	8.40 ± 2.79	96 ± 68	
CsA (2.5 mg/kg)	8.00 ± 5.12	190 ± 109	73 ± 8	9.30 ± 2.52		37 ± 11
Control (cremophor-EL)	10.70 ± 3.62	116 ±59	92 ± 6	8.7 ±2.50		

* = values expressed as mean ± SD
# = since the clearance rate was greater for CsG than CsA, values given represent through levels at 6 hours (2.5 mg/kg CsG) and 12 hours (5.0 mg/kg CsG) after last injection rather than 24 hours for CsA

(ALT), sodium and potassium. Further, no CsG was detected in whole blood 24 hours after the last injection, as compared to CsA treated animals. However, CsG was detected in whole blood 6 and 12 hours after the last injection in animals treated with 2.5 and 5.0 mg/kg of the drug, respectively (table 3).

There was no difference between rabbits treated with either drug and controls with respect to urine volume, urine creatinine, serum creatinine and creatinine clearance (table 3). However, animals treated with 5.0 mg/kg of either CsG or CsA showed a tendency to have a reduced creatinine clearance when compared to controls, but this was not statistically significant ($p < 0.05$).

Morphologically, there were marked changes in the cytoarchitecture of the kidneys from all four groups treated with either CsG or CsA. Light micrographs of the kidney cortex from animals treated with CsG, CsA and controls are shown in figures 9 to 13. All drug treated groups showed the presence of leukocyte infiltrates (figure 10), tubular atrophy (figure 11), and interstitial fibrosis (figure 12), with no pathological changes in controls (figure 9). Further, the presence of arteriolopathy was also observed in rabbits treated with both drugs at doses of both 2.5 and 5.0 mg/kg (figure 13). At the ultrastructural level (figures 14-17), in contrast to controls, the changes observed consisted of numerous vacuoles and lysosomal like structures, loss of cellular integrity, shedding of the proximal tubule brush border, and interstitial fibrosis in the form of numerous collagen fibers. The examination of the liver did not reveal any changes irrespective of the dose and drug used.

The severity of tubular atrophy and interstitial fibrosis was significantly less in animals treated with CsG when

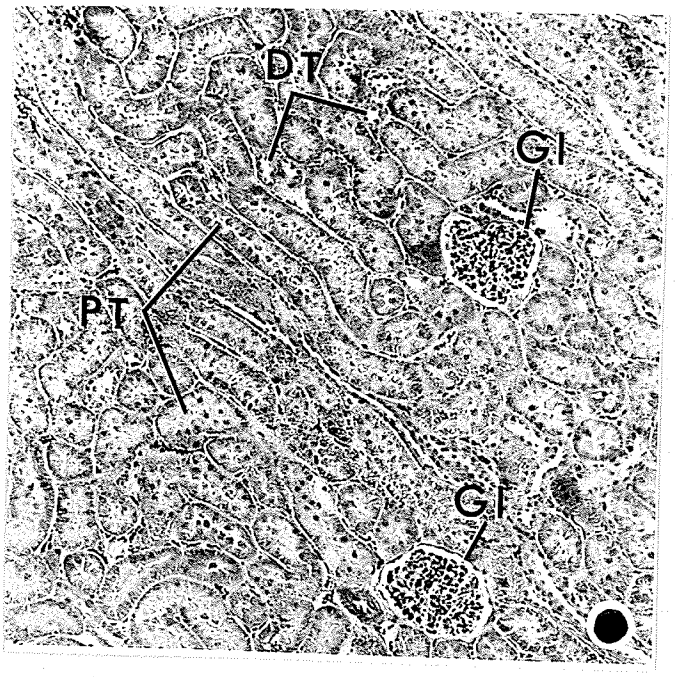


Figure 9. Light micrograph of kidney cortex from a control animal. Note the presence of numerous proximal (PT) and distal (DT) tubules and several glomeruli (Gl). Hematoxylin and eosin Magnification x 100.

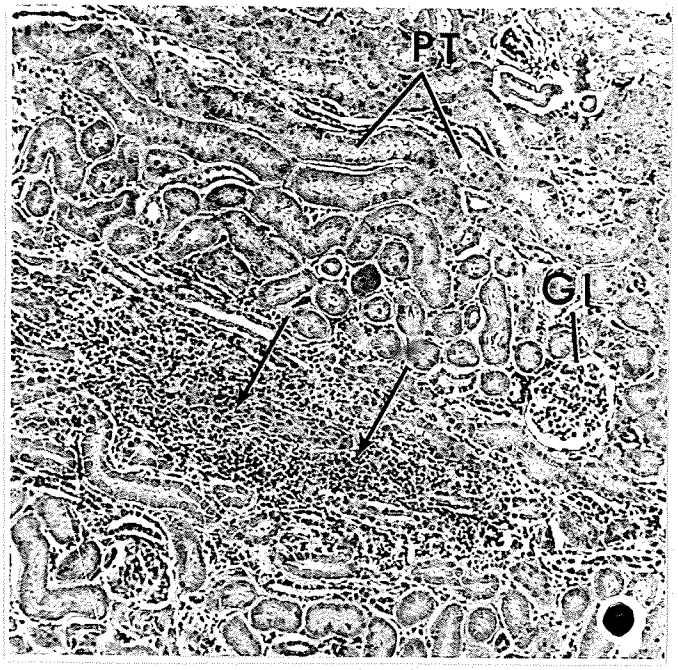


Figure 10. Light micrograph of kidney cortex from an animal treated with 5 mg/kg CsG for 30 days. Note the presence of leucocyte infiltrate (arrows) among normal appearing tubules (PT) and glomeruli (Gl). The same changes were observed in animals treated with 5 mg/kg CsA for 30 days. Hematoxylin and eosin. Magnification x 100.

