A COMPARISON OF THE IMMUNOSUPPRESSIVE EFFICACY OF CYCLOSPORINE A (CsA) AND CYCLOSPORINE G (CsG) *IN VITRO* AND *IN VIVO*

BY

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A Thesis
Submitted to the Faculty of Graduate Studies in
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ABSTRACT

In this study we compared the immunosuppressive effects of Cyclosporine (Cs) A and G, both *in vitro* (human and rabbit) and *in vivo* (rabbit). The 50 % inhibitory concentration (IC_{50}) (mean ± SEM) of CsG was approximately three times greater than that of CsA for mitogen and alloantigen-induced lymphocyte proliferation in Primary Mixed Lymphocyte Culture (1° MLC) = 60 ± 7 ug/l vs 19 ± 4 ug/l respectively; ($p< 0.01$). Kinetics studies in both human and rabbit systems showed that the effectiveness of both drugs was similarly reduced when added at later times after culture initiation. The effects of CsA and CsG in combination appeared to be antagonistic at higher and additive at lower drug doses. *In vivo* studies using skin allografts confirmed *in vitro* findings. Both CsA and CsG at 5 and 10 mg/kg/day significantly ($p< 0.01$) prolonged graft survival compared to control animals. However, at these doses and even at 15 mg/kg/day CsG, CsA was more efficacious at prolonging skin graft survival in rabbits ($p< 0.01$) mean survival time (MST ± SEM, days) at 10 mg/kg/day was >20.5 ± 6.5 vs 15 mg/kg/day CsG, 15 ± 1.9. These results suggest that both *in vitro* and *in vivo* in rabbits CsG is less immunosuppressive than CsA.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
BB	Brattle-Borough
cm^2	centimeter squared
Con A	concanavalin A
CsA	cyclosporine A
CsC	cyclosporine C
CsD	cyclosporine D
CsG	cyclosporine G
°C	degree Celsius
FKBP	FK-506 binding protein
g	gram
GFR	glomerular filtration rate
HPLC	high performance liquid chromatography
hr	hour
IC_{50}	fifty percent inhibitory concentration
IL-1	interleukin-1
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
iv	intravenous
kg	kilogram
l	liter
MHC	major histocompatibility complex
MST	mean survival time

uCi	microcurie
ug	microgram
ul	microliter
mg	milligram
ml	milliliter
NCSS	number cruncher statistical system
NZW	New Zealand white
(Nva^2)	norvaline-2
NF-AT	nuclear transcription factor of activated T cell
PPIase	peptidyl-prolyl isomerase
PBMC	peripheral blood mononuclear cell
PHA	phytohemagglutinin
1° MLC	primary mixed lymphocyte culture
RIA	radioimmunoassay
RBF	renal blood flow
RPF	renal plasma flow
rpm	revolution per minute
SEM	standard error of the mean

To GOD and To my *NANAY* and *TATAY*

I. INTRODUCTION

Cyclosporine A (CsA) is the most widely used immunosuppressive agent in clinical transplantation. This fungal metabolite is the first immunosuppressive agent to selectively act only on a limited population of lymphocytes and to be devoid of myelotoxicity (Reviewed in 1). However, the use of CsA is limited by a number of side effects, the most serious of which is nephrotoxicity.

Cyclosporine G (CsG) is a natural analogue of CsA. It differs from CsA in one amino acid residue at position 2 of the molecule. CsG was shown by Hiestand et al. in animal studies to have potent immunosuppressive activity both in vitro and in vivo (2). The same group also reported that CsG lacks the nephrotoxic side effect of CsA in rats (3). This finding has triggered enthusiasm for the possible use of CsG in clinical transplantation. Its immunosuppressive efficacy has been compared with that of CsA in experimental organ transplantation in various animal models (4-10). However, the results have been contradictory. The effect of CsG in rabbits and most importantly in humans is not clear at the present time. In this study we compared the immunosuppressive effects of CsA and CsG in vitro in humans and rabbits and in vivo in rabbits.

II. LITERATURE REVIEW

A. IMMUNOSUPPRESSION IN TRANSPLANTATION

The major barrier to organ transplantation between individuals of the same species are the antigens of the Major Histocompatibility Complex (MHC). The likelihood of acceptance or rejection of the transplanted graft is closely related to the extent of genetic differences between the donor and the recipient of the graft. Since there are a large number of gene loci in the MHC and much polymorphism within the loci, a normal population will have a very large number of different haplotypes and a chance of a full match between random individuals is very slim, eg. <1/40,000 in MHC class I system (11). When antigens differ between the donor and the recipient of a transplanted tissue (as is the usual case), an immune reaction will be induced against them. This will normally lead to the rejection and destruction of the transplant. A variety of immunosuppressive agents has been used to control tranplant rejection. At first, cytostatic drugs like cyclophosphamide, originally developed to combat tumours were employed (12). This method was very nonspecific such that all proliferating cells, and not just those involved in an immune reaction of the immune system, were damaged. Azathioprine and steroids were next introduced. Both of these compounds have a more

selective activity unlike cyclophosphamide. Azathioprine is a derivative of 6-mercaptopurine. It is an inhibitor of DNA and RNA synthesis and as a consequence it blocks the proliferative response of sensitised lymphocytes. One disadvantage of its site of action is the inhibition of B lymphocyte as well as T lymphocyte proliferation. The main side effect of azathioprine is bone marrow depression, and this is to a large extent dose-related (13). Steroids like prednisone and prednisolone have a marked lymphocytotoxic and anti-inflammatory activity. They depress the immune response in a number of ways, in particular by blocking lymphocyte proliferation probably by preventing activation of the cytokine Interleukin-1 (IL-1) gene (14). Hypertension, sodium and fluid retention, pathologic fracture of long bones, pancreatitis, and suppression of growth in children are among the many adverse effects of steroids (15). Inspite of the fact that azathioprine and steroids are myelotoxic, together they dominated the choice of immunosuppressive medication for two decades (12). Other immunosuppressive agents that have been used in clinical transplantation were antilymphocyte and antithymocyte sera and drainage of the thoracic duct in transplant recipients. These methods were aimed at either reducing the number of lymphocytes or at damaging lymphocytes. These sera are only used for short term treatment, and batch to batch variation of the serum also poses a problem. Now monoclonal antibodies like

OKT3 have been used to prevent transplant rejection. OKT3 is the most widely used preparation so far. It reacts with peripheral T lymphocytes by binding to the CD3 molecule (part of the T cell antigen receptor complex) on T cells. Although effective both in prophylaxis and for the treatment of steroid-resistant rejection, it is no more specific than antilymphocyte serum (14). Therapy with more specific monoclonal antibodies, such as those directed to the IL-2 receptor (IL-2R) are becoming available in the future. One major drawbacks in the use of this treatment is the development of an antibody mediated immune response by the recipient against these antibodies developed in a different species. It was not until the introduction of CsA in the 1980s however, that a major advance in the solid organ transplantation occurred. CsA is a potent immunosuppressive agent that reversibly affects the activation of T lymphocytes and does not affect haematopoietic cells. The initial application of CsA to clinical transplantation was remarkably successful, however long term effects of this drug result in other complications such as nephrotoxicity and hepatotoxicity in transplant patients. CsA has a narrow therapeutic window and utilization of this compound requires careful monitoring of drug levels in the blood to provide adequate immunosuppressive therapy while avoiding potential toxicity. At the present time the combination of cyclosporine,

azathioprine and steroids has become the standard therapy in transplantation for many centres.

B. CsA

CsA is the major metabolic product of the fungus Tolypocladium inflatum Gams and was initially isolated for its antifungal activities, but interest in the drug soon focused on its potent immunosuppressive capabilities. It is a cyclic peptide which consists of 11 amino acids. The structure and chemical properties of CsA have been well characterized (16, 17). The structure of CsA is shown in Figure 1. CsA has a molecular weight of 1,202.6 daltons. It is highly lipophilic. Ten of the amino acids are known aliphatic amino acids and the amino acid in position 1 has 9 carbon atoms which is unique to this compound. The *in vitro* total synthesis of CsA and a number of analogues has now been achieved (17, 18). The amino acids 1, 2, 3, 10, and 11 (19) are important for the immunosuppressive activity of CsA. An essential element is the carbon chain of the C9 amino acid and methylvaline in position 11 (16). Substitution of various amino acid residues reveals that the active site of the molecule appears to be focused about the unique C9 amino acid . Alkyl residues of 2-3 carbon atoms in position 2 of the molecule display good immunosuppression.

C. MECHANISM OF ACTION OF CsA

1. *IN VITRO* STUDIES

Despite a worldwide effort by hundreds of investigators, a complete understanding of the mechanism of action of CsA remains unknown. The effect of CsA on T cell activity is best examined in the context of the complex immunoregulatory relationships that modulate the immune response. Briefly, transplant antigens are presented by antigen presenting cells (APC) such as macrophages, dendritic cells and Langerhans cells to T lymphocytes. This interaction causes the macrophage to secrete IL-1 which induces the T cell production of another cytokine, IL-2 and the simultaneous production of the IL-2R (20). The autocrine association between IL-2 and its receptor (21) drives T cell proliferation and T lymphocyte-mediated help/induction, and cytotoxic activities essential for the induction of allograft rejection. IL-2 may also stimulate B lymphocytes bearing IL-2 receptors to augment antibody production and proliferation (22). It has been shown by various studies (23-34) that CsA effectively inhibited the transcription of IL-2. The initial studies demonstrating the effect of CsA on IL-2 production were performed on primary stimulation with alloantigen or mitogens and have been extended to the responses of sensitized lymphocytes, or

secondary responses. Some studies indicated that IL-2 production was not only inhibited in primary responses by CsA but this agent also inhibited production of IL-2 by sensitized lymphocytes upon specific rechallenge with antigen indicating some efficacy against sensitized cells (24, 35). These findings have some clinical implications in that CsA may be useful in suppressing the response of sensitized individuals or to affect ongoing immune responses such as active graft rejection or autoimmunity. However, studies by Klaus *et al.* (36) showed that CsA had no effect upon help provided by already primed effector cells but will abolish the help provided by unprimed cells. Kinetic studies showed that the proliferation of T lymphocytes as indicated by ^{3}H-thymidine uptake in response to mitogens or alloantigens was not inhibited by CsA more than 36 hours following mitogen stimulation or 72 to 96 hours following alloantigen stimulation (37-39). Studies by Koponen *et al.* (40) indicated that CsA prevented cells from proceeding from G0 to G1 phase in the cell cycle.

The detailed molecular events in this process remain elusive. It appears that CsA immunosuppression is related to the binding of an intracellular receptor protein, cyclophilin (41, 42) which is a major cytosolic constituent of both prokaryotic and eukaryotic cells. The human isoform of this protein has a mass of 17,373 daltons (43). Recently,

cyclophilin (44, 45) and FK-506 binding protein (FKBP) (46, 47) has been reported to have a peptidyl-prolyl isomerase (PPIase) activity. FK-506 is a macrolide (molecular weight of 822 daltons) isolated from the fermentation broth of a strain of Streptomyces tsukubaensis (48). It is structurally distinct from CsA and was shown to be 10-100 times more potent than CsA in inhibiting T cell responses *in vitro* and *in vivo* (49). Like CsA, it exerts powerful inhibitory effects on CD4+ T (helper) cell activation and on the secretion of IL-2 and other cytokines, including various cell growth factors and interferon-gamma (50).

