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Pharmacokinetic Interactions Augment Toxicities of Sirolimus/Cyclosporine Combinations

HEMANGSHU PODDER,* STANISLAW M. STEPKOWSKI,* KIMBERLY L. NAPOLI,* JAMES CLARK,† REGINA R. VERANI,† TING-CHAO CHOU,‡ and BARRY D. KAHAN*

**Department of Surgery, Division of Immunology and Organ Transplantation, and †Department of Pathology and Laboratory Medicine, The University of Texas Medical School at Houston, Houston, Texas; and ‡Molecular Pharmacology and Therapeutics Program, Memorial Sloan-Kettering Cancer Center, New York, New York.*

Abstract. This study correlated the dynamic effects of sirolimus (rapamycin; RAPA) and cyclosporine (CsA) alone *versus* in combination to produce renal dysfunction, myelosuppression, or hyperlipidemia, with their corresponding blood and tissue concentrations. After salt-depleted rats were treated with RAPA (0.4 to 6.4 mg/kg per d) and/or CsA (2.5 to 20.0 mg/kg per d) for 14 d, the GFR, lipid levels, bone marrow cellularity, and CsA/RAPA concentrations in whole blood *versus* liver or renal tissues were measured, and the median effect model was used to discern the type of drug interactions. Compared with vehicle controls (1.98 ± 0.34 ml/min), GFR values were reduced only by large doses of drug monotherapy, namely RAPA (3.2 mg/kg per d = 1.2 ± 0.02 ml/min or 6.4 mg/kg per d = 1.3 ± 0.2 ml/min; both $P < 0.01$) or CsA (10.0 mg/kg per d = 1.2 ± 0.1 ml/min or 20.0 mg/kg per d = 0.8 ± 0.4 ml/min; both $P < 0.01$). In contrast, hosts that were treated with smaller doses of CsA/RAPA combinations showed more pronounced effects in reduction of GFR values: 2.5/0.4 mg/kg per d, modestly (1.5 ± 0.5 ml/min; $P < 0.01$); 5.0/0.8 mg/kg per d, moderately (0.23 ± 0.01 ml/min; $P < 0.001$); and higher-dose groups, markedly. The exacerbation of renal dysfunction seemed to be due to a pharmacokinetic interaction of RAPA to greatly increase CsA concentrations in whole blood and, particularly, in kidney tissue. In contrast, the pharmacodynamic effects of CsA to potentiate two RAPA-mediated toxicities—myelosuppression and increased serum cholesterol/low-density lipoprotein cholesterol—occurred independently of pharmacokinetic interactions. RAPA aggravates CsA-induced renal dysfunction owing to a pharmacokinetic interaction, whereas CsA produces a pharmacodynamic effect that augments RAPA-induced myelosuppression and hyperlipidemia.

The development of agents that produce synergistic immunosuppression with the calcineurin antagonists (CNA)—cyclosporine (CsA) or tacrolimus—is based on the hypothesis that drug-dose sparing mitigates renal dysfunction, as well as the pleiotropic neural and hepatic toxicities associated with CNA administration (1,2). Furthermore, the addition of a synergistic drug may reduce the adverse impact of the large variations in the pharmacokinetic (3,4) and pharmacodynamic (5) behavior of CsA among renal transplant recipients. Although the addition of sirolimus (rapamycin; RAPA) to a CsA-based regimen has fulfilled the expectations of synergistic immunosuppression (6,7), randomized, blinded trials document inferior renal function, compared with CsA/azathioprine (Aza)/prednisone (Pred)- or CsA/placebo/Pred-treated patients (8,9). However, CsA and RAPA show a pharmacokinetic interaction that is due, at least in part, to common metabolism by cytochrome P450 3A4. Therefore, the present study used an animal model to dissect the pharmacokinetic from the pharmacodynamic components of the toxicity produced by CsA/RAPA combinations.

Although the exact mechanisms of CsA-induced nephrotoxicity are not understood fully, important components include increased vascular resistance, which produces decreased renal blood flow (10,11); generation of reactive free radicals, which causes both oxidative stress (12) and cytochrome P450 activation (13); upregulated expression of the profibrogenic principle transforming growth factor-β (14); increased generation and responses of smooth muscle cell calcium to vasoconstrictive stimuli (15); upregulated synthesis and expression of angiotensin II receptors (15); and depressed nitric oxide production by both endothelial and inducible nitric oxide synthases (16). Furthermore, CsA has been reported to promote Fas-mediated (17) apoptosis of LLC-PK1-cultured renal tubular cells *in vitro*, an effect that is blocked by peptide inhibitors of caspases 3, 8, and 9 (18). Thus, increased vasoconstriction and apoptosis characterize CsA nephrotoxicity.

