A comparison of cyclosporine A and cyclosporine G in a rabbit heterotopic cardiac transplant model: graft outcome and histological findings

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Summary. Cervical heterotopic heart transplants were performed on 20 male New Zealand white rabbits comprising 4 treatment groups. Animals in each group were injected daily via the marginal ear vein and received one of the following regimes: Cyclosporine A, 10 mg/kg/day; Cyclosporine G, 15 mg/kg/day; cremophor-El, 3ml/day; or normal saline. Measurement of 24 hour trough blood concentrations revealed no significant differences between the average concentrations of Cyclosporine A and Cyclosporine G. Animals were examined daily and the cervical allografts assessed by palpation for viability/rejection. The duration of the study ended for each animal when the graft stopped beating at which time the animals was euthanized and the transplanted heart and native kidneys harvested and processed for light microscopy evaluation of rejection and drug toxicity, respectively.

Graft survival in the Cyclosporine A group significantly surpassed that seen in the Cyclosporine G group as well as the control groups, whereas in animals treated with Cyclosporine G, graft survival was not different from controls. In the native kidney, there were no differences in glomerular tuft area or volume density amongst drug-treated or control animals. In contrast, tubule atrophy and interstitial fibrosis were markedly greater in Cyclosporine A-treated vs Cyclosporine G-treated animals.

The results of this study indicate that, whereas Cyclosporine G is less nephrotoxic than Cyclosporine A, given equivalent blood concentrations Cyclosporine A delays rejection of a cardiac allograft significantly longer than Cyclosporine G in this animal species.

Key words: Cyclosporine, Heart, Kidney, Morphology

Introduction

Cyclosporine A (CSA) is the most widely used immunosuppresive agent in clinical transplantation. However, the use of CSA is limited by a number of side effects, most notably nephrotoxicity (Myers, 1986; Keown et al., 1987). Recognition of CSA toxicity has led to a search for other agents as effective as CSA but less toxic. One such compound, Cyclosporine G (CSG), a natural analogue of CSA, was proposed by Hiestand et al. (1985) who noted that CSG lacked nephrotoxic side effects in the rat, and moreover, was as effective as CSA in preventing rejection of heart and renal allografts in this species. CSG differs from CSA at the number two amino acid position where nor-valine replaces alpha-aminobutyric acid. The mechanisms of action are similar for both drugs and involves inhibition of interleukin-2 (IL-2) production and release. Both drugs have been shown to inhibit other lymphokines as well (Borel, 1989). Variability in the pharmacokinetics of CSG has been noted between different species (Grant et al., 1987a; Venkataramanan et al., 1987; Dsouza et al., 1988; Faraci et al., 1988). In the rabbit, the half-life of CSG after intravenous injection has been shown to be shorter than that for CSA (Dsouza et al., 1988; Lukowski, 1991). It has been recommended, therefore, that for purposes of comparison, a slightly higher dose of CSG should be administered to achieve 24-hour trough levels equivalent to CSA (Dsouza et al., 1988).

Both CSA and CSG have been administered by various notes. Intravenous administration delivers CSA and CSG most effectively into the systemic circulation (Shah et al., 1988), but peak levels decrease rapidly (Dsouza et al., 1988). Intraperitoneal administration results in a pharmacokinetic pattern similar to intravenous with high peaks and low troughs (Findon and Miller, 1988). With oral administration, absorption is low and incomplete (Ptachinski et al., 1985; Shah et al., 1988), as bioavailability can be as low as 5%. In contrast, bioavailability with subcutaneous

administration is 60%, and variation between peak and trough levels is minimal (Findon and Miller, 1988).

Since the study of Hiestand et al. (1985) a number of species, including the rat (Hoyt et al., 1985; Prop et al., 1987; Grant et al., 1987b), have been used in the evaluation of the immunosuppressive efficacy of CSG, namely the rabbit (Rayat et al., 1993), dog (Calne et al., 1985; Todo et al., 1986; White et al., 1986), and primate (Ogunnaike et al., 1987), using various types of allografts, e.g., skin (Rayat et al., 1993), kidney (Calne et al., 1985; White et al., 1986; Grant et al., 1987b), heart (Hoyt et al., 1985; Ogunnaike et al., 1987; Prop et al., 1987) and liver (Todo et al., 1986). Although both agents were reported nephrotoxic, CSG was touted as being less nephrotoxic than CSA, based on the results of studies using rodents (Hoyt et al., 1985; Collier et al., 1986; Masri et al., 1987; Tejani et al., 1988). The differences in nephrotoxicity reported in these studies were minor and in other studies using larger animal species, namely the dog and monkey, no differences could be detected (Todo et al., 1986; White et al., 1986; Ogunnaike et al., 1987). Some reports suggest that CSG may produce more hepatotoxicity than CSA (Calne et al., 1985; Faraci et al., 1988).