The precise mechanism by which CsA and FK-506 selectively inhibit cytokine gene expression at a pretranscriptional level is not fully understood, but a transcription activator has been implicated as the target. It was proposed that CsA may mediate some of its effects, such as the inhibition of IL-2 and gamma interferon production during T lymphocyte activation, via its inhibitory action on PPIase. PPIase facilitates folding as well as modulating various intracellular signal transduction processes through cis-trans isomerization of the partner molecule. This interconversion of a peptide substrate was reported to be inhibited by CsA (45). The inhibition of isomerase activity by CsA was shown to be due to the fact that CsA (and possibly FK-506) contain a structure termed a "twisted amide surrogate", which is a

transition state mimic of a peptidyl-prolyl bond undergoing isomerization (51). It has been suggested that CsA and FK-506 function by acting as prodrugs becoming active only when they are complexed with their respective receptors collectively known as immunophilins (43). The target for the drug-immunophilin complex was speculated to be calcineurin which is a Ca2+ and calmodulin-dependent protein phosphatase (52). It has been suggested that calcineurin is an essential intermediate in the signaling pathway. This protein carries information from the cell membrane to the nucleus. The ability of calcineurin to dephosphorylate a synthetic peptide derived from the regulatory subunit of cAMP-dependent kinase was shown to be blocked by CsA and FK-506 when bound to their respective immunophilins (53). CsA was also reported to potently inhibit the transcriptional activity of Nuclear Factor of Activated T cells (NF-AT), a nuclear transcription factor that helps regulate the transcription of IL-2 gene (43). More recently, it has been shown that CsA and FK-506 blocked the assembly of NF-AT,but not the synthesis of this protein (54). It remains unclear how these two structurally different compounds could have the same effect on NF-AT.

The effect of CsA on the IL-2 receptor is more controversial with some studies showing that level of IL-2R was decreased in the presence of CsA and some showed no effect of the drug (24, 25, 32, 55-60) . Its effect on suppressor T

lymphocytes is not fully understood, since the measurement of this activity *in vitro* remains difficult to assess. Studies on the direct effects of CsA on the B lymphocytes, monocytes and granulocytes are somewhat difficult to interpret owing to the involvement of T cell products in the normal function of these cells. The initial studies of Borel *et al.* (37, 61, 62) established that CsA was capable of decreasing antibody production and impairing cell-mediated immunity *in vitro* and of attenuating skin graft rejection and graft-vs-disease *in vivo*. These data suggested that CsA might interfere with T lymphocyte activation of B cells. Other reports have indicated that CsA has a direct effect on B lymphocytes (63) and this was shown to occur early in B cell activation (64). Studies on the effects of CsA on the presentation of antigen to T cells by APC are conflicting (32). Earlier studies (65, 66) indicated that CsA impaired the presentation and processing of antigen by macrophages. On the other hand, Muller *et al.* (67) showed that CsA had no effect on the antigen presentation by macrophages using lysozyme specific T cell hybridomas.

2. *IN VIVO* STUDIES

a). ANIMAL

CsA has been shown to prolong allograft survival in a

number of animal models using a variety of different organ grafts for example, skin, liver, heart , kidney, lung (68-72). In some species permanent graft survival is easier to induce than others such that CsA had to be administered for only a short time to achieve long-term and even permanent acceptance of the transplant. In other species however induction of permanent graft survival is more difficult and graft survival is only possible as long as CsA is administered. The type of transplanted organ is also important. Rejection of some organs is easier to suppress than others. For example in rabbits CsA was not effective at inducing permanent immunological unresponsiveness to allogeneic skin grafts even though kidney and heart allografts in these animals were permanently accepted (73). The skin has more APC compared to the heart hence permanent acceptance of this organ is difficult. In the dog, renal and skin grafts remained functional as long as CsA therapy was continued but were rejected shortly after discontinuation of CsA treatment (74). This is in contrast to permanent acceptance of lung allografts in dogs after limited CsA treatment (75,76). Comparable anomalies were observed in cynomolgus monkeys with prolonged survival of orthotopic cardiac allografts after a 14 day course of CsA treatment, whereas renal allografts were rejected within 10 days after the course of therapy (77-79).

b). HUMAN

CsA has been shown in a large number of clinical trials to improve renal, liver, heart, lung and pancreas graft survival (12, 80-83). The compound improves renal graft survival by one to five years post transplant (81). The 30-month survival rate for liver transplant patients taking CsA was almost 60 % compared to 24 % after treatment with other immunosuppressants (12). The overall survival of heart transplantation was improved from 50% to at least 75% after introduction of CsA (84). In bone marrow transplantation, CsA has rapidly become a standard therapeutic agent. In aplastic anemia, an 80 % survival rate one year post transplant was observed in recipients treated with CsA compared to only 30 - 40 % for patients treated with methotrexate. Since it was shown to prevent the onset of diabetes mellitus in Brattle-Borough (BB) rats (85), the usefulness of CsA has been tested in a number of autoimmune diseases. Uveitis, an autoimmune disorder and a feature of Behcet's syndrome, responds very well to therapy with CsA (86). However, as soon as CsA is withdrawn, the patients relapse. Controlled trials are now being conducted to test the effectiveness of CsA in primary rheumatoid arthritis, lupus erythematosus, multiple sclerosis, myasthenia gravis, thyroiditis and psoriasis (87-92). Despite the findings in animal system, CsA for the most part has not

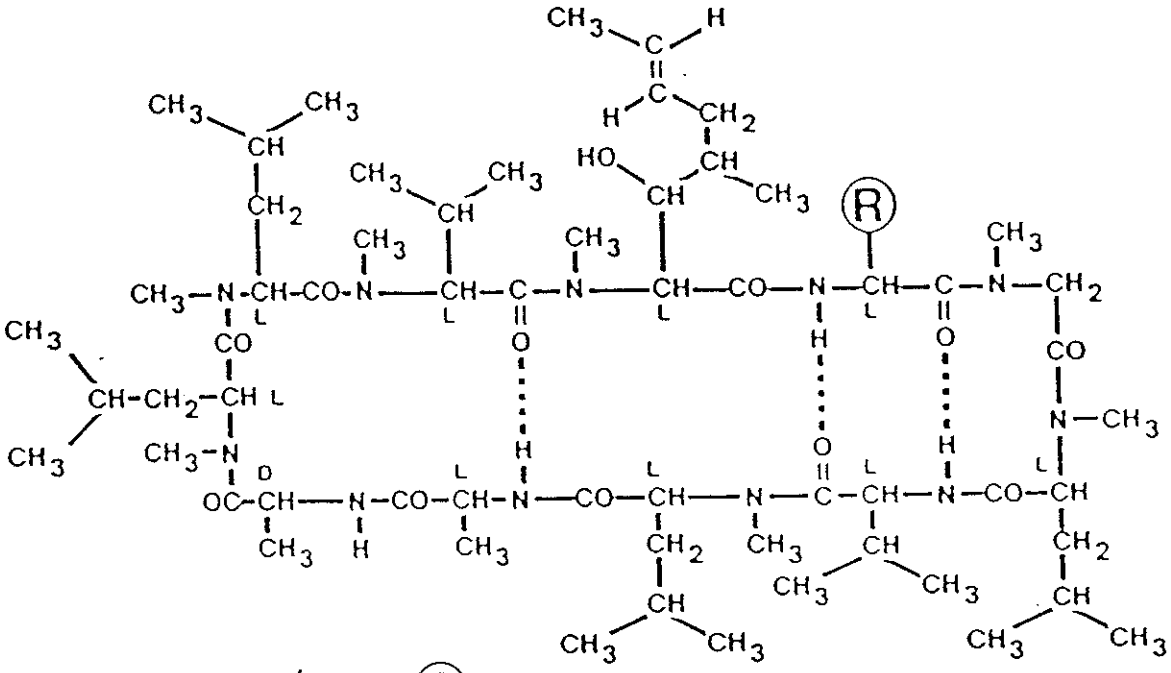
been able to induce tolerance in the clinical setting, thus necessitating continued immunosuppressive therapy.

D. SIDE EFFECTS OF CsA

Like most potent drugs CsA exhibits a number of side effects which limit its use. The most serious of these is nephrotoxicity. Results of studies appearing in the 1980s showed that the incidence of episodes of nephrotoxicity following renal transplantation was 50 - 75 % (93-95). This functional toxicity consists of dose-dependent reductions in renal flow and glomerular filtration rate that are reversed once dose levels are reduced. CsA has a very narrow toxic-to-therapeutic ratio, the therapeutic window being 100-250 ug/l (96) for serum trough levels as measured by the specific monoclonal antibody Radioimmunoassay (RIA) for CsA. Nephrotoxicity is a major problem in renal transplantation because it is difficult to distinguish rejection from toxicity since both induce loss of renal functon. Other important adverse effects of CsA include hepatotoxicity, hyperkalemia, gingival hyperplasia, hirsutism and tremor (97-99). A better correlation between elevated cyclosporine drug levels and hepatotoxicity has been noted than the correlation seen with nephrotoxicity (12). The incidence of hepatotoxicity ranges from 20 to 40 %, and most cases can be successfully managed by

reduction of cyclosporine doses. Anaphylaxis has also been reported (100) after intravenous administration and may be due to reaction to the drug or vehicle.

Figure 1. The chemical structure of CsA and CsG with norvaline replacing alpha amino butyric acid at position 2 of the molecule.



CYCLOSPORIN	Ⓡ
A	$CH_2\ CH_3$
G	$CH_2\ CH_2\ CH_3$

F. CsG

There is a vigorous search for an analogue that possesses the immunosuppressive activity but not the toxic effects of CsA. The fungus Tolypocladium inflatum Gams produces in addition to CsA a number of analogues of the same structural type. A total of 25 natural components has been reported as cyclosporines A to Z (16), and about 750 semisynthetic analogues have been produced and tested in vitro, but only a few of them have been available in sufficient quantity for *in vivo* characterization (17, 101). The first natural analogue to have been studied in detail were dihydro cyclosporine C (CsC) in which the amino acid threonine replaces alpha-amino-butyric acid in position 2 of the molecule, and cyclosporine D (CsD) in which valine replaces the latter amino acid. Dihydro CsC was found to inhibit both cell mediated and antibody mediated immunity (17). CsD has been shown not to inhibit antibody mediated immunity, and affects allograft rejection only weakly as compared to CsA (101). However, it was shown to be similar to CsA in suppressing a variety of cell mediated responses in models of chronic inflammation and in the localized graft vs host assay (17). In addition, CsD has been observed to have no nephrotoxic effect. However, because of other side effects such as hepatotoxicity and hypertension, further clinical studies were abandoned.