Because the synergistic immunosuppressive interactions between CsA and RAPA were first documented in rats (19,20),

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Correspondence to Dr. Barry D. Kahan, Division of Immunology and Organ Transplantation, The University of Texas Medical School at Houston, 6431 Fannin, Suite 6.240, Houston, TX 77030. Phone: 713-500-7400; Fax: 713-500-0785; E-mail: bkahan@orgtx71.med.uth.tmc.edu

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impact of the drug combination on the kidney. The salt-depleted rat model has been reported to produce functional and histopathologic effects that resemble the dose-dependent impairment of renal function observed in CsA-treated kidney transplant patients (1,10,21–23). In contrast, administration of therapeutic doses of RAPA (0.04 to 0.8 mg/kg per d intravenously) caused no significant renal changes in rats (24). At 0.8 mg/kg per d RAPA, normal rats displayed only a marginal elevation in serum creatinine (SCr) values, and spontaneously hypertensive rats showed neither accelerated necrotizing vasculopathy nor tubular atrophy (25). Only supratherapeutic (1.6 to 6.4 mg/kg per d) RAPA doses produced transmural fibrinoid necrosis of vessels in the gastrointestinal submucosa and in the kidney, as well as juxtaglomerular hypertrophy, tubular dilation, basement membrane thickening, vacuolization, and atrophy (25).

Owing to its use in combination with CNA, there is considerable interest in understanding the impact of RAPA on CsA-induced nephrotoxic injuries. Andoh *et al.* (22) reported that subcutaneous coadministration of RAPA potentiated CsA-induced nephrotoxicity in salt-depleted rats and postulated that the effect was due to enhanced hyperglycemia. In contrast, we suggested that increased drug concentrations contribute to the adverse renal effects displayed by the CsA/RAPA combination, because we had documented elsewhere that a portion of the synergistic effect was due to pharmacokinetic interactions (26), particularly after oral coadministration (27). The present study revealed a predominant role of pharmacokinetic interactions to produce toxic renal exposures of CsA and of dynamic effects to potentiate the lipid as well as the myelosuppressive toxicities.

Materials and Methods

Animals

Male Wistar Furth (RT1^u) rats (160 to 200 g), obtained from Harlan Sprague Dawley (Indianapolis, IN), were housed in cages in a temperature- and light-controlled environment. The animals, which were maintained *ad libitum* on either regular or low-salt chow (0.05% sodium; Teklad Premier, Madison, WI) with access to tap water, were weighed and examined daily.

Drugs

Commercial oral formulations of CsA (Sandimmune; Novartis Research, East Hanover, NJ) and RAPA (Rapamune; Wyeth-Ayerst, Princeton, NJ) were administered by oral gavage in a constant volume of 0.2 ml daily for 14 d.

Experimental Groups

After a 7-d conditioning period on low-salt chow, groups of six rats were assigned randomly to treatment for 14 d with CsA alone (2.5, 5.0, 7.5, 10.0, 15.0, or 20.0 mg/kg per d), RAPA alone (0.4, 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d), or CsA/RAPA combinations at a fixed 6.25:1 ratio (2.5/0.4, 5.0/0.8, 7.5/1.2, 10.0/1.6, 15.0/3.2, or 20.0/6.4 mg/kg per d), which had been shown to be the optimal ratio to document synergistic immunosuppression, or at varying ratios of fixed 5.0 or 10.0 mg/kg per d CsA doses with ascending amounts of RAPA. There were two untreated control groups, each composed of six rats: one fed a low-salt diet and the other fed a normal diet.