The rabbit has been used to evaluate CSA more recently and it appears to be a promising model for studying nephrotoxicity (Thliveris et al., 1991, 1994). In this species, CSA has been shown to induce structural and functional changes characteristic of the chronic CSA nephrotoxicity seen in humans namely arteriolopathy, tubule atrophy, vacuolization of proximal tubule cells, mononuclear infiltrates and interstitial fibrosis (Thliveris et al., 1994). These lesions have not been found consistently using other animal models. Establishment of an animal model which demonstrates a consistent histopathologic response to CSA would facilitate studies attempting to identify the mechanisms involved in producing chronic CSA nephrotoxicity.

A practical advantage of using a rabbit model is that it is large enough to allow the performance of a cervical cardiac transplant without the use of magnification. A cardiac allograft as opposed to hepatic or renal, simplifies the monitoring of graft rejection since daily palpation is all that is required. Cessation of graft function in a technically successful cardiac transplant can be confidently attributed to rejection since cardiotoxicity is not a significant problem with most immunosuppressive agents, while deterioration of renal or hepatic allografts may be due to rejection or drug toxicity. Placement of a graft in the neck imparts less of a physiologic insult than placement in the abdomen and, most importantly, does not result in paraplegia, whereas this complication is common with an abdominal approach in this species (Mitchell et al., 1989).

The objectives of the present study are: (1) to compare CSA and CSG, based on their abilities to prevent allograft rejection in a rabbit heterotopic cardiac transplant model when both drugs are maintained at similar blood concentrations, and, (2) to compare the toxicities of these drugs in this model.

Materials and methods

Cardiac allografts from smaller (1.0-2.0 kg) donors were transplanted into the necks of 20 male New Zealand white (NZW) rabbits (3.0-4.0 kg) using the technique described by Heron (1971), and modified in our laboratory (Fryer et al., 1993).

Cardiac allografts which stopped beating within 72 hours of surgery were considered technical failures and were therefore eliminated from the study. Otherwise allografts were palpated daily until study end points i.e. (1) allograft rejection (cessation of beating), (2) animal death, or (3) severe generalized toxicity, at which time living animals were sacrificed by way of a lethal injection of Euthanyl. All animals underwent a post mortem examination which included removal of allografts and native kidneys for histology. Excised cardiac allografts were processed for light microscopy and sections stained with H&E, examined under a microscope, and scored using the histologic grading system recommended by the International Society for Heart Transplantation (Billingham et al., 1990). Kidneys were similarly prepared and stained with H&E and were examined for mononuclear cell infiltrates, tubular atrophy, arteriolopathy and interstitial fibrosis and scored semiquantitatively on a scale of 0-4+ (absent, minimal, moderate or severe; respectively) for tubule atrophy and interstitial fibrosis as previously described (Thliveris et al., 1991). Additional sections were stained with periodic acid-Schiffs (PAS) for the detection of arteriolopathy. Quantification of glomerular tuft area and volume density was determined using a ZIDAS (Zeiss Interactive Digital Analyses System, Carl Zeiss, Oberkocken, Germany) image analysis system. To rule out misinterpretation of pathological changes seen in the kidney due to indigenous microorganisms, in this instance *Encephalitozoon cuniculi*, potentially present in control and immune-suppressed animals, blood was obtained at the time of sacrifice and subjected to serologic testing, i.e., indirect immunofluorescent antibody and ELISA (Institut Armand-Frappier, Laval, Quebec, Canada).