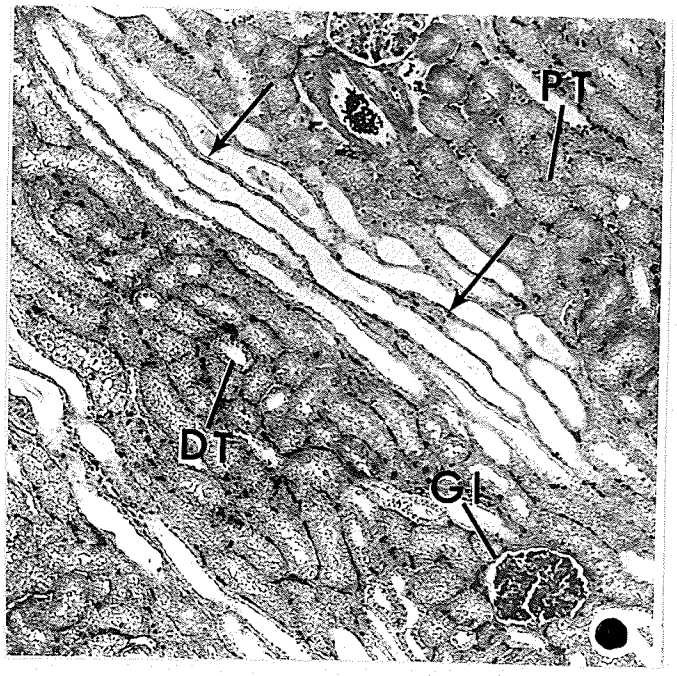


Figure 11. Light micrograph of kidney cortex from an animal treated with 5 mg/kg CsG for 30 days, showing several examples of tubular atrophy (arrows). PT, DT = normal appearing proximal and distal tubules, Gl = glomerulus. The same changes were observed in animals treated with 5 mg/kg CsA for 30 days. Hematoxylin and eosin. Magnification x 100.

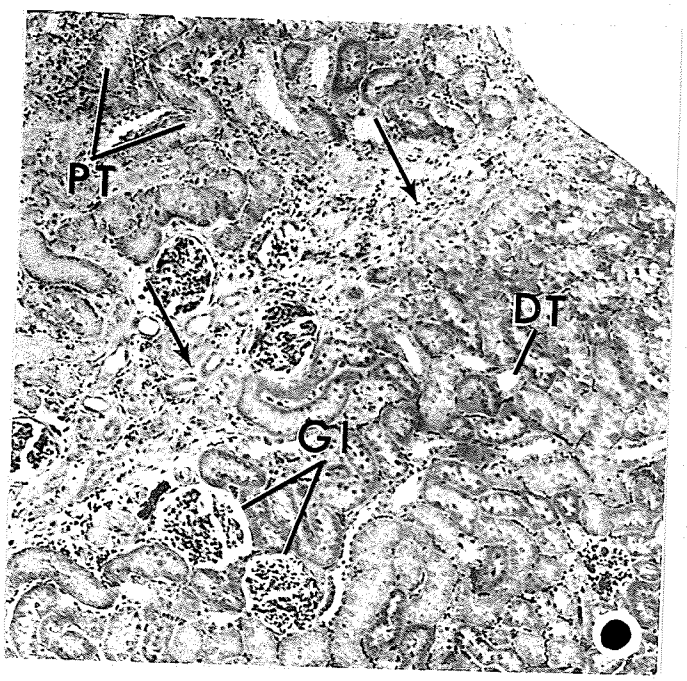


Figure 12. Light micrograph of kidney cortex from an animal treated with 5 mg/kg CsG for 30 days. Note the loss of histological integrity and the presence of interstitial fibrosis (arrows). The same changes were observed in animals treated with 5 mg/kg CsA for 30 days. Hematoxylin and eosin. PT, DT = normal appearing proximal and distal tubules, Gl = glomerulus. Magnification x 100.

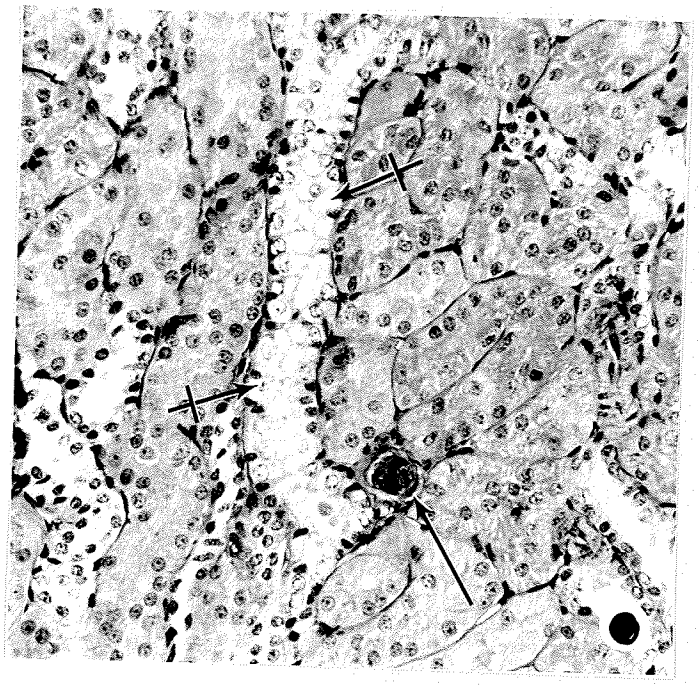


Figure 13. Light micrograph of kidney cortex from an animal treated with 2.5 mg/kg CsG for 30 days. Shown here is a typical example of CsA-associated arteriolopathy (arrow) with nodular protein deposits in the vascular wall and narrowing of the vascular lumen. Adjacent to the affected arteriole is a longitudinal section of a proximal convoluted tubule showing isometric vacuolization of the epithelial cells (crossed arrows). The same changes were observed in animals treated with 2.5 mg/kg CsA for 30 days. Periodic acid - Schiff. Magnification x 400.

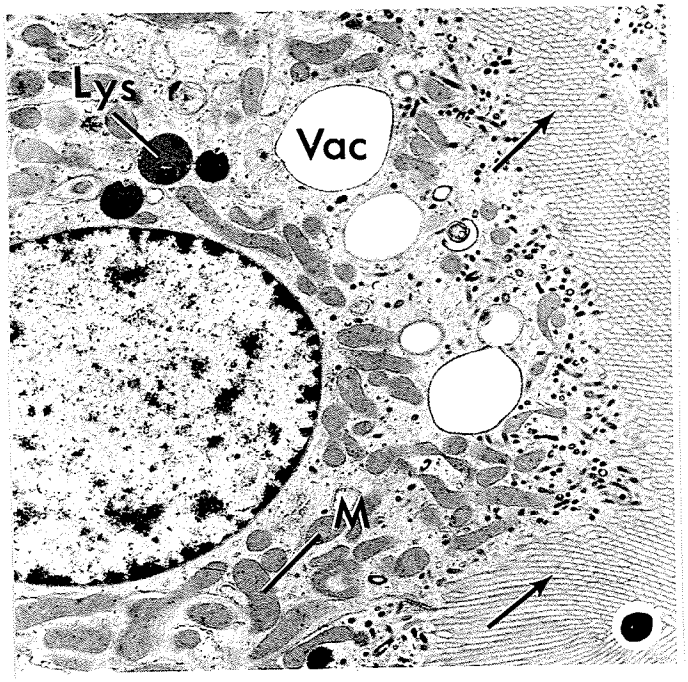


Figure 14. Electron micrograph of a kidney proximal tubule cell from a control animal. Note the presence of several vacuoles (Vac), numerous mitochondria (M) and lysosomal-like structures (Lys). Arrows = brush border. Magnification x 7000.