(Nva^2) cyclosporine otherwise known as CsG has norvaline substituted for amino-butyric acid in position 2 of the cyclic peptide (as can be seen in Figure 1). It has a molecular weight of 1,217 daltons. The compound was first introduced in 1984 as an analogue that has potent immunosuppressive activity both *in vitro* and *in vivo* (2) and lacks the nephrotoxic side effect of CsA in rats (3). This finding has created enthusiasm for the possible use of CsG in clinical transplantation and lead to many studies comparing its immunosuppressive efficacy with that of CsA. *In vitro*, CsG was shown to have similar immunosuppressive profile as CsA in its ability to inhibit mitogen and alloantigen-induced production of gamma interferon, lymphotoxin, and Tumor Necrosis Factor (TNF) (102). *In vivo*, CsG has been studied in a variety of animal models however the results have been conflicting (2-10, 103-105). CsG at equivalent doses to CsA displayed comparable efficacy in prolonging survival of skin allografts in both mice and rats (2). Furthermore, both CsG and CsA were shown to be equally effective in preventing kidney as well as heterotopic heart allograft rejection in rats (3). In dogs, Venkataramanan et al. (105) reported that CsG has similar immunosuppressive properties to CsA in inhibiting liver allograft rejection. In contrast to these findings, Calne et al. (4) showed that CsG has significantly greater immunosuppression, in terms of renal allograft functions over

CsA in dogs. They suggested that this could be explained by the higher blood levels of CsG detected compared to CsA. Other studies have shown that CsG was a less effective immunosuppressant than CsA, for example in cynomolgus monkeys with orthotopic heart transplants, CsA was better at prolonging graft survival than CsG (5). Interestingly this was so inspite of higher blood CsG levels than CsA. Prop et al.(6) has reported that CsG was also less efficacious than CsA in suppressing rejection of lung and heart allografts in rats.

Toxicity studies have yielded even more confusing results. CsG was found to be less nephrotoxic than CsA in mice and rats (106-110). In Sprague-Dawley rats treated with either CsA or CsG (25 mg/kg for 21 days), there was no change in RPF (renal plasma flow) and GFR (glomerular filtration rate) for the CsG treated rats as compared to controls (111). 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. Furthermore, CsG treated animals showed lack of tubular atrophy upon examination of the kidney whereas this effect was seen in 40% of CsA treated animals. Faraci *et al.* (106) have demonstrated in Wistar rats that CsG was less nephrotoxic but more hepatotoxic than CsA as measured by plasma creatinine and bilirubin levels. CsG gave higher cyclosporine plasma levels

than CsA with same oral dose (10 mg/kg for approximately 30 days) and CsG caused glucose intolerance which was less evident than CsA treated animals. Others have reported that both CsA and CsG were devoid of nephrotoxicity in rats, dogs and primates (4, 5, 8, 9, 10, 103, 112). Todo *et al.* (10) have shown in dogs with liver transplants that neither drug (20 mg/kg for 30 days) ever caused an increase in BUN (blood urea nitrogen) or creatinine. Some investigators have demonstrated CsG to be more hepatotoxic than CsA (4, 103, 107, 111) while others showed that both drugs are both nephrotoxic and hepatotoxic in rats. On the basis of acute studies, Paller *et al.* (113) found renal dysfunction attributable to CsG to be at least equal in magnitude to that produced by CsA. Infusion of CsG in a dose of 20 or 10 mg/kg/day caused a 53 % fall in GFR and a 50% in RPF and RBF (renal blood flow). The discordance among various studies may in part be due to lack of standardized procedures for assessing toxicity as well as variation in strain and species of animals used in the investigation.

The effect of CsG in humans remains not clear. Most recently, clinical trials in renal transplantation have been performed and results were once again contradictory (114-116). Preliminary data from a randomized, prospective, double-blinded trial of CsA vs CsG in cadaveric renal transplantation suggested that the efficacy of CsG was similar to that of CsA.

Recipients treated with CsG tended to have better renal function and improved blood pressure control with fewer antihypertensives than those patients on CsA (114). Adams *et al.* (115) have also reported that CsA and CsG were comparably effective in reducing frequency of early renal transplant rejection. In addition, they suggested that CsG may be less nephrotoxic than CsA as demonstrated by trends towards lower serum creatinine, and other parameters despite higher trough levels. On the contrary, Lindholm *et al.* (116) concluded that CsG did not present significant advantages over CsA as an immunosuppressant for renal transplantation since CsG was found to be significantly more hepatotoxic than CsA and not convincingly less nephrotoxic eventhough there was no difference in the immunosuppressive efficacy of the two drugs.

III. RATIONALE AND OBJECTIVES

Preliminary data suggest that CsG may be as immunosuppressive as CsA and possibly less nephrotoxic although a number of conflicting reports do exist. Much of the controversy can be attributed to the lack of a suitable animal model for investigating nephrotoxicity as well as standardized procedures for studying the pharmacokinetics, immunosuppressive activity, and for measurement of the drug.

In this study, we investigated the effects of CsA and CsG *in vitro* using human and rabbit cells, and *in vivo* in rabbits. *In vitro* studies included the determination of the fifty percent inhibitory concentrations (IC_{50}) for inhibition of mitogen and alloantigen stimulated rabbit and human peripheral blood monuclear cell (PBMC) responses, as well as a study of the kinetics of inhibition by the two drugs. The interactions of the combined suboptimal concentrations of CsA and CsG were also studied to determine whether the effects of the two compounds on PBMC proliferation were synergistic, additive or antagonistic. Finally, the efficacy of CsA and CsG was studied *in vivo* by comparing the skin graft survival time in the CsA and CsG treated New Zealand White (NZW) rabbits.

IV. MATERIALS AND METHODS

A. *IN VITRO* STUDIES

1. IC_{50} and KINETICS OF INHIBITION

PBMC were isolated from the heparinized blood of healthy hospital personnel and NZW rabbits by Ficoll Hypaque density gradient centrifugation. The isolation of PBMC has been well described (117, 118), but minor modifications have been made in our laboratory. Briefly, human blood was diluted with an equal amount of saline at room temperature. Thirty milliliters of diluted blood was laid over twelve milliliters of Ficoll Hypaque (specific density = 1.076 g/ml) and centrifuged at 1500 rpm for 30 minutes. The lymphocyte layer was aspirated, washed three times with saline, counted, and resuspended at the indicated dilutions in complete medium which consisted of RPMI-1640 (Gibco, New York, U.S.A.) supplemented with 10% AB serum and 1% penicillin/streptomycin for human studies. For rabbit studies, minor modifications were made to maximize the amount of PBMC collected. Blood was drawn from the ear artery and centrifuged at 1500 rpm for 25 minutes. The buffy layer ie., the portion between the plasma and the red blood cells, was aspirated and diluted with equal amount of RPMI-1640. Eight milliliters of diluted blood were laid over 5

milliliters of Ficoll Hypaque and then centrifuged at 1500 rpm for 30 minutes. The lymphocytes were collected, washed three times with RPMI-1640 and resuspended in complete medium containing 10% Fetal Calf Serum (Bocknek, Canada) and antibiotics.

Assessment of immunosuppression was performed in both mitogen (human and rabbit) (119) and alloantigen (human only) stimulated cultures (102). All assays were performed in quadruplicate cultures in a final volume of 200 ul/well in 96-well round-bottomed microtitre plates (Becton Dickinson, New Jersey, U.S.A.).

a). Mitogen Stimulation: $1x10^5$ PBMC were cultured with either Concanavalin A (Con A; Sigma, St. Louis, MO, U.S.A), at a final concentration of 10 ug/ml or Phytohemagglutinin (PHA; Sigma), at a final concentration of 5 ug/ml in complete medium for 72 hours at 37°C in a 5% humidified atmosphere with a 4-6 hour terminal pulse of ^{3}H-thymidine (0.3 uCi/well, Amersham, U.S.A.). The cells were harvested onto glass-fiber filter paper, dried and assayed in a liquid scintillation counter (Cambridge Technology Inc., U.S.A.) to measure the incorporation of ^{3}H-thymidine as an index of proliferation.

b). Primary Mixed Lymphocyte Cultures (1° MLC): Responder PBMC ($1x10^5$ cells/well) were cultured with equal numbers of a

pool (10 donors) of irradiated (2500 rads, Atomic Energy Canada) stimulator PBMC for 6 days in complete medium with a 4-6 hour terminal pulse of ^{3}H-thymidine.

c). Secondary Mixed Lymphocyte Cultures (2° MLC): Responder PBMC at $1x10^{6}$ /ml were cultured with equal numbers of irradiated stimulator PBMC. Following a 6 day incubation, responder cells ($1x10^{5}$ /well) were restimulated with equal numbers of the original stimulator cells for a further 3 days including a 4-6 hour terminal pulse with ^{3}H-thymidine.

CsA or CsG at concentrations of 0.1 to 1000 ug/l were added to the respective well at the beginning of the culture. The percentage of inhibition of ^{3}H-thymidine incorporation after exposure to the drugs was calculated using the formula outlined below.

$$\text{PERCENT SUPPRESSION} = 1 - \frac{\text{CPM WITH DRUGS}}{\text{CPM WITHOUT DRUGS}} \times 100$$

The kinetics of inhibition of rabbit and human responses were investigated in the three assays described above. CsA or CsG at a dose which gave approximately 90-95 % suppression of

PBMC proliferation (ie., 250 ug/l of each drug for humans; 5 ug/l of CsA or CsG for rabbits) were added at time 0 (culture initiation), 2, 6, 18, or 24 hr for mitogen stimulation. For 1° MLC, CsA or CsG were added at day 0, or at 2 days, 4 days, or 5 days after culture initiation, and for 2° MLC the drugs were added at either 0, 1 or 2 days after initiation of the culture.

2. DRUG INTERACTIONS

The *in vitro* interactions of CsA and CsG were analyzed in mitogen- and alloantigen- stimulated PBMC responses. Three trials in quadruplicate using cells from one donor were performed. The IC_{50} of each drug in each assay was determined as described above. Once the 50% inhibitory concentration of each drug was established two fold dilutions of this dose e.g. 1/2 IC_{50} (CsA=35 ug/l, CsG=75 ug/l for PHA; CsA=1.05 ug/l, CsG=25 ug/l for 1° MLC; CsA=28.5 ug/l, CsG=42.5 ug/l for 2° MLC), 1/4 IC_{50} and 1/8 IC_{50} were added either alone or in combination to the culture. Concentrations of CsA used in combination with CsG ranged from 4.8-70 ug/l (PHA), 0.27-2.1 ug/l (1° MLC), 9.4-59 ug/l (2° MLC). The CsG concentrations ranged from 9.4-150 ug/l (PHA), 6.3-50 ug/l (1° MLC), 10.6-85 ug/l (2° MLC). Expected values were determined by adding together the values for immunosuppression observed when each

drug was given alone. The observed value was the actual amount of immune suppression detected in experiments when combinations of the two drugs was used.

B. *IN VIVO* STUDIES

The *in vivo* efficacy of CsA and CsG were investigated in rabbit skin allografts (73). Outbred male NZW rabbits weighing 2.5 to 3.0 kg were used as donors and recipients. Full thickness (2x2 cm^2) skin grafts were exchanged between pairs of anaesthetized rabbits. The grafts were sewn on all sides and dressed with gauze then covered with adhesive tape. Animals received CsA or CsG or drug vehicle (Cremophor EL and ethanol) intravenously via the marginal ear vein daily until the time of graft rejection. Rejection was defined as the day on which induration occurred on 100% of the skin allograft. Animals were housed in single cages and kept on pellet diet and water *ad libitum*. They were inspected daily for signs of allograft rejection. Animals were weighed once a week and blood collected weekly via the ear artery for measurement of trough drug concentrations. Blood concentrations of CsA and CsG were measured with a monospecific radioimmunoassay kit (Sandoz, Basel, Switzerland) or high performance liquid chromatography (120).