Drug administration

CSA, CSG and their vehicle cremorphor-EL were obtained as gifts from Sandoz Pharmaceuticals Corporation. The 20 recipients were divided into 4 treatment groups with each animal receiving a daily injection. Intravenous dose of CSA, CSG, vehicle or normal saline were injected into the marginal ear veins. Treatment groups were: (1) CSA 10 mg/kg (n=5), (2) CSG 15 mg/kg (n=5), (3) cremopohor-EL 3 ml (n=5), and (4) normal saline 3 ml (n=5). Doses were selected for their anticipated, resultant blood levels, based on knowledge of the pharmacokinetics of CSA and CSG in the rabbit (Dsouza et al., 1988; Rayat et al., 1993).

Laboratory monitoring

Blood samples were obtained preoperatively for baseline assessment and 24-hour trough drug levels were subsequently determined weekly. CSA and CSG levels were determined from whole blood using reverse-phase high pressure liquid chromatography (HPLC) (Copeland and Yatscoff, 1988). Interassay coefficient of variation using this method is less than 10%. Serum was analyzed for sodium, potassium, chloride, total CO_2, urea, creatinine, bilirubin, total protein, albumin, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) as per routine procedure in the Clinical Chemistry Laboratory of the Health Sciences Centre. Animals were placed in metabolic cages for 24-hour urine collections preoperatively and subsequently at biweekly intervals for determination of creatinine clearance. Body weights were determined preoperatively and then weekly.

Statistical analysis

Statistical assessment was performed using analysis of variance, analysis of covariance and Kaplan-Meier estimates using a log rank test, where appropriate.

Results

The average 24-hour trough blood concentrations of animals administered CSA and CSG are shown in Figure 1. The CSG levels tended to be slightly higher than those obtained with CSA, but this was not statistically significant.

Graft survival in the CSA group greatly surpassed that seen in the CSG group (p=0.003) as well as the controls (p<0.003) (Fig. 2). Graft survival in the CSG group was not statistically different from controls.

Changes occurring in creatinine clearance during the course of the study were determined in each of the
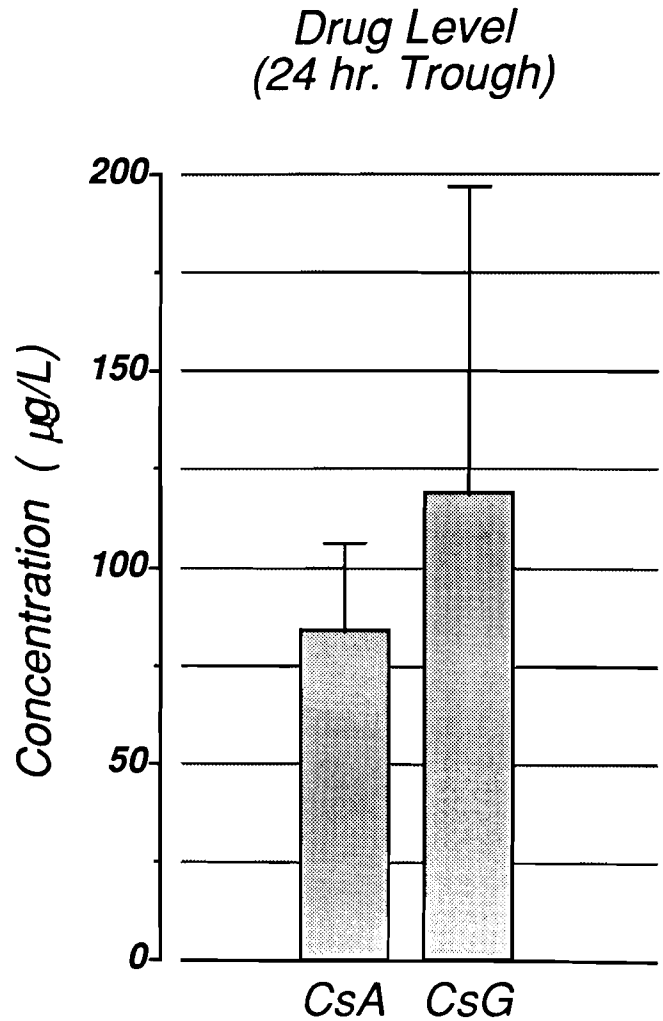