After receiving the final drug doses on day 14, the animals were placed in metabolic cages for 24-h urine collections and GFR measurements, by use of iohexol in the Renalyzer PRX 90 (Provalid AB, Lund, Sweden). Inman *et al.* (28) documented in rats that this method provides an accurate and reliable measure of GFR, compared with inulin determinations. In addition, urinary sodium, potassium, magnesium, calcium, phosphate, and creatinine levels were quantified by use of established methods in our clinical chemistry laboratory. Upon completion of the urine collection, the animals were anesthetized with intraperitoneal pentobarbital (Abbott, Chicago, IL) to obtain whole-blood samples for complete blood counts, CsA and RAPA concentration measurements, and serum aliquots for sodium, potassium, magnesium, calcium, phosphate, uric acid, cholesterol, creatinine, and lipoproteins. The laboratory results, presented as mean values ± deviants, were compared for statistical significance by use of ANOVA; $P < 0.05$ was accepted as significant.

The left kidney was removed and split in half. One half of the left kidney and a 2-g sample of the right lateral lobe of the liver were used for drug concentration measurements. The other half of the left kidney was fixed in buffered 10% formalin and processed overnight; 3-μm histologic sections were stained with progressive hematoxylin-eosin, periodic acid-Schiff, or Masson's Trichrome reagents. Two independent pathologists (J.C. and R.V.), who were blinded to treatment assignments, used semiquantitative scales of light microscopic criteria to assess the degree of vasculopathy, glomerular changes, and tubulointerstitial damage in multiple kidney sections. Tubular and glomerular changes were graded separately as follows: 0, no changes; 1+, <5%; 2+, 5 to 25%; 3+, 26 to 50%; and 4+, >50% involvement. A similar vascular scale included the following: 0, none; 1+, minimal; 2+, mild; 3+, moderate; and 4+, severe. Although the scores generally were concordant, when they were disparate, a mean value was chosen as the histopathologic grade.

Renal Function

GFR was measured by the iohexol method (29). The femoral vein and artery, as well as the transplant ureter, were cannulated individually by use of 10-0 silicone tubing (Baxter, Deerfield, IL). BP, heart rate, and urine output were monitored with the use of a Micro-Med apparatus (Louisville, KY) and analyzed with the use of a DMSI 2004 computer program (Micro-Med). BP was recorded automatically every 30 s and urine output every 5 min. A loading dose of 1000 mg/kg iohexol (Omnipaque, 300 mg/ml; Nycomed, Inc., Princeton, NJ) was administered intravenously over 5 min, followed by infusion of 600 mg/kg over 90 min, as recommended by Inman *et al.* (28). Urine samples, collected at 20-min intervals after completion of the loading dose, were analyzed for iohexol concentrations. Whole-blood samples were obtained at the midpoints of the urine collections. GFR values (ml/min) were calculated by the formula $(U \times V)/P$, where U is urinary iohexol concentration (mg/ml), V is urine output (ml/24 h), and P is plasma iohexol concentration (mg/ml). The results were presented as mean ± deviants, and statistical significance was assessed by *t* test.

Bone Marrow Cellularity

The right femur was harvested, fixed in 10% buffered formalin, decalcified in formic acid for approximately 1 wk, sectioned (3 to 5 μm), and stained with hematoxylin and eosin by use of standard techniques. Hematopoiesis was estimated as the percentage of the

elements. The average number of megakaryocytes in four high-power fields (40×) was used to estimate the effects of RAPA with or without concomitant administration of CsA on platelet formation.

Drug Concentration Measurements

For CsA measurements, whole-blood samples (0.5 ml) were collected into ethylenediaminetetraacetate-containing tubes (Becton Dickinson, Mountain View, CA); 1- to 2-g aliquots of hepatic and renal tissues were disrupted by use of an Ultrasound Homogenizer (Fisher Scientific, Pittsburgh, PA). CsA determinations were performed by use of an automated fluorescence polarization immunoassay (TDx; Abbott, Chicago, IL). In contrast to human specimens and on the basis of supporting data of others (30–35), we documented elsewhere that the TDx technology provides results similar to HPLC because rats do not produce CsA metabolites that cross-react significantly in the TDx assay (36). CsA concentrations were expressed as ng/ml for whole blood or ng/g for wet-tissue weight. The intra-/interassay coefficients of variation for blood and tissue CsA measurements were 3.2% at 150 ng/ml and 1.7% at 800 ng/ml, and 2.3% at 150 ng/ml and 1.6% at 800 ng/ml, respectively (Napoli KL, Kahan BD, unpublished observations).