All animals were sacrificed by intravenous injection (via

the marginal ear vein) of lethal dose of anaesthetics or euthanol at the end of the study. Samples of skin tissues were formalin fixed and parafin embedded for light microscopic evaluation. The tissue sections were stained with hematoxylin and eosin kindly performed by Dr. Jim Gough of the Department of Pathology, Health Sciences Centre who was not aware of the treatments the animals received.

C. CYCLOSPORINE PREPARATIONS

Both CsA and CsG (OG-37-325) were kind gifts of Sandoz, Canada. For *in vitro* studies, the compounds were dissolved in 80% ethanol plus 20% Tween 80 and diluted with RPMI-1640 to obtain the desired concentrations. A diluent which contain 0.1% ethanol and Tween 80 but without drug was prepared for use as a control. The diluent at the highest concentration and comparable to the most concentrated cyclosporine sample did not inhibit the proliferation of the PBMC. The drug concentrations were confirmed by HPLC prior to further dilutions. For *in vivo* studies, both CsA and CsG were dissolved in Cremophor EL and ethanol (vehicle) to a concentration of 50 mg/ml. The drugs were further diluted in saline to yield the desired concentrations such that animals received a constant volume of the drug preparation (1.0 ml).

The anaesthesia used was a mixture of ketamine (50

mg/dose) plus xylazine (10 mg/dose) and acepromazine (7.5mg/ml).

D. STATISTICAL ANALYSIS

All data are presented as mean ± SEM.

Statistical differences between samples were sought using a two-way analysis of variance program (NCSS) or paired T tests. Non-parametric one-way analysis of variance (Kruskal-Wallis rank sum test) was used to test difference in the skin transplant experiments. A p value of less than 0.05 was considered statistically significant.

V. RESULTS

A. *IN VITRO* STUDIES

1. The Effect of CsA and CsG on Proliferation of human and rabbit PBMC. The effect of the drugs on the proliferation of human and rabbit PBMC in response to mitogens and alloantigens was evaluated. Both drugs inhibited the proliferation of PBMC in a dose-dependent manner. A typical dose response curve is shown in Figure 2. Maximum inhibition was achieved between 100 and 1000 ug/l. The fifty percent inhibitory concentration of CsA was 0.55 ug/l and 1.30 ug/l for CsG.

The IC_{50} of both drugs was determined in both human and rabbit systems. As shown in Table 1, significantly more (up to three times) CsG than CsA was required to inhibit proliferation e.g. the IC_{50} of CsG in Con A stimulated rabbit PBMC was 2.8 ± 0.5 ug/l compared to 0.9 ± 0.2 ug/l of CsA ($p \leq 0.02$). Likewise in humans, the IC_{50} of CsG required to inhibit the same mitogen response was 66.0 ± 20.0 ug/l compared to 23.0 ± 5.0 ug/l of CsA ($p \leq 0.05$). Similar findings were observed in alloantigen-induced human PBMC (IC_{50} 1° MLC CsA = 19.0 ± 4.0 ug/l vs IC_{50} CsG = 60.0 ± 7.0 ug/l $p \leq 0.05$ and for 2° MLC IC_{50} CsA = 18.0 ± 7.0 ug/l vs IC_{50} CsG = 35.0 ± 9.0 ug/l $p \leq 0.005$). Interestingly, the IC_{50}s of both drugs using rabbit

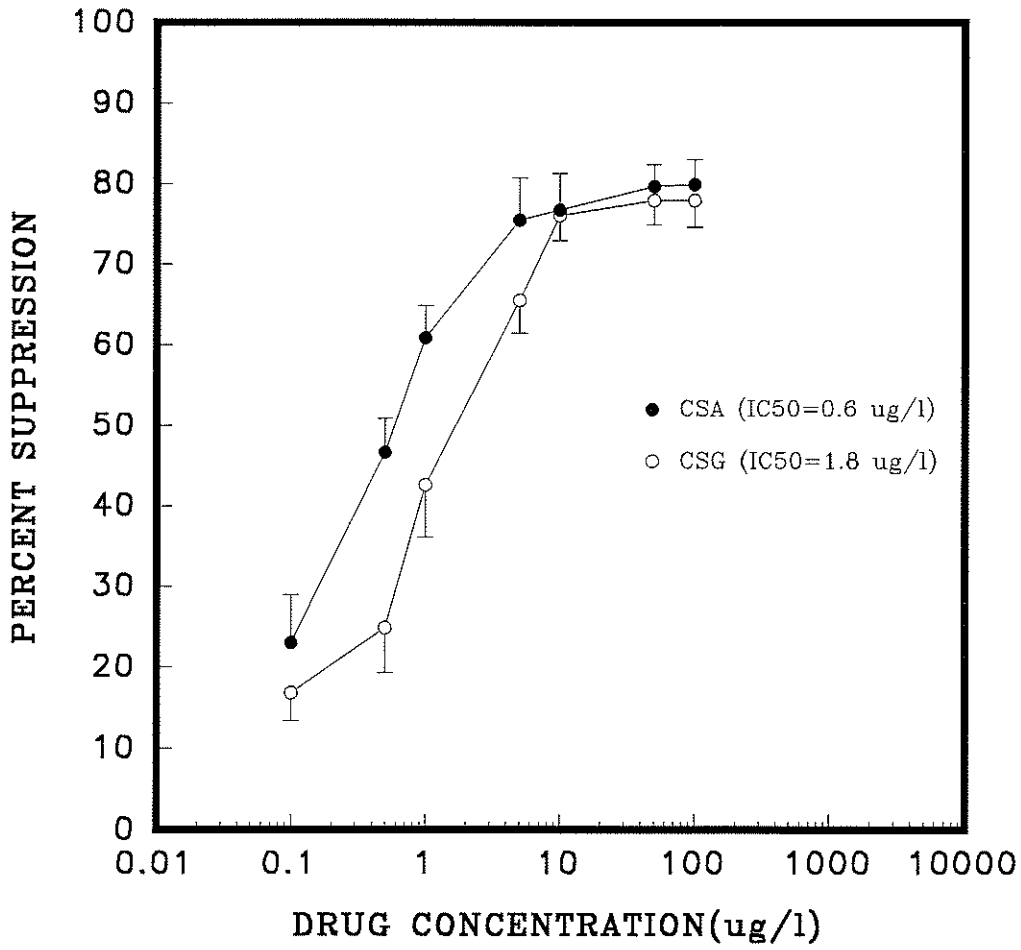
PBMC were much lower than those obtained in human experiments e.g. in PHA stimulated rabbit PBMC the IC_{50} of CsA and CsG were 1.3 ± 0.6 ug/l and 1.8 ± 0.4 ug/l (not significant) respectively compared to 24.0 ± 7.0 ug/l (CsA) and 69.0 ± 17.0 ug/l (CsG), $p \leq 0.01$ in human responses.

CsA and CsG were most effective when added early to mitogen stimulated cultures (Figure 3 and 4). Suppression of the responses was reduced by almost 50% (human cultures) or completely disappeared (rabbit cultures) if the drug was added 24 hours after culture initiation. Similar results were seen for alloantigen stimulated cultures (Figure 5 and 6) i.e. both CsA and CsG had to be added soon after culture initiation to suppress the response.

2. <u>The Effect of Combinations of CsA and CsG on Mitogen and Alloantigen Induced Responses</u>. In clinical transplantation, combining of immunosuppressants to increase their effectiveness in preventing graft rejection is a common practice. We attempted to mimic this *in vitro* by examining the effects of combinations of high, intermediate and low doses of CsA and CsG on the proliferation of human PBMC (Figure 7a, b, and c respectively). At higher doses of these drugs their combined effect eppeared to be antagonistic (Figure 7a) e.g. 35 ug/l CsA alone gave 49.4 ± 6.1 % suppression of the PHA response while 75 ug/l of CsG alone gave 31.8 ± 3.1%

suppression of the response. Thus the expected suppression of the drugs combined would be 81.2% if the effects of both drugs were additive. The actual result was 50.3 ± 4.2% suggesting that the two drugs were in fact antagonistic when used in combination. Similar results were seen for the 1° and 2° MLC. However, when lower doses of the combinations of CsA and CsG were used then the expected and observed suppression were similar suggesting that the effects were additive (Figure 7b and c).

Figure 2. The effect of CsA and CsG on the proliferation of rabbit PBMC. 50 ul of CsA or CsG (from 0.1 - 1000 ug/l) was added to $1x10^5$/well PBMC cultured in complete medium with 5 ug/ml PHA. After incubation at 37°C in 5% humidified atmosphere for 72 hours, proliferation was measured by ^{3}H-thymidine uptake. The percentage of inhibition was calculated as described in the Materials and Methods section.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DRUG CONCENTRATION(ug/l)
CSA (IC50=0.6 ug/l)
CSG (IC50=1.8 ug/l)

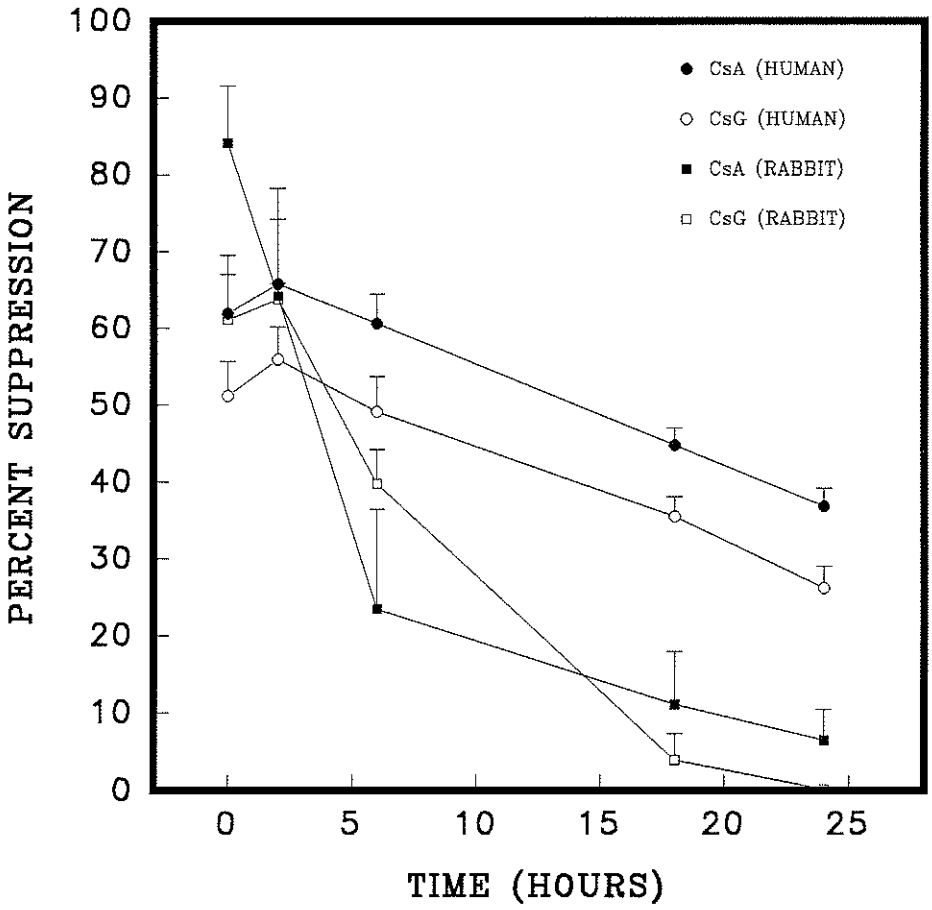
Table 1. Comparison of the fifty percent inhibitory concentrations (IC_{50}) of CsA and CsG on responses of human and rabbit lymphocytes to phytohemagglutinin (PHA), concanavalin A (Con A), in primary (1° MLC) and secondary (2° MLC) mixed lymphocyte cultures.