Fig. 1. Average 24 hour trough levels for CSA and CSG (µg/L±SD) in male New Zealand white rabbits receiving CSA intravenously (IV) 10 mg/kg/day and CSG (IV) 15 mg/kg/day.
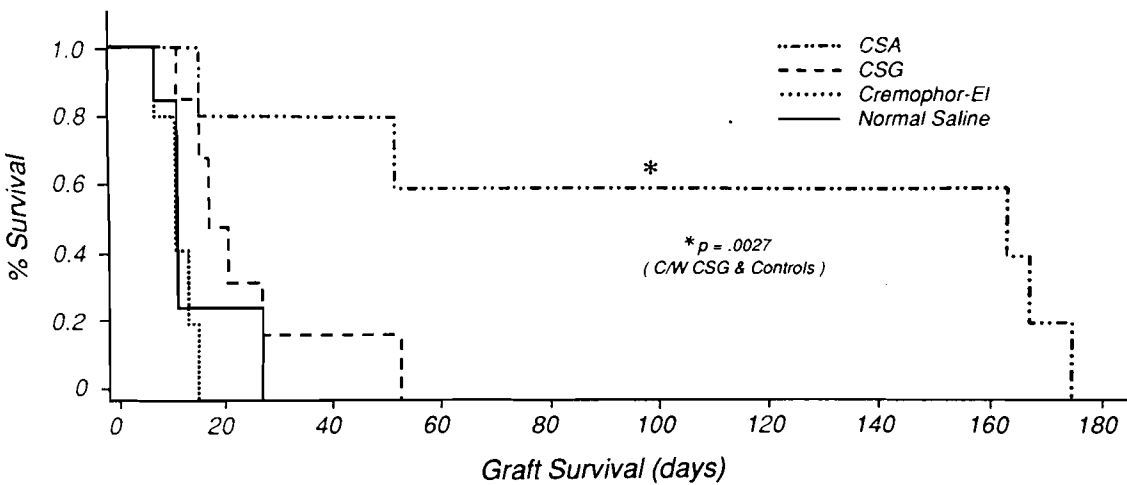

Fig. 2. Survival distribution of cardiac allografts in rabbits receiving CSA (IV) 10 mg/kg/day, CSG (IV) 15 mg/kg/day, cremophor-El (IV) 3 ml/day, and normal saline (IV) 3 ml/day.

treatment groups (Fig. 3). Although the creatinine clearances in the groups given CSA and CSG decreased and those in the control groups increased, no significant changes between drug treated animals and controls could be demonstrated (p>0.1115), nor was there a significant

difference between CSA and CSG. Since some of the change in creatinine clearance may be attributed to dehydration or to muscle wasting, both of which should correlate with weight loss, a co-variant analysis using weight change and creatinine clearance change was performed but again no significant difference between

Table 1. Cardiac allograft survival and histology.

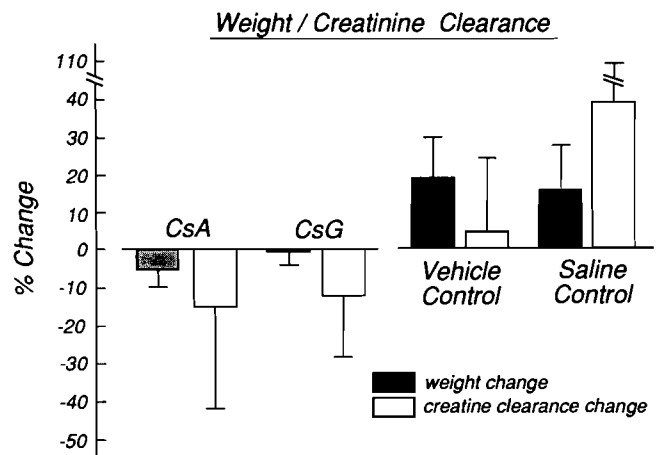| GROUP	GRAFT SURVIVAL (days)	REJECTION SCORE
CSA	13	3B
	165	3A
	173	3A
	162	3A
	49	3B
CSG	19	4
	26	3B
	15	3B
	14	3A
	51	3A
Normal saline control	9	4
	10	4
	10	3B
	25	4
	7	4
Cremophor control	7	4
	10	4
	9	4
	13	4
	11	4