RAPA concentrations were estimated by use of our published method of HPLC with ultraviolet detection (37). Owing to the photosensitivity of RAPA, left kidney and liver samples (1 to 2 g) had to be protected from light during ultrasonic disruption. Briefly, 1 ml of 0.1 M sodium carbonate and 20 ml of methanolic-estradiol-3-methyl ether, an internal standard, were added to 1 ml of whole blood. After double extraction with 10 ml of t-butyl methyl ether, the pooled supernates were evaporated, reconstituted twice with 150 μl of absolute ethanol, and finally suspended in 100 μl of mobile phase buffer composed of an 85:15 ratio of methanol/water. After centrifugation, 85-μl aliquots of supernates were injected onto tandem Supelosil C18 columns (Supelco, Bellefonte, PA) heated to 40°C. During elution at a flow rate of 0.5 ml/min, ultraviolet absorbance was monitored at 276 nm. RAPA concentrations were estimated on the basis of a calibration curve consisting of 8 drug-free whole-blood (or tissue) samples that

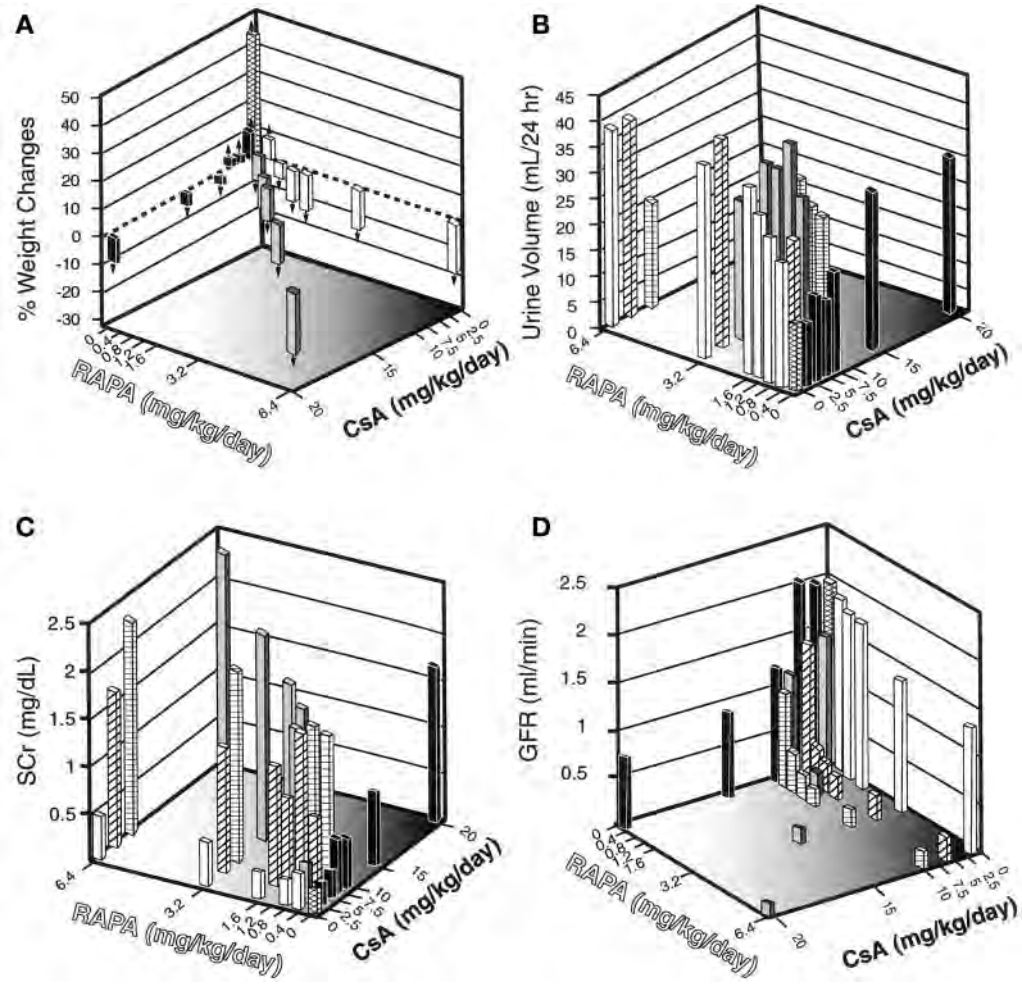

Figure 1. Effect of cyclosporine (CsA) and rapamycin (RAPA) alone or in combination on animal weight and renal function. Animals that were fed a low-salt diet either were untreated (▨) or were treated for 14 d with CsA alone at doses of 2.5, 5.0, 7.5, 10.0, 15.0, or 20.0 mg/kg per d (■); RAPA alone at doses of 0.4, 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d (□); or CsA/RAPA combination at doses of 2.5/0.4, 5.0/0.8, 