			IC_{50} (ug/l)		
STIMULUS	CELL SOURCE[a]	n	CsA	CsG	SIGNIFICANCE[b]
Con A	RABBIT	11	0.9 ± 0.2	2.8 ± 0.5	$p \leq 0.02$
PHA	RABBIT	11	1.3 ± 0.6	1.8 ± 0.4	NS
Con A	HUMAN	8	23 ± 5.0	66 ± 20.0	$p \leq 0.05$
PHA	HUMAN	8	24 ± 7.0	69 ± 17.0	$p \leq 0.01$
1° MLC	HUMAN	8	19 ± 4.0	60 ± 7.0	$p \leq 0.05$
2° MLC	HUMAN	8	18 ± 7.0	35 ± 9.0	$p \leq 0.005$

[a] PBMC ($1x10^6$ /ml) were cultured with either mitogen (human and rabbit; 3 days) or alloantigens (human only; 6 days) at 37 °C in the presence of varying doses of CsA and CsG.

[b] Significant differences between the IC_{50} of CsA and CsG were sought by paired T- tests.

Figure 3. Kinetics of inhibition of PHA induced proliferation by CsA and CsG. Results are from 4 separate experiments. Rabbit or human PBMC were stimulated with PHA (5 ug/ml) for 3 days. CsA or CsG (5 ug/l in rabbit experiments and 250 ug/l in human experiments) was added to cultures at 0, 2, 6, 18 or 24 hours after culture initiation. Proliferation was measured by ^{3}H-thymidine uptake.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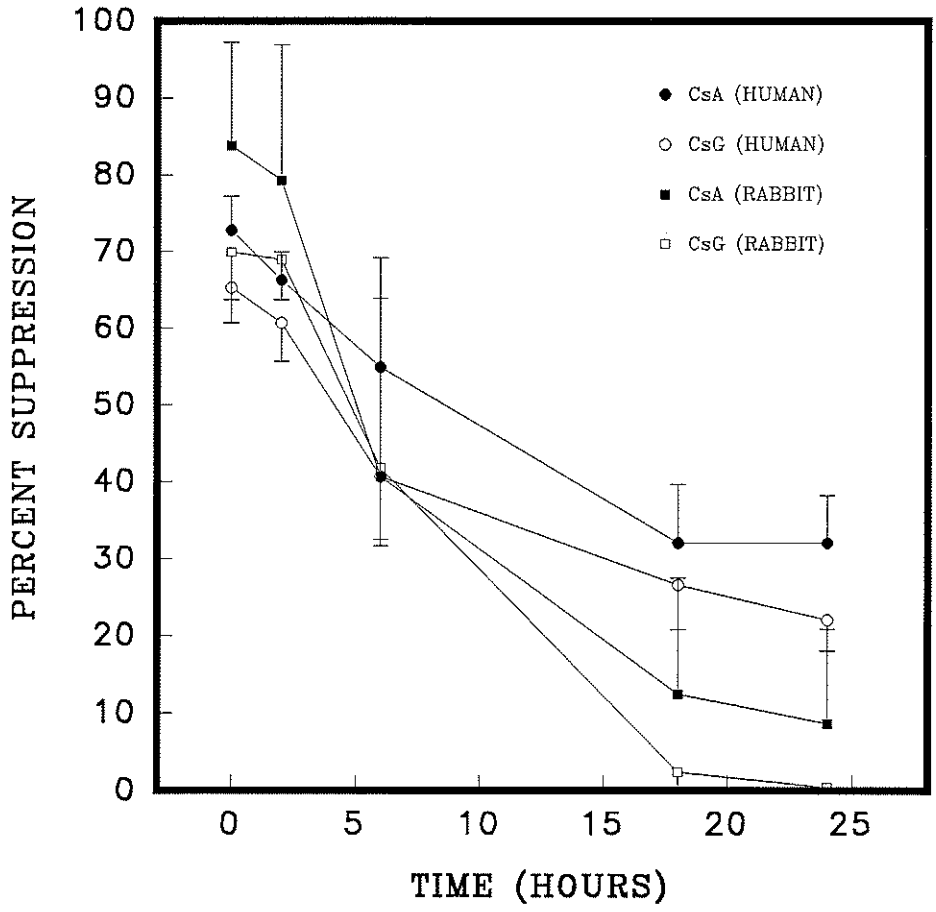
TIME (HOURS)
CsA (HUMAN)
CsG (HUMAN)
CsA (RABBIT)
CsG (RABBIT)

Figure 4. Kinetics of inhibition of Con A stimulated PBMC by CsA and CsG in 4 rabbits and 4 volunteers. PBMC ($1x10^6$/ml) were cultured with Con A (10 ug/ml) for 72 hours in complete medium. CsA or CsG was added to cultures at 0, 2, 6, 18 or 24 hours after culture initiation.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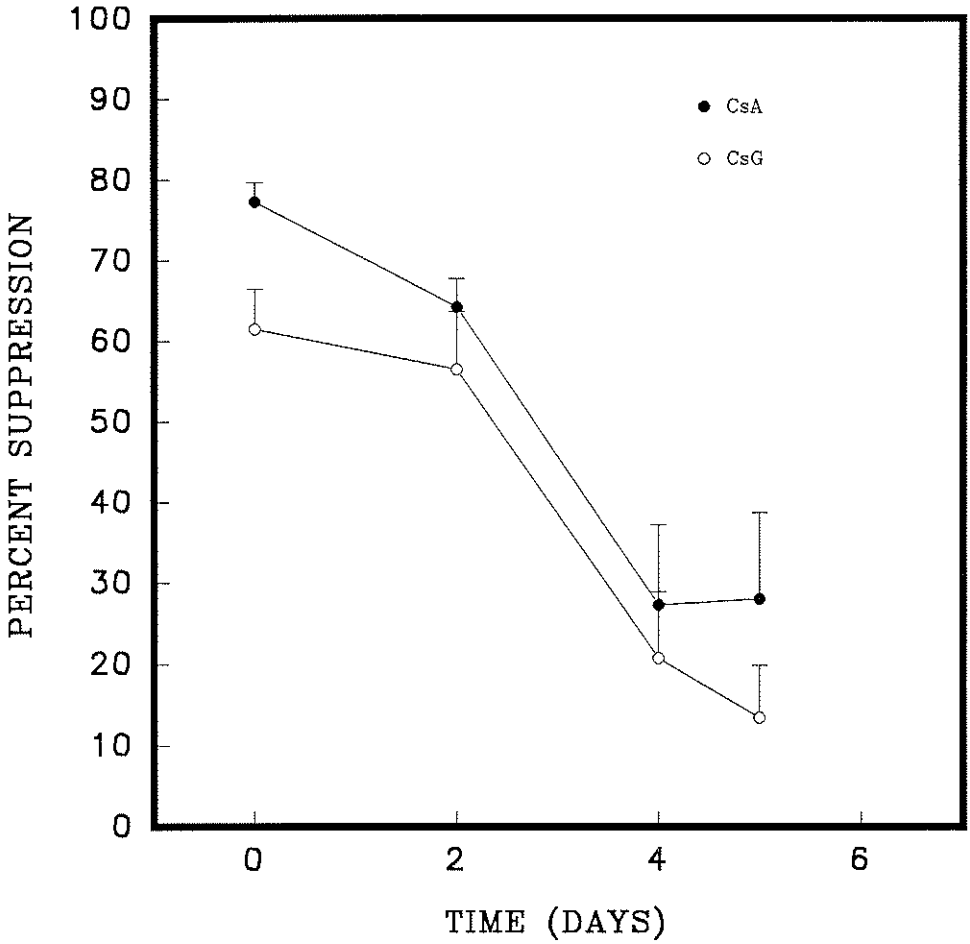
CsA (HUMAN)
CsG (HUMAN)
CsA (RABBIT)
CsG (RABBIT)

Figure 5. Kinetics of inhibition of CsA and CsG in primary mixed lymphocyte culture (1° MLC). Each point represents the results of 4 independent experiments. Human PBMC ($1x10^5$/well) were cultured with an equal number of irradiated stimulator cells and incubated at 37°C in 5% humidified atmosphere for 6 days. CsA or CsG were added on day 0, 2, 4 or 5.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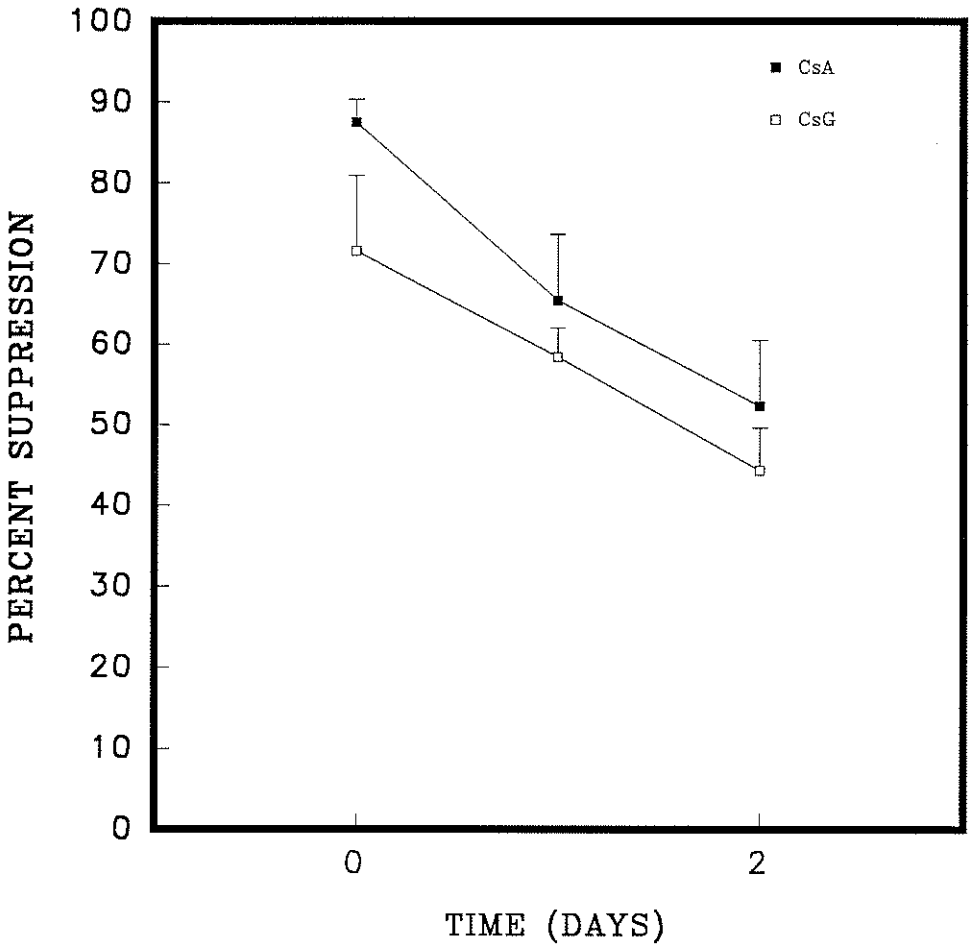
TIME (DAYS)