Fig. 3. Percentage change in average creatinine clearance and average body weight (% change from baseline ± SD) during the study in rabbits receiving CSA (IV) 10 mg/kg/day, CSG (IV) 15 mg/kg/day, cremophor-El (IV) 23 ml/day, or normal saline (IV) 3 ml/day.
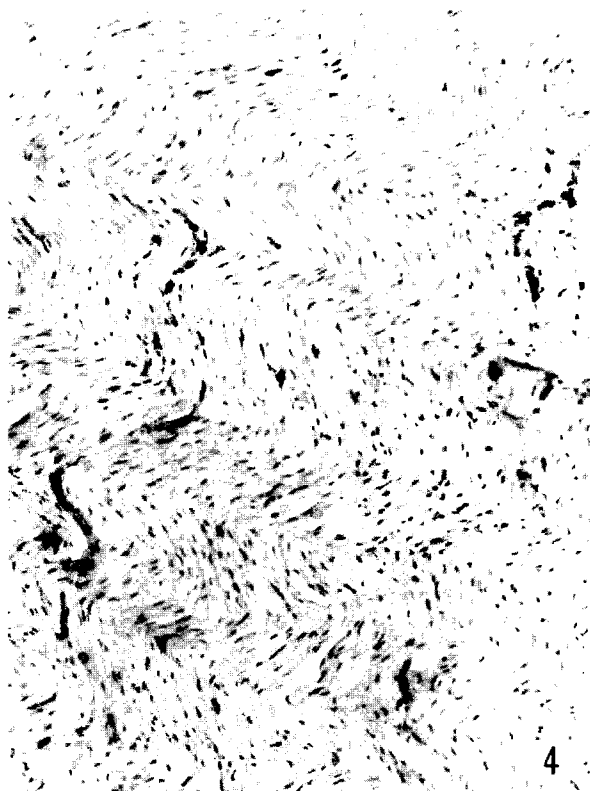

Fig. 4. Micrograph of typical myocardial fibers from a native heart of a saline control animal. H&E. x 500

Fig. 5. Micrograph of allograft heart from a saline control animal. Note marked inflammatory infiltrate (arrows) and myocyte damage. H&E. x 500

CSA and CSG was demonstrated.

No significant changes in sodium, potassium, chloride, total CO_2, urea, creatinine, bilirubin, total protein or albumin occurred during the course of the study in any of the treatment groups. Changes in the liver enzymes (data not shown) were noted, however, namely increases in ALT ($p<0.01$) compared with vehicle and normal saline controls in both groups of cyclosporine treated animals and an increase in ALP ($p<0.05$) in the CSG group compared with controls.

An analysis of cardiac allograft histology (Figs. 4-6) revealed that all animals whose allografts stopped beating at a point beyond 72 hours following surgery, had a rejection score of 3A or greater (Table 1).

Renal morphology for the native kidneys is shown in Table 2 and Fig. 7. As can be seen, there were no differences in glomerular tuft area or glomerular tuft volume density among the four groups of animals. In contrast, statistical significance was found only in the animals receiving CSA but not CSG in terms of tubule atrophy and interstitial fibrosis. This was due to the fact that four of five CSA-treated animals showed these lesions, whereas they were seen in only one of five animals receiving CSG. Arteriolopathy was also noted in all lesion-positive animals, was focal and affected only a few arterioles. Results of serological testing for opportunistic pathogens were negative for all animals in the study.

Table 2. Morphological assessment (mean±SD) of kidneys from rabbits treated with cyclosporine A, G, vehicle or saline.

GROUP	GLOMERULAR TUFT AREA (mm^2 x 10^{-3})	GLOMERULAR TUFT VOLUME DENSITY (mm^3 x 10^{-2})	TUBULE ATROPHY*	INTERSTITIAL FIBROSIS*
CSA	6.91±1.21	4.46±0.53	1.32±0.82**	1.75±0.96**
CSG	6.35±0.66	4.65±1.05	0.35±0.55	0.51±0.46
Vehicle	5.57±0.83	5.58±0.82	0	0
Saline	6.38±0.60	5.10±0.80	0	0