7.5/1.2, 10.0/1.6, 15.0/3.2, or 20.0/6.4 mg/kg per d (▨). In addition, some rats were treated with a constant dose of 5 (▨) or 10 (▤) mg/kg per d CsA with ascending RAPA doses, namely 0.4, 0.8, 1.2, 1.6, 3.2, and 6.4 mg/kg per d. After 14 d, we measured percentage of weight change in comparison with the weight at the beginning of therapy (A), 24-h urine output (B), serum creatinine levels (SCr; C), and GFR (D). For details,

had been spiked with 0, 2, 5, 10, 20, 30, 40, or 50 ng of RAPA. The assays for tissue concentrations added 0.5 ml of drug-containing (or, for calibrators, exogenously spiked) homogenates to 0.5 ml of sodium carbonate. The intra-/interassay coefficients of variation for blood and tissue RAPA measurements were 6.4% at 4.0 ng/ml and 4.2% at 32 ng/ml, and 7.8% at 4.0 ng/ml and 5.6% at 32 ng/ml (38).

Statistical Analyses

Because all immunosuppressive agents studied to date (39) have obeyed the median-effect equation of Chou and Talalay (40), which relates dose (or concentration) to biologic effect, this model was chosen to assess the nephrotoxic interactions between CsA and RAPA. The relationship is described by the following equation:

$$(fa/fu) = (D/Dm)^m \quad (1)$$

where *fa* is the fraction affected, the percentage of inhibition (reduction from the normal value), *fu* is the uninhibited fraction (1 − *fa*), *D* is the administered drug dose (concentration), *Dm* is the dose (concentration) required for 50% inhibition (the median effect), and *m* is