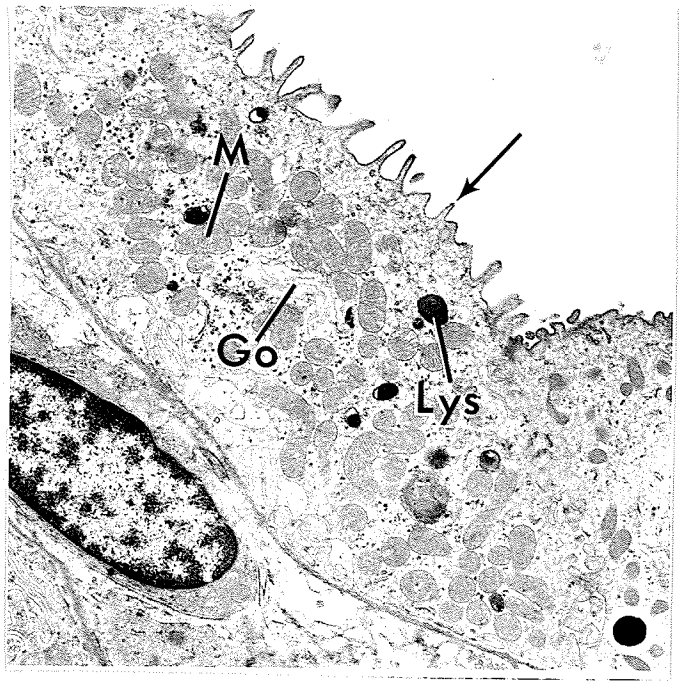


Figure 15. Electron micrograph of a kidney distal tubule cell from a control animal. Note the presence of numerous mitochondria (M), lysosomal-like bodies (Lys), and sparse numbers of microvilli (arrow) at the adluminal border of the cell. Go = Golgi complex. Magnification x 7000.

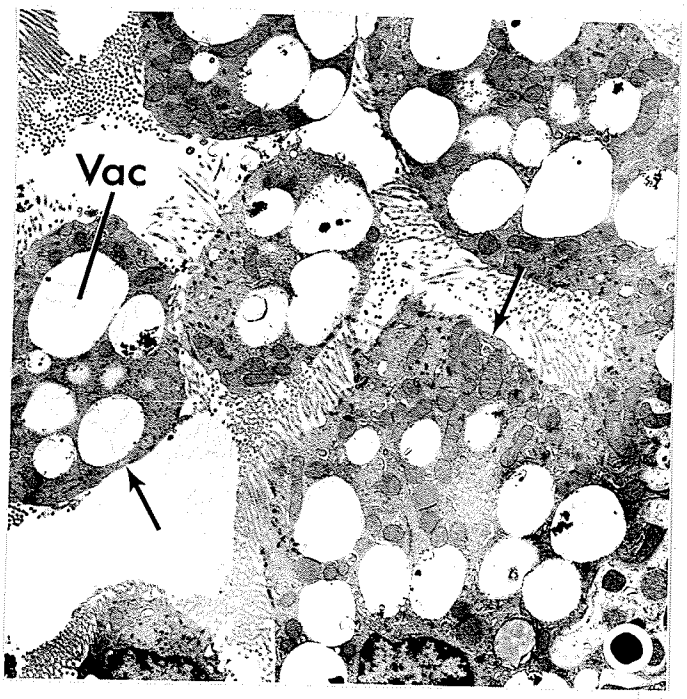


Figure 16. Electron micrograph of several kidney proximal tubule cells from an animal treated with 5 mg/kg CsG for 30 days. Note the numerous vacuoles (Vac) and reduction of the brush border (arrows). The same changes were observed in animals treated with 5 mg/kg CsA for 30 days. Magnification x 4500.

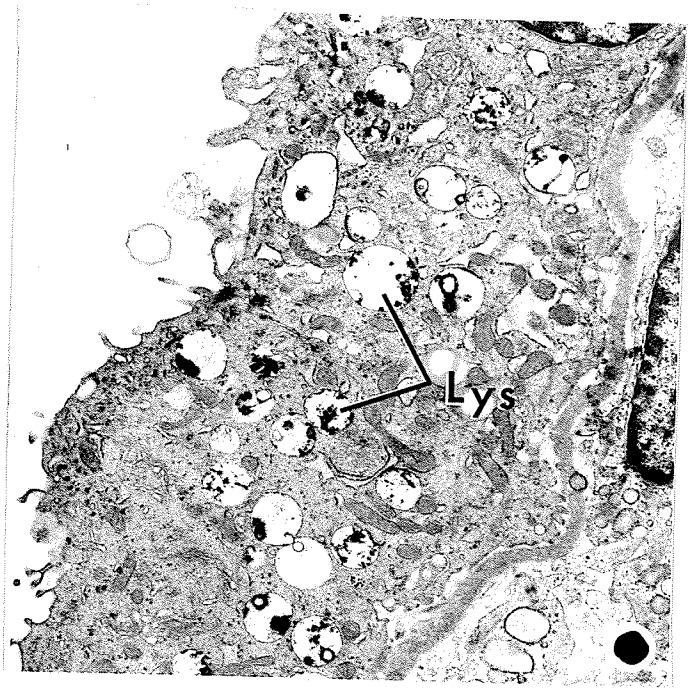


Figure 17. Electron micrograph of a kidney distal tubule cell from an animal treated with 5 mg/kg CsG for 30 days. The numerous vacuoles seen here containing dense material probably represent autolysosomes. The same changes were observed in animals treated with 5 mg/kg CsA for 30 days. Magnification x 7000.

compared to those treated with CsA (table 4). An increased amount of leucocyte infiltration was only observed in rabbits treated with 5.0 mg/kg of CsA as compared to the same dose of CsG. There was no statistical difference in leucocyte infiltration between rabbits treated with 2.5 mg/kg of either CsG or CsA. Additionally, there were no differences among the five groups with respect to glomerular tuft area and density or arteriole wall thickness.

E. TISSUE CONCENTRATIONS

The concentrations of CsG or CsA in the fat, heart, kidney, liver and spleen in rabbits receiving 5.0 mg/kg of either drug for 30 days is shown in table 5. The concentrations of CsG in the heart, kidney and liver were significantly lower when compared to the same tissues of rabbits treated with CsA. No statistical difference was observed in the case of fat and spleen.