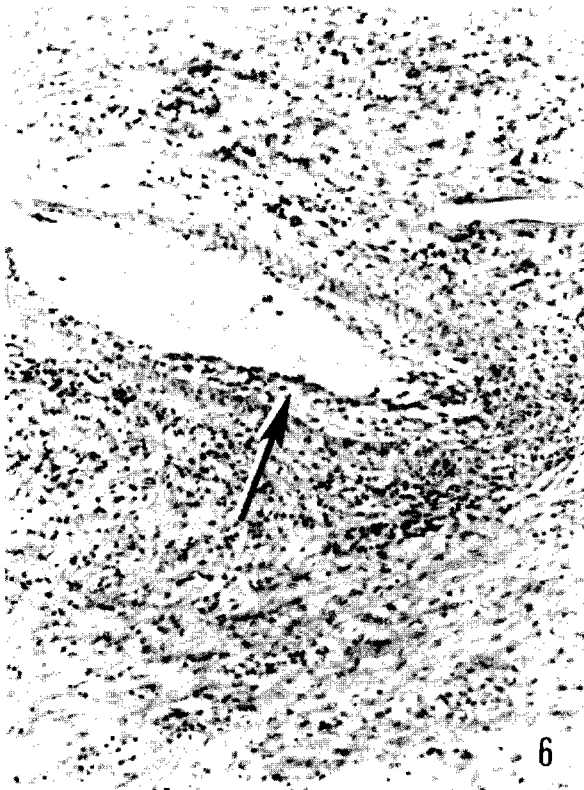*: semiquantitation score 0-4; **: $p<0.05$ greater than animals receiving CSG and controls.

Fig. 6. Micrograph of allograft heart from a saline control animal. showing an example of vasculitis (arrow). H&E. x 500
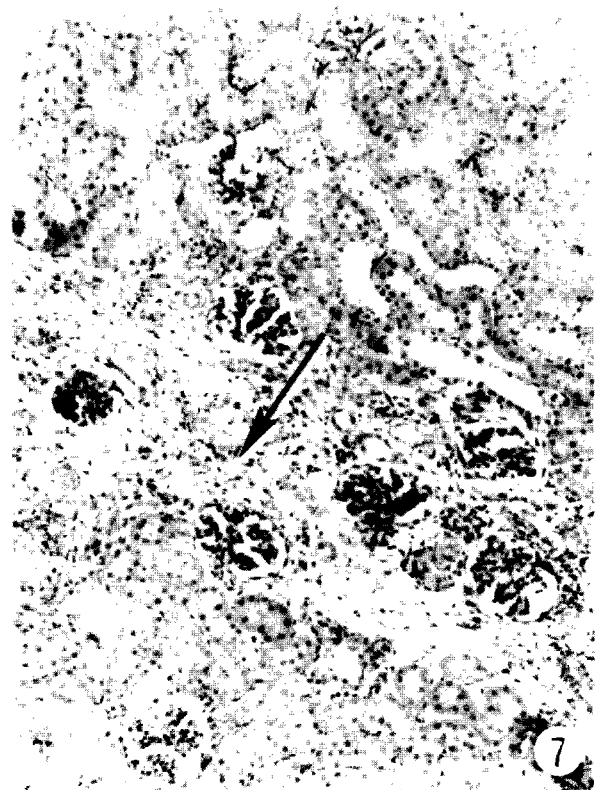

Fig. 7. Micrograph of renal cortex from a CSG-treated animal receiving a heterotopic heart transplant. Note loss of histological integrity and the presence of interstitial fibrosis (arrow). H&E. x 500

Discussion

Graft survival in the CSA treated animals significantly surpassed that occurring in the CSG treated group. Only one of six allografts in the CSG group survived beyond 30 days. No statistically significant difference was shown to exist between CSA and CSG blood levels. The average CSG level was only slightly higher than that seen for CSA. The similarity of the levels in these groups indicates that the selection of a CSG dose of 15 mg/kg/day for comparison with CSA 10 mg/kg/day was therefore reasonable. The fact that the average CSG level was slightly higher than the average CSA level reinforces the suspicion that at comparable blood levels, CSG is less effective than CSA in its ability to prevent allograft rejection in this particular animal model. In a parallel study (unpublished observations), in which animals were administered CSA or CSG subcutaneously, much higher blood levels were obtained for each drug, but not without significant adverse effects. Severe generalized toxicity necessitating euthanasia occurred in most animals. Despite this, graft survival in the viable animals given CSA subcutaneously was similar to that seen with the animals administered the drug intravenously. In animals given CSG subcutaneously, graft survival was much shorter compared with CSA (p<0.001) but was better than controls (p<0.02). Furthermore, in preliminary work in our laboratory (Lukowski, 1991), it was noted that concentrations of CSG in the heart, liver, and kidney were significantly lower (p<0.05) when compared to tissue CSA levels in CSG and CSA treated animals, respectively. This finding indicates that lower tissue concentrations of CSG can exist despite equivalent blood levels and this may have played a role in CSG's inferior ability to prolong allograft survival in the present study. These observations support those reported from a number of studies (Hoyt et al., 1985; Grant et al., 1987b; Ogunnaike et al., 1987; Prop et al., 1987) but they contradict the results of others (Calne et al., 1985; Hiestand et al., 1985; Todo et al., 1986; White et al., 1986). Interspecies variability (Calne et al., 1985; Grant et al., 1987a; Dsouza et al., 1988; Faraci et al., 1988; Borel, 1989) may be largely responsible for the differences noted and, therefore, one should use caution in comparison of data between species.