Figure 6. Kinetics of inhibition of CsA and CsG in secondary mixed lymphocyte culture (2° MLC). After stimulation of human responder cells by equal number of irradiated stimulator cells for 6 days, $1x10^6$/ml responder cells were restimulated again with irradiated stimulator cells for an additional 3 days. CsA or CsG was added to the cultures at 0, 1 or 2 days after culture initiation. Results are from 4 experiments in different individuals.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CsG
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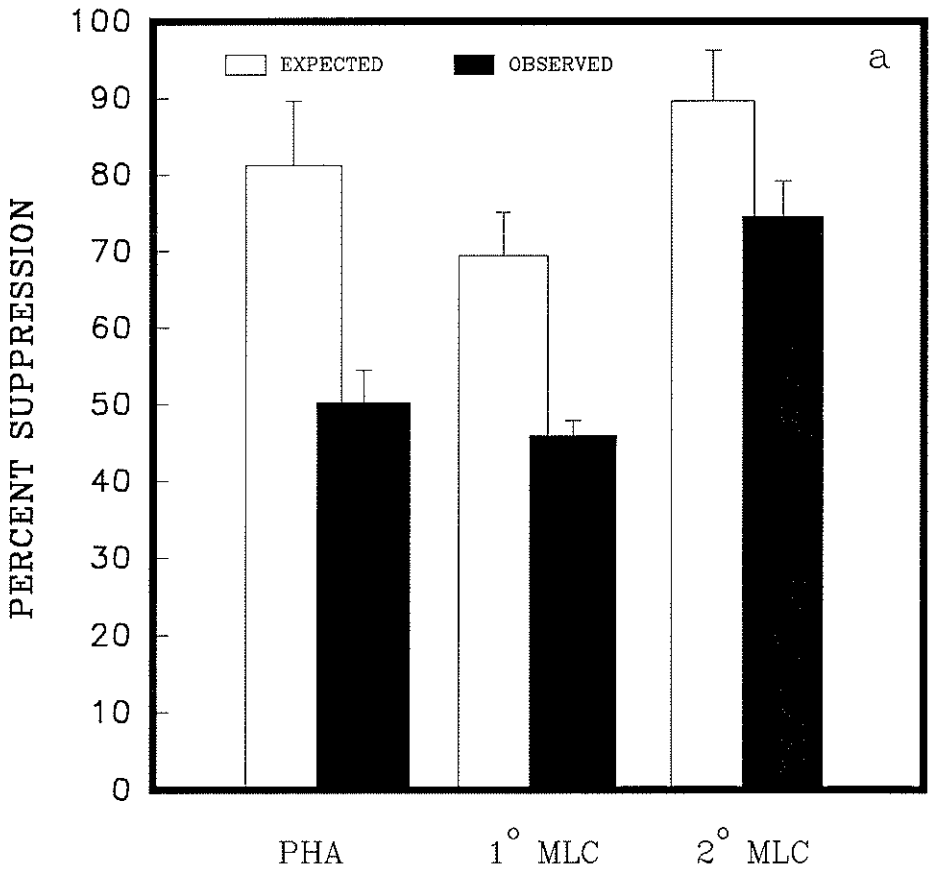
TIME (DAYS)

Figure 7. Effect of combinations of CsA and CsG on the inhibition of mitogen and alloantigen responses. Expected was the sum of the percentage of inhibition of two drugs when added alone to the cultures, and observed was the percentage of inhibition of CsA and CsG when added together to the cultures. Each point represents the mean ± SEM of 3 separate experiments. Experiments were performed with combinations of CsA and CsG expected to yield high (7a), intermediate (7b) and low (7c) amounts of suppression.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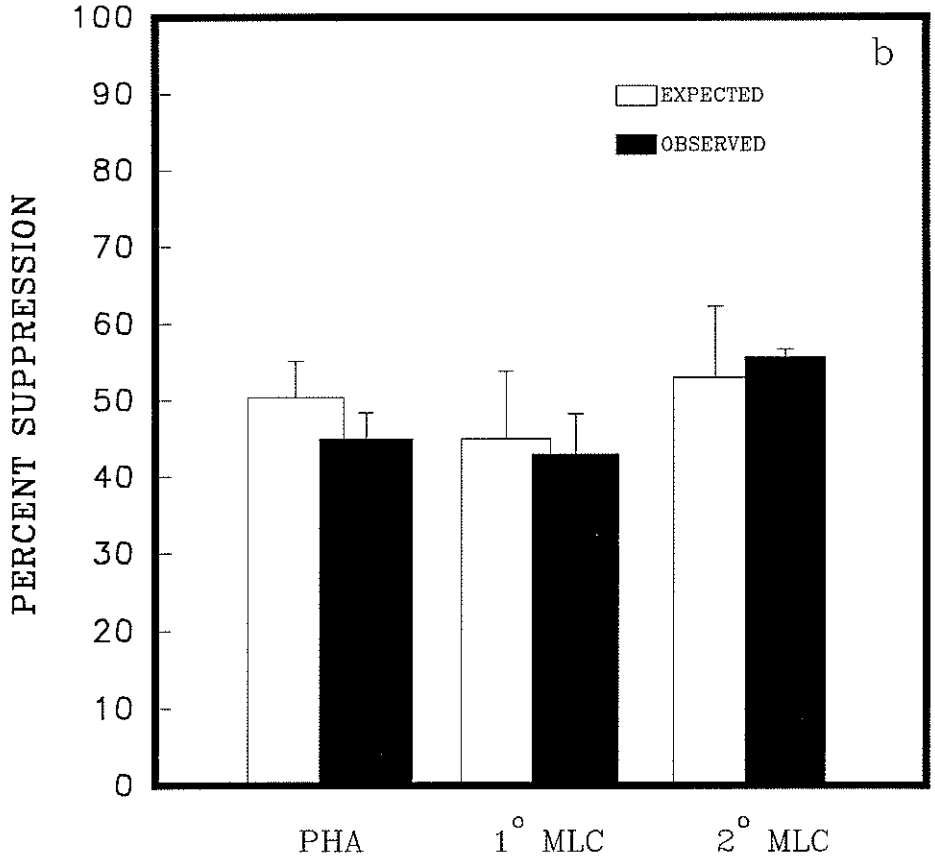
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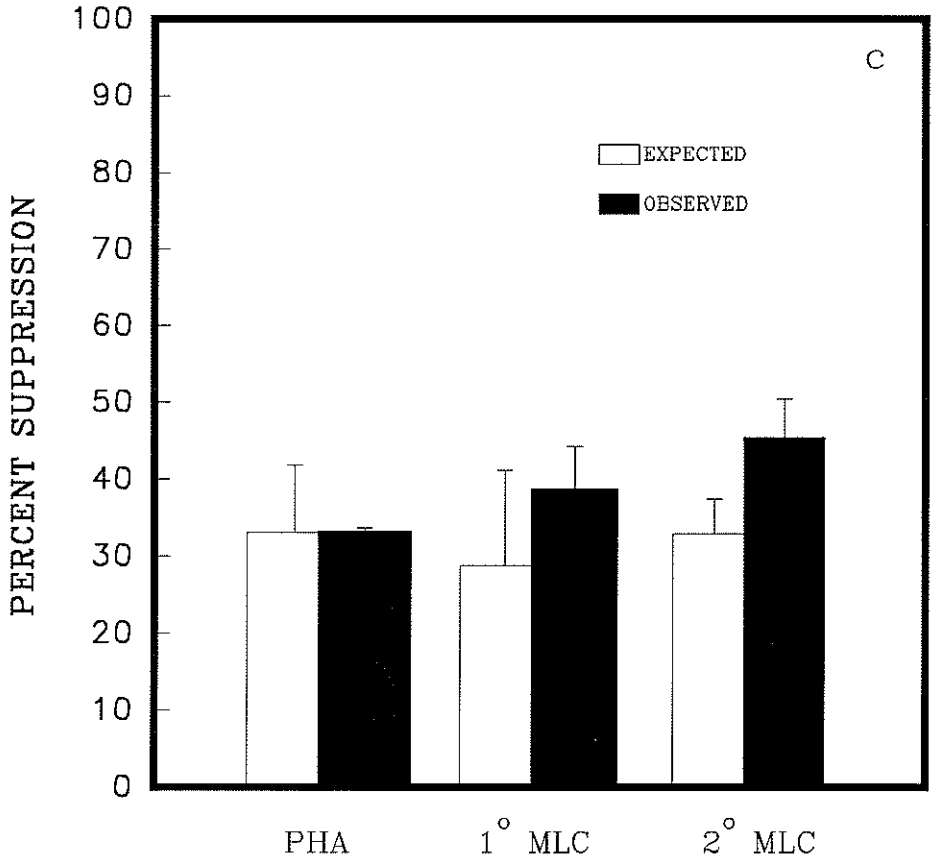
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B. *IN VIVO* STUDIES

1. The Effect of CsA and CsG on Skin Graft Survival. We next compared the effects of CsA and CsG on skin graft survival in rabbits. Rejection of the transplanted skin grafts occurred in all animals in the control group (MST ± SEM = 10.6 ± 1.7 days). Examples of rejected grafts are shown in Figures 8 and 9.

Both CsA and CsG significantly prolonged skin graft survival in rabbits compared to control animals (Figure 10) when given at 5, 10 (CsA, CsG) or 15 (CsG) mg/kg/day. All animals in the control group lost their grafts by day 15 whereas rabbits treated with CsA or CsG kept their grafts beyond this point. However at both 5 and 10 mg/kg CsA was significantly more efficacious than CsG at equivalent doses ($p< 0.05$). Even when animals received 15 mg/kg/day of CsG they did not achieve the same mean survival time compared to rabbits receiving CsA at 10 mg/kg/day (ie., 15 ± 1.9 days for 15 mg/kg CsG vs >20.5 ± 6.5 days for 10 mg/kg CsA). We also monitored the trough blood levels of each drug. It can be seen from Table 2 that animals receiving the same dose of CsG as CsA had lower trough levels of drug (e.g. at 5 mg/kg/day CsG trough level was 36.2 ± 5.3 ug/l versus CsA; 46.2 ± 12.1 ug/l). However, even when the trough levels of CsG were three times higher than CsA we still did not find that CsG was as

effective as CsA. During these experiments we noticed that 5 rabbits in the 10 mg/kg/day CsA treated group sufferd greater than 10% weight loss (12-23%) (data not shown). This was not observed in the CsG treated animals. Two rabbits receiving CsA (10mg/kg/day) died before the end of these experiments as did 2 animals treated with CsG at 15 mg/kg/day. Cause of death was not clear; no autopsies were performed.