F. IN VITRO STUDIES

The porcine renal epithelial cell line LLC-PK_1 was chosen to investigate the toxicity of CsG and CsG metabolites, to allow for comparison to studies previously performed with CsA and its metabolites. The effect of CsG and metabolites GM1 and GM9 on the growth of LLC-PK_1 cells is shown in figure 18. CsG was found to be much more toxic to the cells than either of the two metabolites at 5000 to 20000 ug/L. Figure 19 shows the effect of these agents on DNA synthesis. All values were standardized for protein concentration. No metabolite was as effective as CsA in

Table 4. Morphological Assessment of Kidneys from Rabbits Treated Daily for 30 Days Intravenously with CsG or CsA.

Group (n = 5)	Glomerular Tuft Area ($mm^2 \times 10^{-3}$)	Glomerular Tuft Density ($mm^3 \times 10^{-3}$)	Arteriole Area ($mm^2 \times 10^{-4}$)	Leucocyte Infiltrates	Tubular Atrophy	Interstitial Fibrosis
CsG (5.0 mg/kg)	6.35 ± 0.66	4.65 ± 1.05	5.78 ± 0.45	1.05 ± 1.33	0.35 ± 0.55#	0.50 ± 0.50#
CsA (5.0 mg/kg)	7.21 ± 1.11	4.46 ± 0.53	5.64 ± 0.76	1.80 ± 1.15	1.60 ± 1.08	1.70 ± 1.61
CsG (2.5 mg/kg)	6.88 ± 1.02	5.58 ± 0.41	6.92 ± 1.10	0.35 ± 0.34#	0.10 ± 0.14#	0.15 ± 0.14#
CsA (2.5 mg/kg)	6.38 ± 0.60	5.10 ± 0.80	5.74 ± 0.53	2.10 ± 0.89	1.90 ± 1.08	0.80 ± 0.76
Control (Cremophor-EL)	5.57 ± 0.83	5.58 ± 0.82	5.36 ± 0.70	0	0	0

* = values expressed as mean ± SD
# = $p < 0.01$ less than in animals receiving CsA at the same dose

Table 5. CsG and CsA concentrations[@] in rabbit tissues.

Tissue	CsG (ug/g)	CsA (ug/g)
Fat	996 (796 - 1335)[#]	1129 (166 - 1741)
Heart	195 (147 - 257)[*]	650 (171 - 1240)
Kidney	231 (74 - 365)[**]	852 (436 - 1387)
Liver	119 (42 - 248)[*]	306 (137 - 409)
Spleen	318 (223 - 386)	3938 (460 - 9224)

@ = values are expressed as mean
# = range in brackets
* = $p < 0.05$ less than in animals receiving CsA
** = $p < 0.01$ less than in animals receiving CsA

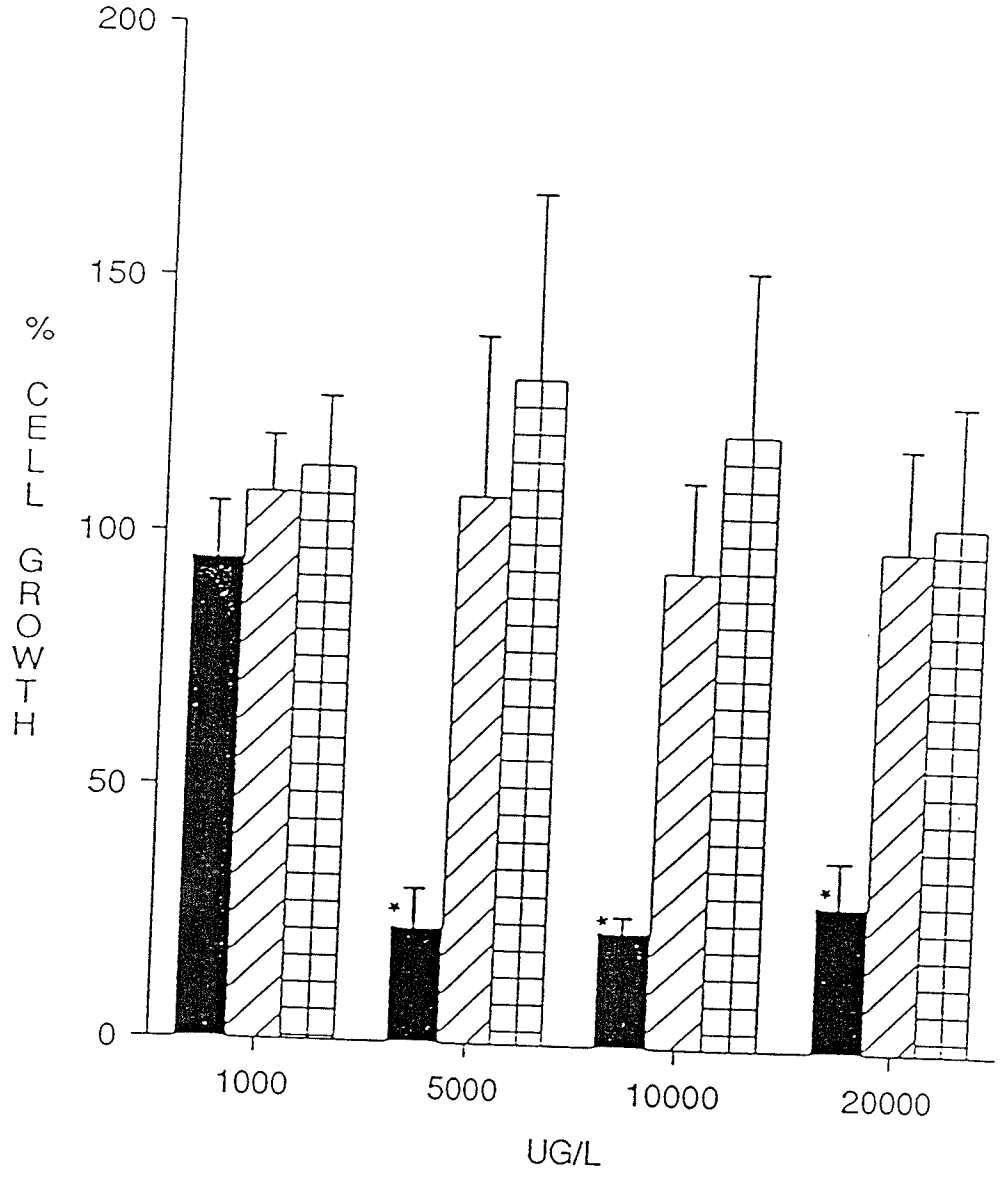


Figure 18. The effect of CsG and CsG metabolites on cell growth of LLC-PK_1 cells. CsG (▬), GM1 (▨), GM9 (▦). Results are expressed as mean ± SD (n = 3) of percent inhibition of cell growth as compared to control cultures. * = $p < 0.01$ less than control.