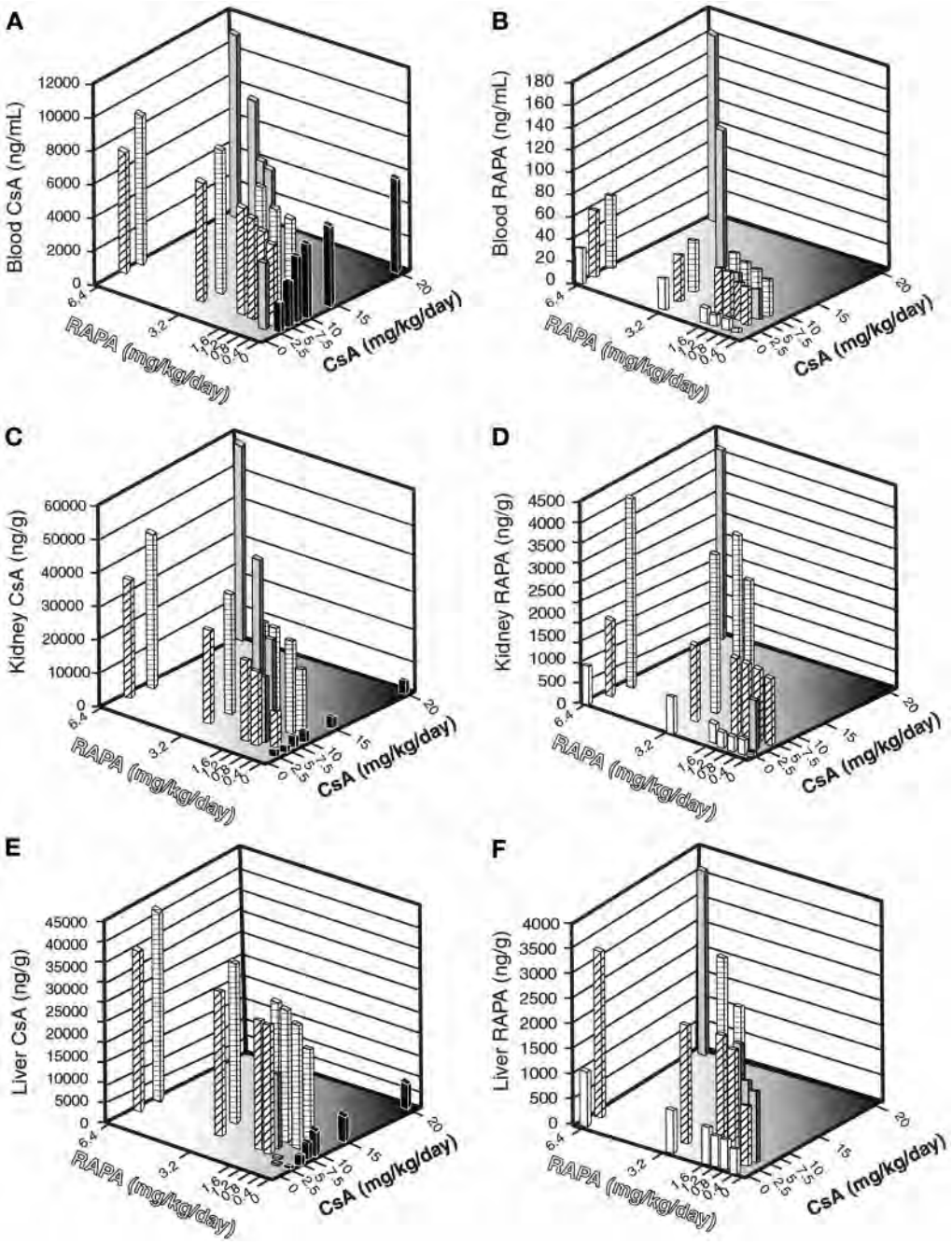

Figure 2. Pharmacokinetic interactions between CsA and RAPA. Whole-blood levels of CsA (A) and RAPA (B), kidney tissue levels of CsA (C) and RAPA (D), and liver tissue levels of CsA (E) and RAPA (F) were measured after a 14-d course of immunosuppressive therapy with

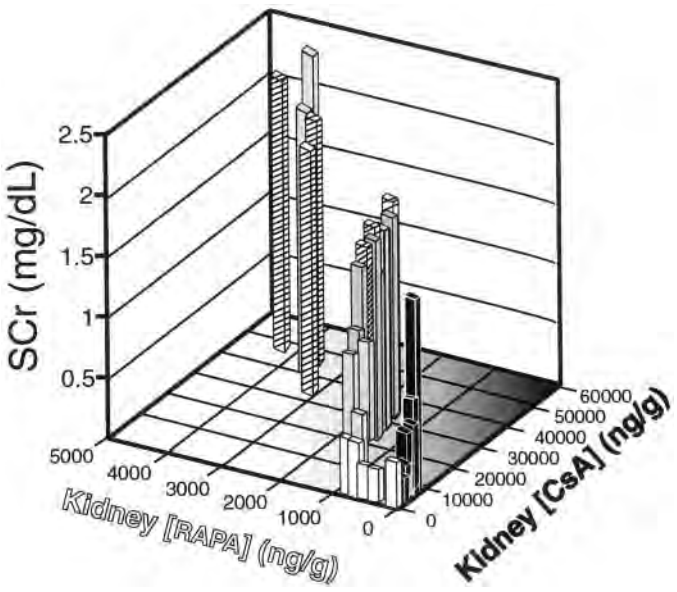

Figure 3. Correlation between CsA and RAPA tissue concentrations and SCr levels. Kidney tissue levels were measured after a 14-d course of immunosuppression with CsA alone at doses of 2.5, 5.0, 7.5, 10.0, 15.0, or 20.0 mg/kg per d as shown sequentially on the z-axis; RAPA alone at doses of 0.4, 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d as shown sequentially on the x-axis; or CsA/RAPA combination at doses of 2.5/0.4, 5.0/0.8, 7.5/1.2, 10.0/1.6, 15.0/3.2, or 20.0/6.4 mg/kg per d (▨). In addition, some rats were treated with a constant dose of 5 or 10 mg/kg per d CsA with ascending RAPA doses, namely 0.4, 0.8, 1.2, 1.6, and 3.2 mg/kg per d. Kidney drug concentrations (ng/g wet tissue) either for treatment with each drug alone or in combination are plotted *versus* creatinine levels.