Figure 8. Skin grafts on NZW rabbit. Skin grafts (2x2 cm^2) were placed on the shaved back of the animal and inspected daily for signs of rejection. The skin allograft in (a) has started to become indurated as indicated by its dark red color and hard texture. This was a rabbit in the control group 7 days post transplant. A rejected skin graft is shown in (b). This graft was on another rabbit in the control group at 14 days post transplant. The dark red color of the graft has given way to black with rejection. Notice the sutures on one side have started to come off. The graft in (c) was from a rabbit in the 10 mg/kg/day CsA group which retained its graft until the end of the experimental period. Notice hair growing back on the graft. Photo was taken on day 25 post transplant.

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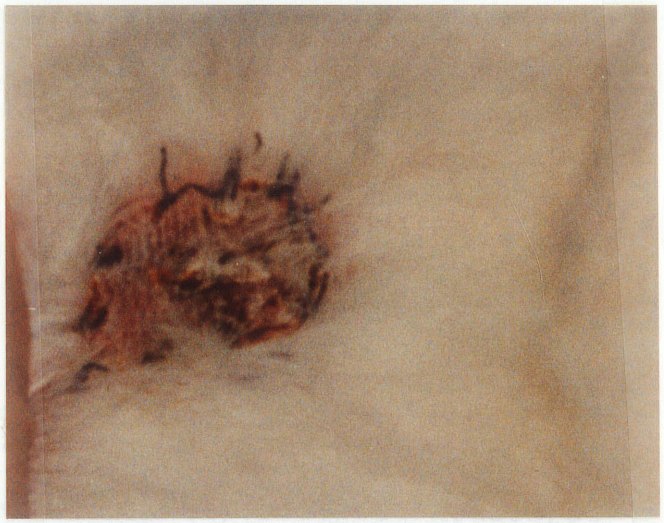

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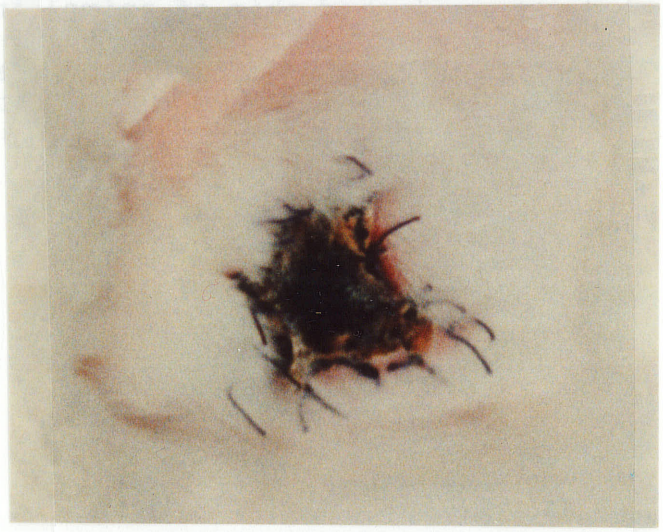

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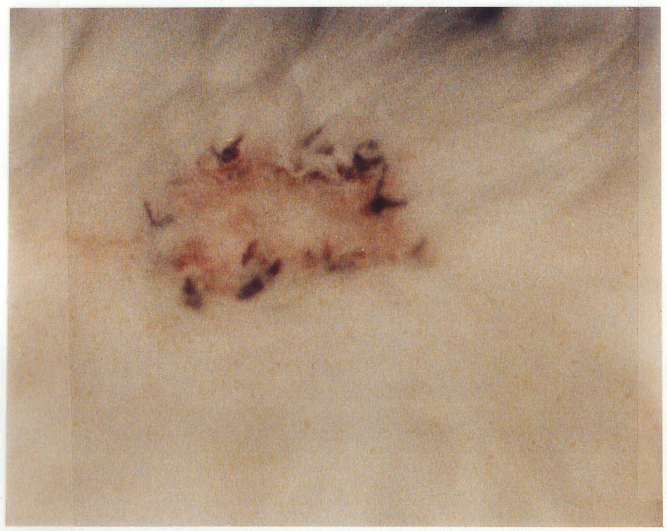

Figure 9. Hematoxylin and eosin sections of skin allografts and normal skin of rabbit. Samples of skin tissue were collected at the end of the study period (i.e. 30 days post transplant) or on the day graft rejection occurred.(a) Mononuclear cell infiltrate in the rejected skin of animal in the control group. (b). Section of normal untouched rabbit skin. Note the absence of mononuclear cells. (c). A skin graft taken from an animal who had not rejected the graft (30 days post transplant). This rabbit received 10 mg/kg/day CsA.

a.

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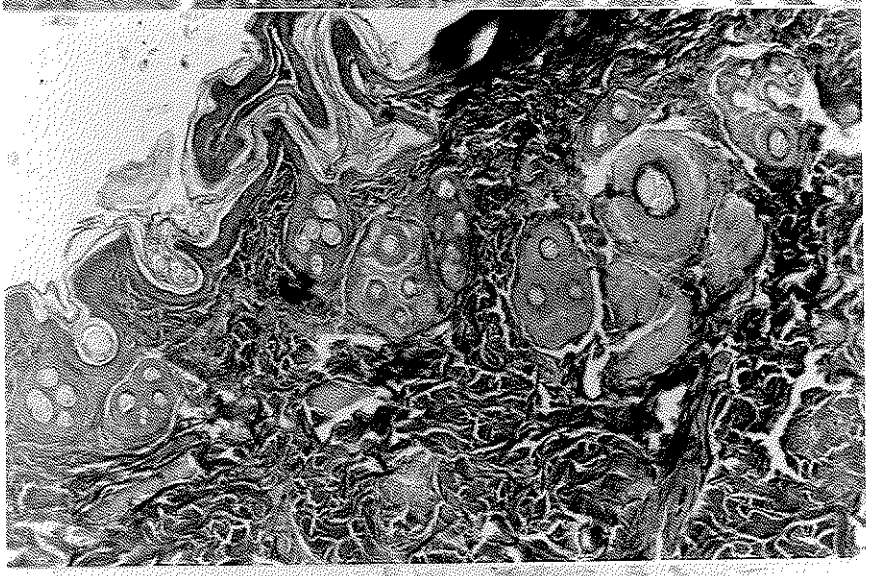

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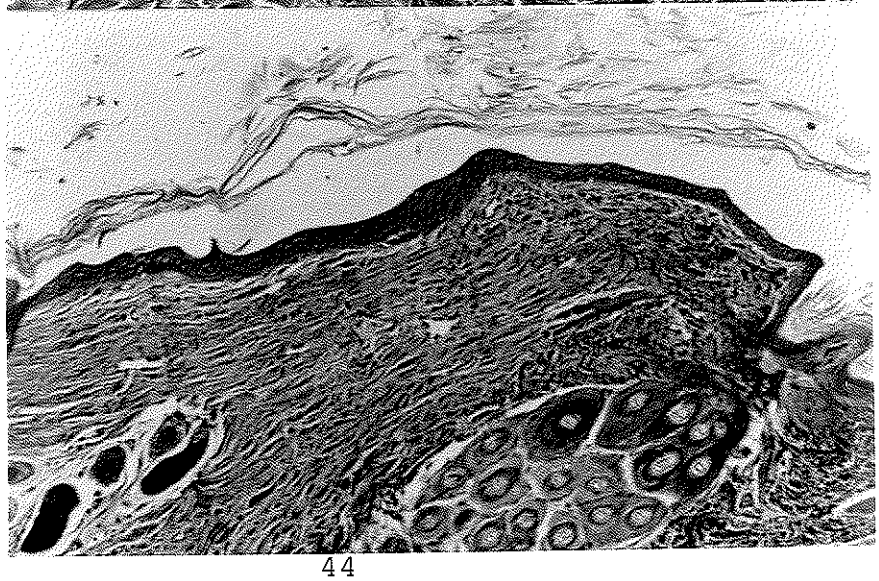


Table 2. Comparison of the skin graft survival times and cyclosporine trough concentrations in rabbits given varying doses of CsA or CsG.

DOSE[a] (mg/kg/day)	n	SURVIVAL TIMES (DAYS)	MST[b] ±SEM	TROUGH LEVELS[c] (ug/l)
CONTROL	14	8,9,9,10,10,10, 11,11,12,12,12, 10,15,10	10.6±1.7	
2.5 CsA	3	10,12,16	12.7±2.5	45.0±1.0
2.5 CsG	3	13,14,18	15.0±2.2	<25
5 CsA[d]	9	10,14,15,>25,>25, >30,17,20,14	>18.9±6.2	46.2±12.1
5 CsG	9	12,12,13,14,16, 25,17,18,19	16.2±3.9	36.2±5.3
10 CsA[e]	11	13,>30,>30,>25,>26 ,12,13,14,21,18,23	>20.5±6.5	89.6±20.0
10 CsG	10	16,17,19,13,14, 15,18,17,17,15	16.1±1.8	77.9±8.0
15 CsG	4	13,14,15,18	15.0±1.9	98.0±9.8

[a] CsA and CsG were injected iv until rejection of the graft. Control animals received the drug vehicle (cremophor EL and ethanol).

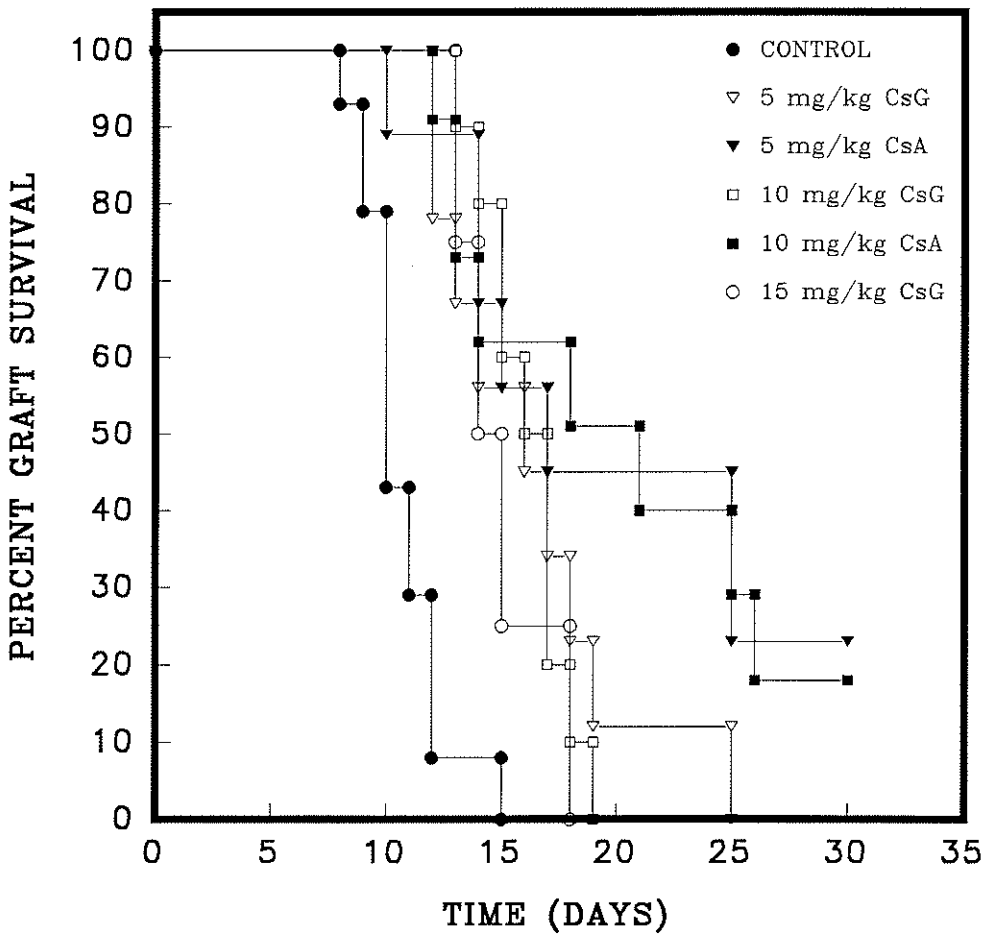
[b] Mean survival time (days).