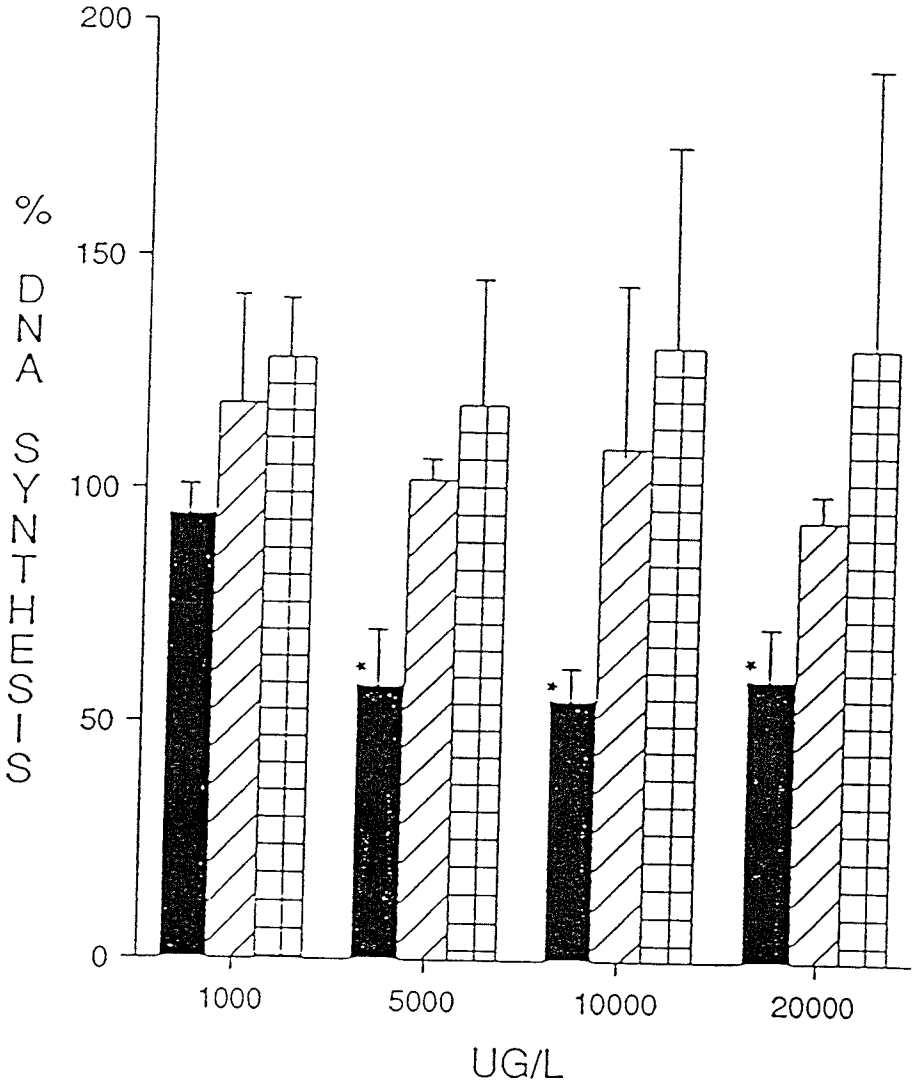


Figure 19. The effect of CsG and CsG metabolites on DNA synthesis of LLC-PK$_1$ cells. CsG ( ), GM1 ( ), GM9 ( ). Results are expressed as mean ± SD (n = 3) of percent inhibition of DNA synthesis as compared to control cultures. * = < 0.01 less than control.

inhibiting DNA synthesis at concentrations ranging from 5000 to 20000 ug/L.

The effect of CsG, GM1 and GM9 on the cell growth and DNA synthesis of isolated rabbit mesangial cells is shown in figure 20 and 21. CsG had a slight stimulatory effect on cellular proliferation at 1000 ug/L (figure 20). However, it suppressed cell growth at between 5000 and 20,000 ug/L in a concentration dependent manner. Both metabolites, GM1 and GM9, had a slight stimulatory effect between 1000 and 10000 ug/L. None of these effects on cellular proliferation were statistically different from those observed in untreated cells ($p < 0.05$).

CsG, GM1 and GM9 all had a significant effect on DNA synthesis of rabbit mesangial cells (figure 21). CsG inhibited DNA synthesis by 50% at 1000 ug/L and 80% at 20,000 ug/L. GM1 and GM9 inhibited DNA synthesis at all concentrations tested, and exhibited 65% and 75% inhibition at 20,000 ug/L, respectively.