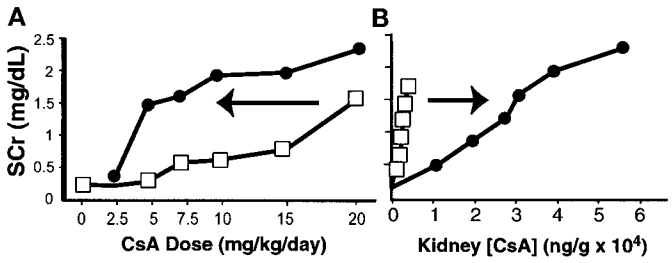

Figure 4. Impact of RAPA on CsA-induced nephrotoxicity. The arrow indicates the impact of addition of RAPA to shift the SCr values. (A) Increase in SCr as a function of CsA dose in the absence (□) or presence (●) of 0.8 mg/kg RAPA. (B) Increase in SCr as a function of CsA kidney concentrations. With use of regression lines to assess statistical differences between RAPA Yes/No, the *P* values are 0.0001 for A and 0.002 for B.

the slope coefficient. Logarithmic conversion of the median effect equation linearizes the following relationship:

$$\log(fa/fu) = m\log(D) - m\log(Dm) \quad (2)$$

When the data display a Pearson's correlation coefficient (r) >0.75, the equation is believed to predict the drug dose D (or concentration, C) necessary to achieve any arbitrary effect level. The nature of the index (CI) analysis, which assessed the dose of each drug necessary to achieve x% inhibition:

$$CIx = \frac{D_{1C}}{D_{1A}} + \frac{D_{2C}}{D_{2A}} + \frac{D_{1C} \times D_{2C}}{D_{1A} \times D_{2A}} \quad (3)$$

where D_{1C} and D_{2C} are the doses (or concentrations) of drugs when used in combination and D_{1A} and D_{2A} are the corresponding doses (or concentrations) of drugs used alone. CI values <1 reflect synergistic interactions, CI values =1 reflect additive interactions, and CI values >1 reflect antagonistic interactions.

Results

Body Weight Changes

Control (untreated) animals that were maintained on a low-salt diet for 21 d showed a mean weight gain of 41.5 ± 22.8% (Figure 1A). In contrast, hosts that were treated with 2.5 or 5.0 mg/kg per d CsA showed less weight gain, and those that were given 10.0, 15.0, or 20.0 mg/kg per d showed a maximal weight loss of 10% (*versus* untreated hosts; all groups, $P < 0.001$). Although animals that were treated with 0.4 mg/kg per d RAPA showed a slight weight gain, those that received 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d RAPA showed a maximal weight loss of 20% (all groups, $P < 0.001$). Rats that were treated with CsA/RAPA drug combinations displayed a maximal weight loss of 27% (all groups, $P < 0.005$). The weight loss seemed more likely to reflect metabolic causes than anorexia, because all experimental animals had food in their stomachs and stool in their colons. Furthermore, there was no evidence of drug-induced diarrhea or dehydration, as evident on examination and by blood chemistries (*vide infra*).

Renal Function Changes

Animals that were treated with either CsA or RAPA monotherapy showed greater urine output than hosts in the control groups (Figure 1B; $P < 0.002$), suggesting the presence of renal injury. Because blood glucose levels were similar among rats in each group, there was no evidence that hyperglycemia was producing a diuretic effect. In contrast to the control group, which showed a mean SCr value of 0.25 ± 0.05 mg/dl, treatment with ascending 2.5 to 20.0 mg/kg per d doses of CsA produced serial increases in SCr values (all groups, $P < 0.0007$; Figure 1C). In contrast, rats that were treated with the smaller RAPA doses (0.4, 0.8, or 1.6 mg/kg per d) showed insignificant changes; only animals that received 3.2 or 6.4 mg/kg per d showed significantly increased SCr values ($P = 0.001$). The CsA/RAPA groups showed higher SCr concentrations than either monotherapy group, increasing from 0.35 ± 0.05 mg/dl for the 2.5/0.4 mg/kg per d group to 2.35 ± 0.37 mg/dl for the 20.0/6.4 mg/kg per d group. Interestingly, ascending RAPA doses added to fixed amounts of CsA (5 or 10 mg/kg per d) produced less adverse effects ($P = 0.005$) than those observed in groups with simultaneously increasing doses of both drugs ($P = 0.001$).

The SCr results were confirmed by the GFR values. In comparison to the normal GFR values (1.98 ± 0.34 ml/min; Figure 1D), animals that were treated with CsA doses of 5.0

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