One study which found CSG to be equally effective to CSA in its ability to prevent allograft rejection did not report drug levels (Hiestand et al., 1985) while others achieved CSG levels significantly greater than the CSA levels in the comparison group (Calne et al., 1985; Todo et al., 1986; Grant et al., 1987b), and noted no significant increase in renal toxicity with these higher levels. What is needed is a detailed study comparing CSA with CSG at equal blood concentrations as well as with incremental increases in CSG levels to the point of toxicity, to clarify how high CSG concentrations can be increased in the pursuit of improving immunosuppression without causing a greater degree of toxicity than that seen with CSA.

Administration of either CSA and CSG resulted in decreases in creatinine clearance which were not significantly different from controls. A factor which must be considered is the length of time that animals were receiving each of the drugs. Since animals administered CSG rejected earlier, they received less drug and therefore were at less risk of developing chronic nephrotoxicity, yet there was no statistically significant difference between the decreases in creatinine clearance incurred with CSA and CSG. These findings are consistent with those of most other investigators (Calne et al., 1985; Todo et al., 1986; White et al., 1986; Ogunnaike et al., 1987). Some studies have evaluated the relative alterations in renal function produced by CSA and CSG using methods more sophisticated than creatinine clearance. Tejani et al. (1988) determined glomerular filtration rate (GFR) using inulin and renal plasma flow (RPF) using para-aminohippuric acid and found significant reductions in these parameters with CSA but not with CSG. Drug levels were not reported in this study nor were the immunosuppressive efficacies of the doses used evaluated. Paller and Ferris (1987) compared CSA and CSG in acute and chronic studies by measuring inulin clearance and calculating GFR and renal blood flow (RBF), and concluded that CSG had adverse effects on GFR and RBF similar to those seen with CSA. Two recent studies in man (Huser et al., 1992; Henry et al., 1993) were contradictory with respect to renal toxicity on the part of CSG. Both studies noted that CSG was as effective in CSA in promoting kidney graft survival. However, Henry et al. (1993) reported less nephrotoxicity by CSG as determined by serum creatinine and GFR, whereas Huser et al. (1992) noted that CSG induced the same degree of histological damage as CSA. Moreover, both of these studies noted hepatotoxicity on the part of CSG, but not by CSA; liver enzymes were persistently elevated in those patients administered CSG.

Renal histopathologic changes consistent with chronic cyclosporine nephrotoxicity were noted in rabbits receiving CSA (4 of 5 animals) as well as those receiving CSG (1 of 5 animals) but not in control animals. In previous studies from our laboratory, lesions were seen at 30 days following daily treatment with CSA and CSG (Lukowski, 1991; Thliveris et al., 1991) but not before this time. This finding was corroborated in the present study where lesions were not noted in animals rejecting prior to 30 days. Moreover, in a recent study from our laboratory (Thliveris et al., 1995) we noted that tubule atrophy and interstitial fibrosis were markedly greater (p<0.02) in CSA-treated versus rapamycin-treated animals subjected to heterotopic heart transplantation. In contrast there were no differences in glomerular tuft area or tuft volume density. In addition, in this study drug efficacy with respect to heart allograft viability was similar between CSA and rapamycin.

In summary, CSG is less effective than CSA in preventing cardiac allograft rejection in the rabbit model

given the comparable 24-hour trough levels achieved in this study. With higher levels of CSG, results may differ from those seen in the present study. Additional animals studies as well as extended clinical trials are necessary to further evaluate CSG's immunosuppressive potential and associated nephrotoxicity.

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