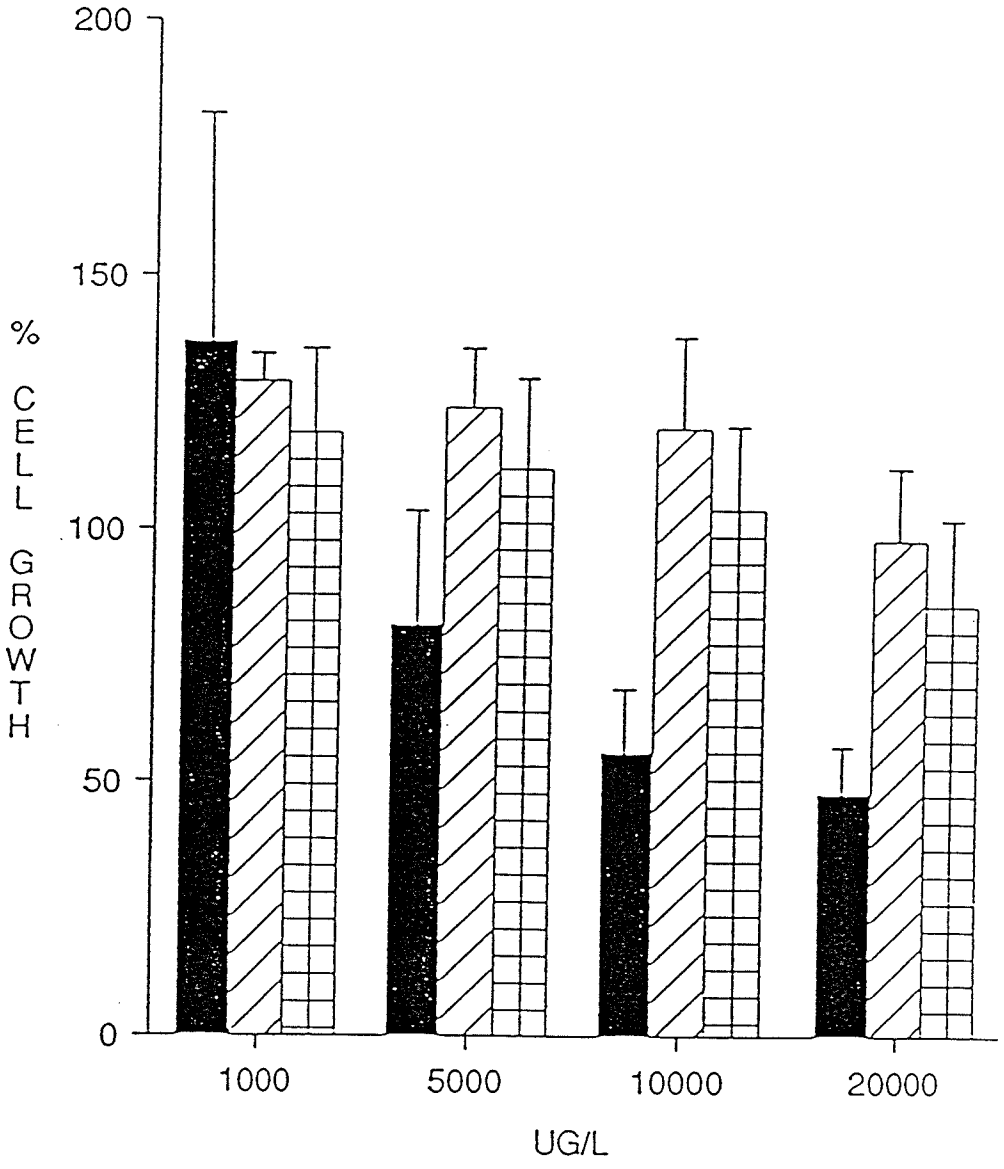


Figure 20. The effect of CsG and CsG metabolites on cell growth of isolated rabbit mesangial cells. CsG (■), GM1 (▨), GM9 (▦). Results are expressed as mean ± SD (n = 3) of percent inhibition of cell growth as compared to control cultures.

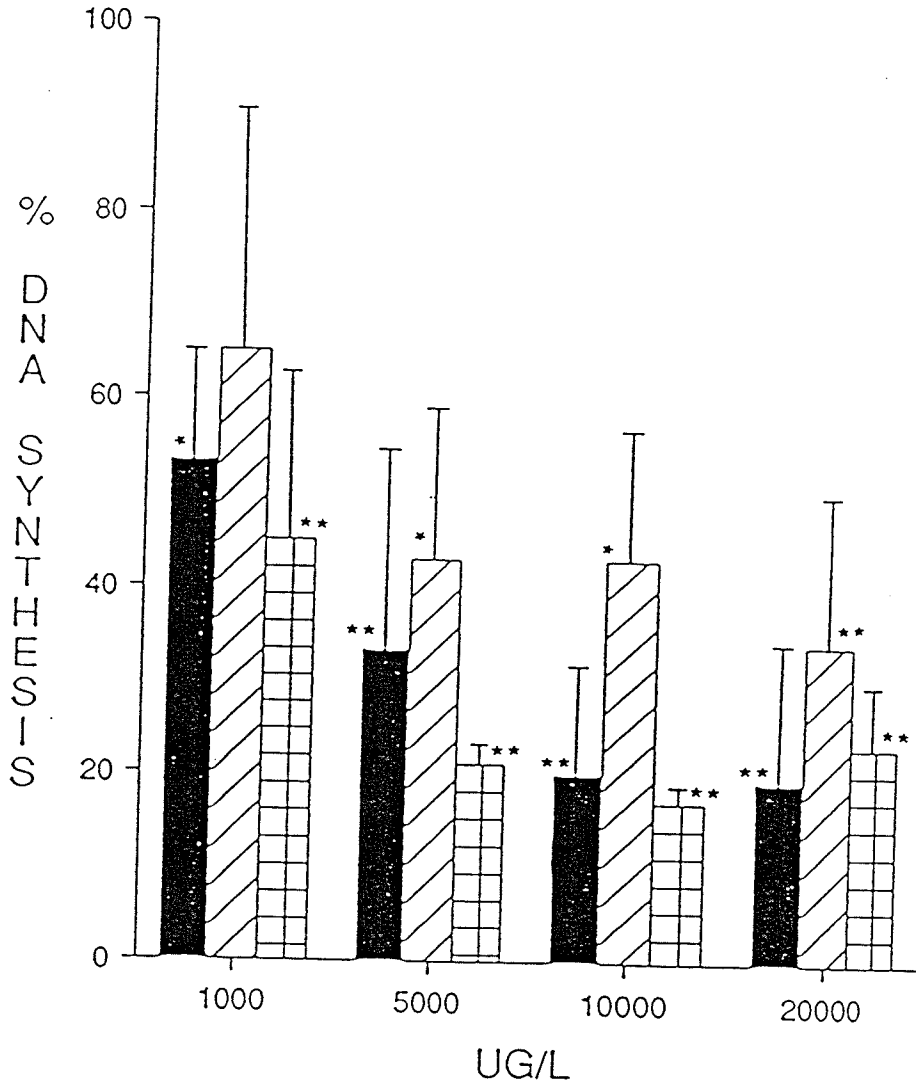


Figure 21. The effect of CsG and CsG metabolites on DNA synthesis of isolated rabbit mesangial cells. CsG (■), GM1 (▨), GM9 (▦). Results are expressed as mean ± SD (n = 3) of percent inhibition of DNA synthesis as compared to control cultures. * = < 0.05 less than control. ** = $p < 0.01$ less than control.

V. DISCUSSION

Blood level monitoring of CsG has been suggested to provide a guide for dosage adjustment to maintain the appropriate drug concentrations to maximize immunosuppression while preventing rejection. However, for therapeutic monitoring to be clinically relevant, there should ideally exist uniformity with respect to controllable factors such as the medium of analysis, sample preparation technique and analytical methodology used. Two recent reports have adressed the advances in the therapeutic monitoring of CsA and based on existing research data have made specific recommendations for the methods of analysis (57,86). Little has been published regarding the sample preparation for and the method of quantitation of CsG. We therefore studied the distribution of CsG and two CsG metabolites in blood, and compared various methods of analysis of this drug.

CsG was shown to have a temperature dependent distribution in whole blood. Maximum recovery of CsG in plasma, as measured by HPLC, was obtained after a 10 minute period of re-equilibration at 37°C. Prolonging the length of re-equilibration at 37°C upto 3 hours did not alter the maximum recovery of CsG in plasma. These results confirm the findings of Yatscoff and Jeffery (42), where the redistribution of CsG between erythrocytes and plasma at 37°C was studied on samples obtained from renal transplant patients receiving CsG, and maximum recovery was observed within 10 minutes of incubation of samples at 37°C. However, to ensure complete equilibration of CsG in all

samples, we recommend a 30 minute re-equilibration period.