[c] 24-hour CsA or CsG whole blood trough levels (mean ± SEM) were measured by HPLC.

[d] $p \leq 0.01$ vs 5 mg/kg/day CsG.

[e] $p \leq 0.01$ vs 10 and 15 mg/kg/day CsG as determined by Mann-Whitney U-test.

Figure 10. Skin graft survival of rabbits treated with CsA or CsG. Full thickness skin grafts were exchanged between outbred NZW rabbits. Rabbits received either 5 mg/kg/day, or 10 mg/kg/day (CsA or CsG) or 15 mg/kg/day (CsG only) intravenously. Grafts were inspected daily and rejection was defined as the day on which 100% induration of the graft occurred.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TIME (DAYS)
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5 mg/kg CsG
5 mg/kg CsA
10 mg/kg CsG
10 mg/kg CsA
15 mg/kg CsG

C. SUMMARY

1. *IN VITRO* STUDIES

i. Both CsA and CsG were immunosuppressive.

ii. In rabbits, the IC_{50} of both drugs was much lower compared to humans.

iii. More CsG than CsA was required to inhibit mitogen-induced responses of human and rabbit PBMC.

iv. More CsG than CsA was required to inhibit alloantigen-induced proliferation of human PBMC.

v. Both drugs were most effective in inhibiting proliferation of PBMC when added at the initial stages of cell activation.

vi. CsA and CsG combinations appeared to be antagonistic at high but not low drug doses.

2. *IN VIVO* STUDIES

i. Both CsA and CsG significantly prolonged skin graft survival in rabbits.

ii. At the same dose, CsA was significantly more efficacious than CsG in prolonging graft survival.

iii. Trough levels of CsG tended to be lower than CsA in rabbits receiving similar drug doses.

iv. At comparable trough levels of both drugs CsA was significantly more efficacious in prolonging skin graft survival.

VI. DISCUSSION

In 1985 Hiestand and colleagues (2, 3) first demonstrated the immunosuppressive potency of CsG. They also reported that this compound exerted fewer side effects than CsA in rats. At equivalent doses, CsG showed no detrimental effects on kidneys, blood pressure and liver of these animals compared to CsA. This finding made CsG a potential substitute for CsA in clinical transplantation. In this study we compared the immunosuppressive activities of CsG and CsA both *in vitro* (rabbit and human cells) and *in vivo* (skin allografts in rabbits). Our studies *in vitro* showed that CsG was less effective than CsA in inhibiting both rabbit and human responses. The IC_{50} for CsG was up to three times greater than that of CsA for both mitogen and alloantigen-induced lymphocyte proliferation. Our results are similar to the findings of Calne *et al.* (4), where they found that dose for dose, CsG was less potent than CsA in inhibiting mitogenesis of human lymphocytes *in vitro*. These results also confirmed our previous study where we found that more CsG than CsA was required to inhibit alloantigen-induced cytokine production (102).

The IC_{50} of both drugs required to inhibit mitogen-induced rabbit immune responses was much less than the IC_{50} for humans which suggests that rabbits may be more sensitive to the

cyclosporines than humans. It has been reported in both *in vitro* and *in vivo* studies that there is a differential sensitivity among species to the effects of both CsA and CsG (17, 121).

Our finding that significantly more CsG than CsA was required to compete with radiolabelled CsA for binding to PBMC may explain in part the reduced immunosuppressive potency of CsG compared to CsA (Rayat G, Yatscoff R, Silverman R, and McKenna R, in press). There are at least two possibilities to explain these findings, (1) that CsG does not cross the cell membrane as readily as CsA or (2) that CsG has a lower binding affinity for its intracellular ligand which is presumably cyclophilin (41). The almost identical structure of CsA and CsG would suggest that these two drugs bind the same immunophilin in the cell. It is also possible that CsA not only competes with CsG for interaction with functionally relevent binding site(s) within the cells, but it may also be able to displace CsG from the majority of such binding site(s).

The results from the *in vitro* kinetic studies confirmed our previous observations (102) that CsG like CsA must be added early to cultures to effect maximum suppression suggesting that CsG also acts at an early stage of T cell activation.

We attempted to examine possible interactions i.e.

synergy, additivism, or antagonism between the two drugs when used in combination. We found that at high doses of the drugs less immunosuppression than expected was observed. The reasons for this are not clear as presumably both drugs will bind the same immunophilin. It is possible that at higher doses of the two drugs, CsG has occupied all of the binding sites since the CsG concentration was at least three times more than CsA to get comparable immunosuppression. Therefore, the inhibition of PBMC proliferation observed was probably due to the action of CsG only and not to the combined effect of the two drugs since both drugs would compete for binding to cyclophilin. Another possibility may be attributed to a qualitative difference, that would not be detectable by a whole cell binding assay. This is the distribution of CsG within the cells where CsG would be translocated to a very minor binding component and could not be removed by CsA. Similar findings for two other immunosuppressive drugs, rapamycin (122, 123) and FK-506 (which bind to the same FK binding protein) have also been observed (124, 125). The macrolide rapamycin (molecular weight = 913 daltons) is the closest known structural analogue of FK-506 and synergistic in effect with CsA (14). It is considerably more potent than CsA in suppressing organ allograft rejection in animals (126, 127) and at least 10 times more potent than CsA in inhibiting murine thymocyte proliferation driven by PHA and IL-1 (127). Unlike CsA and FK-

506 rapamycin did not inhibit IL-2 production, but inhibited the responses of T cells (128). Such differential effects of FK-506 and rapamycin are surprising, given the high degree of similarity in their chemical structures. The functional disparities between FK-506 and rapamycin are also particularly intriguing in view of the recent finding that both immunosuppressants bind equally well to the intracellular protein FKBP. However, the fact that rapamycin behaved as a powerful antagonist of the inhibitory effects of FK-506 on programmed cell death (apoptosis) of T cells (129) and IL-2 production and IL-2 driven proliferation (125) suggests that the two compounds share a common site within the cell. This has important clinical implications which might mean that certain combinations of immunosuppressive drugs should not be used to enhance graft survival.

In vivo we also found CsG to be less immunosuppressive than CsA. At equivalent doses of the drugs, better graft survival was observed in the CsA-treated animals compared to animals treated with CsG. In the majority of *in vivo* studies, CsG was found to be equipotent to CsA in the prevention of allograft rejection, although in other studies a lesser potency has been reported. The reasons for these conflicting data is not known. It could possibly reside in the variation of dosage and route of administration of drug as well as species and organ transplantation differences from study to

study. The apparent discrepancy between this study and previous reports that concluded that CsG and CsA were equipotent might be explained by the relative overdose used in other studies. It might be argued that in order to obtain similar graft survival, equivalent trough levels of the drugs would be required assuming the CsA and CsG have the same mechanism of action. However, we found even with similar and up to three times higher trough levels (e.g. Table 2, 5 mg/kg/day CsA versus 15 mg/kg/day CsG) that CsA was still better at prolonging graft survival. These studies confirm similar findings in a variety of animal species which compared the efficacy of CsA and CsG including heart transplants in cynomolgus monkeys, and heart, lung and renal allografts in rats (5-8). In this study and in other studies where we compared the pharmacokinetics of the 2 drugs, rabbits administered the same dose of CsA and CsG would consistently have lower trough levels of CsG due in part to a more rapid clearance of CsG from the blood (105, 130, Honcharik N, Yatscoff R, manuscript in preparation). This may explain in part the difference in the efficacy of the two drugs in prolonging survival of the skin grafts. Other animal studies have suggested that the efficacy of CsG *in vivo* correlates with the ability to obtain high levels of drug in the blood and that CsG could be as effective as CsA at preventing graft rejection when higher blood levels of CsG than CsA were

attained (5, 6, 8-10, 19, 103). White *et al.* (103) found that *in vitro* the immunosuppressive activity of CsA was consistently greater mg per mg than CsG, however *in vivo* assessment in dogs with renal transplants showed that CsG and CsA were immunosuppressively equipotent. This apparent contradiction may be explained by the substantially higher blood levels of CsG in these animals. In contrast to this finding, Grant *et al.* (9) found that CsG was a less immunosuppressive agent than CsA in Mongrel dogs despite higher serum concentrations. It appears that there are both species and strain differences in the absorption, metabolism and excretion of CsA and CsG. The conflicting levels of CsG may also be due to differences in the medium used for analysis (plasma vs whole blood). It has been shown that the blood/plasma ratio of CsG is smaller(1.23) than that observed for CsA (1.5) (131). This suggests that although the whole blood concentration of the drug would be similar, the plasma concentration of CsG would be higher than that of CsA. The whole blood distribution of CsG also differs from CsA. It appears that CsG is more tightly bound to erythrocytes than CsA thus requiring longer incubation times at 37°C for plasma-erythrocytes re-equilibration of the drug to occur (132). Some of the discordance in the results in monitoring CsA and CsG blood/plasma concentrations may also be related to the method of analysis. CsA and CsG have been measured by HPLC, in which

the parent drugs are quantitated, and by RIA, in which both parent drugs (specific) and a number of metabolites (non-specific) are measured (133). While increased absorption could account for higher CsG blood concentrations, its poor water solubility (similar to CsA) makes improved absorption of CsG over CsA unlikely. Altered distribution of CsG, due to variable binding to red cells or plasma lipoproteins, or differences in hepatic drug clearance, are equally likely to have produced higher CsG than CsA blood concentrations.

Nephrotoxicity caused by the cyclosporines was not investigated in this study. Data presently available are limited and the toxic effects of CsG have not been well established, hence it is difficult to speculate on 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 (131). In NZW rabbits treated with 2.5 or 5 mg/kg/day for 30 days of CsA or CsG, CsG was found to be less nephrotoxic than CsA (134). From the data presented in this study, the therapeutic index of CsG would probably be much higher than CsA. Although CsG appears to be less immunosuppressive than CsA, it may yet be a valuable drug for, if CsG has few side effects in humans as it seems to have in rabbits, its dosage could be safely raised above those used in

this study. Alternatively, its effect could be potentiated with low doses of other drugs such as azathioprine, corticosteroids or even rapamycin. Whether CsG acts as specifically as CsA does remains elusive and future research will show if CsG is another step forward in the prevention of allograft rejection.

VII. CONCLUSION

In summary, we conclude that CsG is less efficacious than CsA both *in vitro* (humans and rabbits) and *in vivo* (rabbit skin allograft). This may be due at least in part to a reduced binding of CsG to their target immune cells as well as a more rapid clearance from the blood. Thus the clinical use of CsG would be dependent upon its possessing much less toxicity than CsA.

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