Time dependent re-equilibration has been observed in whole blood samples from renal transplant patients who are administered CsA as part of their immunosuppressive regimen (53). However, the plasma to whole blood ratio of CsG after re-equilibration is quite different from that of CsA. In normal subjects or renal transplant patients, the plasma to whole blood ratio of CsA is approximately 2.0 (87), whereas our study showed a ratio of 1.0 for CsG. This ratio remained constant for CsG concentrations in whole blood between 100 and 1000 ug/L. At 5000 ug/L of CsG the plasma-whole blood ratio increased to 1.5, suggesting saturation of the erythrocyte fraction. This concentration-dependent distribution is also seen with CsA (52).

Data that describe the distribution of individual CsG metabolites among plasma and cells of whole blood has not been available. This is primarily due to the lack of isolated metabolites. However, a recent report describes the isolation and structural determination of CsG metabolites (23). Here we reported on the distribution of two hydroxylated metabolites of CsG, GM1 and GM9, where concentrations were determined by HPLC. We have found that the plasma to erythrocyte ratio of both CsG hydroxylated metabolites were 0.4 and and increased to about 0.9 at concentrations exceeding 1000 ug/L. This concentration dependent distribution is similar to the corresponding CsA metabolites AM1 and AM9, which are also hydroxylated at position 1 and 9 of the amino acid. The relative concentrations of AM1 and AM9 in plasma remains constant up to 500 ug/L and increases when concentrations in whole blood range between 500 to 1000 ug/L (52). These results suggest that the major hydroxylated CsG

metabolites are sequestered in the cellular fraction in whole blood. The observed significantly greater cellular binding of both GM1 and GM9 when compared to the parent CsG, as well as the concentration dependent distribution of CsG, GM1 and GM9 in blood, would suggest the use of whole blood as compared to plasma for clinical monitoring of either CsG or its metabolites.

The cellular affinity for CsG appears to be influenced by its polarity. CsA, with alpha-aminobutyric acid in amino acid 2 position, is more tightly bound to cells (plasma to blood ratio of 2.0) than CsG (plasma to blood ratio of 1.0), which has a less polar norvaline at this amino acid position. It is interesting to note that the more hydrophilic metabolites, namely those that are hydroxylated appear to be more highly associated with cells than is the parent drug. This is demonstrated by the lower plasma to erythrocyte ratio of CsG metabolites as compared to CsG parent drug. Hydroxylated metabolites of CsA also show a higher affinity for cells than parent drug, with 90% of metabolites being found in the erythrocyte fraction, compared to only 45% of CsA (52).

We have also monitored the concentrations of parent drug and metabolites in whole blood from rabbits treated with CsG or CsA. Using the monoclonal selective antibody, the RIA gave CsG values in whole blood that were 10% lower than those obtained by HPLC. In contrast, whole blood CsA levels were 10% higher when measured by RIA as compared to HPLC. These differences may be accounted for by the imprecision of both methods. CsA whole blood levels measured using the non-selective monoclonal antibody, which exhibits considerable crossreactivity with CsA metabolites, were 3.2 fold higher as compared with the selective RIA method. The non-selective assay gave CsG values that were only 1.4 fold higher when compared with the selective RIA method. Whole blood levels of CsA in patients measured with

the non-selective antibody are usually about 50% higher than those measured by selective methods (82). Only in patients with liver or heart transplants do CsA levels measured by non-selective methods exceed 50% (82). A correlation of CsG metabolite concentration with clinical events would provide the strongest practical justification for monitoring of specific metabolites in routine practice. CsG is currently being evaluated in clinical pilot trials, although it is only recently that metabolites of this drug have been identified (23). Therefore, the use of the selective antibody or HPLC appear to be the methods of choice for the monitoring of CsG, since the overall importance of the immunosuppressive and toxic effects of CsG metabolites are not yet known.

To determine pharmacokinetic parameters of a drug (absorption, distribution, biotransformation and elimination), it is important to establish a dosage schedule that will rapidly produce and maintain a desired concentration range at the site of action. This is particularly important in the case of CsA, which has a very narrow therapeutic index. We have compared pharmacokinetic parameters in the the rabbit to investigate effects of CsG and CsA. It is felt that the results obtained with this animal model would be very similar to that obtained in humans since the CsA pharmacokinetics of both species are very similar (88,89)

The elimination of CsG appears to occur faster than that of CsA. After a single intravenous bolus of CsG, the clearance at both doses is larger than that of CsA. The volume of distribution, which provides an estimate of the extent of drug distribution through body-fluid compartments and of its uptake by tissue, was not different between the two drugs. In contrast, the half-life of CsG which exhibits

a proportional increase with dose was shorter than that of CsA in all cases.

Our results are similar to those obtained by D'Souza et al, where significant differences in half-life and clearance between CsG and CsA with an i.v. bolus of 15mg/kg in rabbits were shown (50). CsG has been shown to be less immunosuppressive in in vitro and in vivo (90). Thus, the higher clearance of CsG could account for the lower immunosuppressive potency as compared to the same dose of CsA. Clinically, this would make it necessary to increase the dose of CsG as compared to CsA in order to establish comparable level of immunosuppression.

Since its introduction, CsA has gained universal acceptance as an immunosuppressive agent for organ transplantation, and its application is extending to treatment of several immunologically mediated disorders (91-93). Early toxicological studies showed that CsA is well tolerated by various animal species and is without systemic side-effects at therapeutic doses. No carcinogenic, mutagenic or teratogenic effect was observed (59). The first clinical trials in 1978 with renal (7) and bone-marrow transplants (94) gave excellent results, which were confirmed in patients with other organ transplants (95-97). However, since then significant CsA-induced nephrotoxicity has been recognized. Nearly all patients on CsA demonstrate some degree of renal impairment which in the most severe cases, has led to end stage renal failure (98).

In humans CsA-induced nephrotoxicity is characterized by interstitial fibrosis, tubular atrophy and arteriolopathy. The lack of a suitable animal model has hampered the study of chronic CsA nephrotoxicity. The rat is the most commonly used animal, however the characteristic lesions observed in man, especially the interstitial fibrosis are not seen in this animal. Acute

changes in kidney function in the rat have been observed, as measured by serum creatinine or by creatinine clearance (75,99). In contrast, two recent reports in which rats were administered CsA at 100 mg/kg s.c. for 10 days and 25 mg/kg i.p. for 28 days resulted in renal damage, characterized by the presence of tubule atrophy and interstitial fibrosis. However, the doses of CsA required to induce these changes are somewhat higher than those in clinical use, which are usually <10 mg/kg/day.

The rabbit has also been used as a model to study CsA nephrotoxicity, but far less frequently than the rat (100). Rabbits treated with an immunosuppressive dose of CsA for a prolonged period of time develop a clinically distinct toxic syndrome characterized by wasting, loss of weight, reduced food and water consumption and movement (77). Increases in serum creatinine were seen suggesting that nephrotoxicity was present, although no significant morphological changes at the light microscopic level were observed (77). We have found that as low as 2.5 mg/kg CsA administered i.v. over a period of 30 days can result in morphological changes consistent with chronic nephrotoxicity seen in man. Moreover, tissue changes were observed at both microscopic and ultrastructural levels. Our findings are in contrast to those reported by Gratwohl et.al. (77), where rabbits did not show any renal structural changes after 60 days of treatment with up to 25 mg/kg (i.v.) of CsA. The reason for the differences observed between our study and Gratwohl et.al. (77) are unclear. The same species of rabbits were used and serum CsA levels in the latter study were even greater than those reported in our study.

The doses of CsA in the present study required to induce renal histologic changes consistent with chronic nephrotoxicity were comparable to clinical doses, which are usually < 10 mg/kg and it

was therefore felt that the rabbit is more more promising model to study CsA as well as CsG nephrotoxicity. In comparing the degree of histopathological damage to the kidney of rabbits treated with the same dose of either drug we found CsG to show significantly less damage with respect to the occurence of leucocyte infiltrates, tubular atrophy and interstitial fibrosis. Arteriolopathy was also present in animals treated with CsG, but its occurence was not statistically different when compared to rabbits receiving CsA. It appears that on a dose to dose comparison CsG is less nephrotoxic than CsA.

Previous reports in mice, rats and dogs have shown CsG to be less nephrotoxic than CsA or to be devoid of toxicity at all (18,20,21,38). However, none of these models were able to show all the histological changes representative of those occuring in patients receiving CsA. A report in which two groups of rats were administered either CsG or CsA at 50 mg/kg for 50 days, resulted in renal interstitial fibrosis in animals treated with either drug, but no other morphological changes which are characteristic for CsA were observed (17). Faraci et.al. demonstrated that both CsG and CsA can cause glucose intolerance in rats treated with 10 mg/kg/day (19). An increased plasma creatinine was observed in the male rats treated with CsA as compared to controls, and no other signs of nephrotoxicity were detected in either the CsG or CsA teated groups. However, plasma bilirubin was elevated in rats treated with CsG as compared to controls and creatinine was elevated in CsA treated rats. We were not able to confirm any hepatotoxicicty of CsG as measured by serum AST and ALT. The activity of these two enzymes in serum are the most frequently measured indicators of hepatocellular damage. The serum AST of rabbits treated with 2.5 and 5.0 mg/kg of CsA were elevated when compared to controls, but this was not statistically

significant. An increase in either of the two enzymes is usually indicative of tissue damage or change in cell membrane permeability that allows for leakage of enzymes into the serum. However, no histopathological changes in the liver were observed in animals treated with either drug. There were no statistical differences in either serum creatinine or creatinine clearance in rabbits treated with either drug as compared to controls. However, there appeared to be a trend towards a decreased creatinine clearance of rabbits which received 5.0 mg/kg of either CsG or CsA as compared to rabbits which received the vehicle alone.

The most important observation of the current investigation is the fact that arteriolopathy, which has been reported in clinical studies and is one of the hallmarks of nephrotoxicity in man, has not previously been demonstrated in any animal studied to date. The histopathology seen in the arterioles has been reported in clinical studies to be associated with decreased creatinine clearance and elevated serum creatinine in transplant patients, subsequently followed by structural damage to the nephron (101,102).

Distribution studies revealed that CsA has a high tissue affinity and that it remains in tissues for several months after discontinuing drug administration (54,103). Among the six tissues studied, we found CsG and CsA to be most prevalent in the fat and spleen. The kidney, liver and heart had all levels of CsG that were less than those found in animals treated with comparable doses of CsA. This result is in contrast to the observed similar volume of distribution of both CsG and CsA. However, it appears to coincide with the reduced nephrotoxicity of CsG observed after histological examination of the kidney.

Cyclosporine nephrotoxicity has been examined using a number of <u>in vitro</u> cell systems (24,64,66-68,72). Cell

cultures allow for the study of the direct effect of drugs on cells in the absence of any hemodynamic alterations present in *in vivo* experiments. However, studies investigating the toxic effects of CsG on renal cell lines have not been previously reported.

CsA has been shown to inhibit proliferation of cultured rabbit and rat renal mesangial cells (24,66) as well as porcine proximal renal epithelial cells (LLC-PK_1) (24,67), demonstrating a suppressive effect of CsA on non-lymphocytic cells. We have shown that CsG is less effective than CsA at inhibiting the cellular proliferation as well as DNA synthesis in both the mesangial and proximal tubule cell lines. CsG inhibits cell growth by 75% between 5000 and 20000 ug/L in the LLC-PK_1 cells. CsA causes close to 100 % inhibition of cell growth at these concentrations (24). The mesangial cell also appears to be less affected by CsG when compared to CsA. Inhibition of cell growth by 50% occurs at a concentration of 20000 ug/L when CsG is used, compared to only 5000 ug/L of CsA needed to achieve a similar inhibition. DNA synthesis is suppressed by as little as 1000 ug/L of CsG to give 50% inhibition as compared to CsA which requires 7000 ug/L to achieve this. This is in contrast to the study by Martin et.al. (66), reporting CsG to be slightly more effective in its ability to inhibit DNA synthesis, although this was not significant.

VI. CONCLUSIONS

1. The distribution of CsG in blood was changed with changes in temperature and concentration. The incubation of whole blood at 37°C caused the re-equilibration of CsG and resulted in its increased recovery in plasma. CsG metabolites are more highly associated with red blood cells than the parent drug.

2. CsG has a faster clearance rate and a shorter half-life than CsA when administered to the rabbit. There appears to be no difference in the volume of distribution between the two drugs.

3. The rabbit was shown to exhibit renal lesions after treatment with CsG, which are consistent with chronic CsA nephrotoxicity seen in man. Using the rabbit as a model we demonstrated CsG to be less nephrotoxic than CsA on a dose per dose comparison.

4. CsG is less effective than CsA at inhibiting the cellular proliferation as well as DNA synthesis in both mesangial and proximal tubule cell lines. Metabolites GM1 and GM9 have no effect on the proximal tubule cell line, but inhibit DNA synthesis in the mesangial cell line by up to 75%.

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