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TOXICITY OF RAPAMYCIN—A COMPARATIVE AND COMBINATION STUDY WITH CYCLOSPORINE AT IMMUNOTHERAPEUTIC DOSAGE IN THE RAT

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Sprague-Dawley rats were treated for 14 days with rapamycin (RAP; 1.5 mg/kg/day i.p.), cyclosporine (15 mg/kg/day by gavage), both drugs in combination, or appropriate drug vehicles. Hematological parameters and biochemical indices of renal and hepatic function were determined throughout the experimental period, at the end of which the rats were killed and tissues examined histologically. There was a significant reduction in weight gain in RAP- but not CsA-treated animals, while rats given both drugs showed a reduction in body weight over the 14-day experimental period. There were no significant alterations in absolute or differential white blood cell counts or in T or B cell numbers, except in the drug combination group, in which an absolute lymphopenia was detected on day 14. Small but significant increases in urinary flow rate (UFR) were found with either drug alone, and there was a marked (4-fold) increase in UFR in response to drug combination. Both RAP and CsA caused a small elevation in serum creatinine concentrations, but only with CsA was there a significant elevation in urinary enzyme activity and reduction in ^{51}Cr. EDTA clearance. The drug combination exacerbated renal impairment, the extent of which was greater than the additive effect of either drug alone. Hyperbilirubinemia of similar magnitude was observed in rats receiving either CsA alone or in combination with RAP. In contrast to its effect on renal function, however, the CsA+RAP combination was without additional effect on liver function compared with the minor changes seen with either drug alone. Plasma and urinary glucose levels were elevated in all drug treatment groups and especially in animals given both drugs. RAP administration did not significantly affect whole-blood CsA concentrations, although the possibility of a pharmacokinetic interaction cannot be totally excluded. Histological studies revealed striking thymic medullary atrophy in all drug-treated animals. In addition, all rats given RAP showed focal myocardial necrosis of overall mild-moderate severity. Kidneys of RAP-treated rats appeared normal, whereas mild, focal, acute tubular necrosis was evident in all CsA-treated animals. Pancreases of all drug-treated animals were normal.

First reports of the successful use of the novel macrolide antibiotic FK506 in the prevention (*1*) and reversal (*2*) of human liver allograft rejection have aroused considerable interest in the potential of this and possibly other related macrolides as clinical immunosuppressive agents. FK506 (mw 822d), and its closest known structural analog rapamycin (RAP;* mw 913), are derived from two distinct species of *Streptomyces*. Both drugs are more potent than cyclosporine in suppressing T cell proliferation in vitro triggered by lectins or antibodies to CD3 (*3*) and in prolonging allograft survival in experimental animals (*4–8*). Like CsA, FK506 selectively inhibits $CD4^+$ T helper lymphocyte activation and the expression of genes encoding interleukin-2 (IL-2), IL-4, and other cytokines (*3, 9–11*). FK506 also appears to inhibit IL-2 receptor expression (*3, 12*). By contrast, RAP does not inhibit IL-2 production or IL-2 receptor expression, but interferes with the responses of T cells to IL-2 and IL-4 (*3*). Despite each macrolide binding to a cytosolic peptidyl-prolyl isomerase (*12*)—distinct from cyclophilin, which may also play a key role in signal transduction within T cells—the two drugs act as reciprocal antagonists in vitro (*10*). The potential value of these macrolide immunosuppressants depends on their toxicity profile in relation to their acknowledged immunosuppressive potency. In a single-center study of liver and other organ transplant patients, no major side effects of FK506 have been reported, and it appears that in these subjects the drug may offer a number of benefits in comparison with CsA (*1, 13, 14*). No detailed toxicological studies of RAP have been documented in any species, although in a recent report, Calne et al. found that, in allografted animals, the toxicity profile of RAP compared favorably with that of FK506 (*4*). In this study, we have examined the pathological effects of RAP at immunotherapeutic dosage, in direct comparison with those of CsA. We have used a rat model, which in this laboratory has provided considerable insight into the efficacy and toxicity of CsA and other therapeutic agents, including FK506 (*15, 16*).

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats (mean initial body weight 220 g) were obtained from the University Animal Department, Foresterhill, Aberdeen. They were maintained in a light-, air-, and temperature-controlled environment on CRM diet (Labsure Animal Foods, K & K Greeff Ltd., Croyden, UK) and with free access to tap water.

Drugs. Rapamycin (Wyeth-Ayerst Research, Princeton, NJ; batch 19) was provided in powder form and suspended in sterile 1% (w/v) carboxymethyl cellulose in saline (low viscosity, c-8758, Sigma, St Louis, MO) at 20°C. The RAP suspension was freshly prepared each day immediately before intraperitoneal injection (1.5 mg/kg body weight). Cyclosporine (Sandimmun oral solution, Sandoz Ltd., Basel, Switzerland) was diluted in olive oil (Boots Ltd., Nottingham, UK) and

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* Abbreviation: RAP, rapamycin.

administered by gavage (15 mg/kg/body weight), using a 4 FG intravenous cannula (Portex Ltd., Hythe, Kent, UK).

Experimental protocol. Rats were randomized into six groups of 4–6 animals and treated from days 0–13 inclusive, with RAP, CsA, RAP+CsA, or either drug vehicle; one group received no treatment. Hematological tests and investigations of urine and plasma biochemistry were undertaken immediately before treatment and on days 7 and 14, as described previously (*17*). Whole-blood CsA levels were determined on days 7 and 14. In addition, the clearance of ^{51}Cr.EDTA, an estimate of glomerular filtration rate, was measured on day 13, 4 hr following drug treatment, using the method described by Ryffel et al. (*18*).

On day 14, the animals were killed by terminal ether anesthesia and tissues removed for histological examination (thymus, spleen, lymph nodes, bone, kidney, liver, heart, skeletal muscle, lung, stomach, small and large intestine, pancreas, skin, and brain).

Preparation of mononuclear cell suspension. Mononuclear leukocytes were isolated from blood anticoagulated with acid citrate dextrose as previously described (*19*). The washed cells were resuspended at 10^7/ml in RPMI-1640 supplemented with 10% v/v fetal bovine serum (Gibco, Paisley, Scotland, UK).

Immunofluorescence staining and flow cytometric analysis. An indirect immunofluorescence procedure was used to demonstrate cell phenotypes using appropriate primary mouse monoclonal antibodies (Serotec Ltd., Bicester, Oxfordshire, UK) as described elsewhere (*19*). The following antibodies were used: W3/25 ($CD4^+$ helper/inducer T cells, macrophages); OX-8 ($CD8^+$ cytotoxic/suppressor T cells, NK cells); OX-8 ($CD8^+$ helper/inducer T cells, macrophages); OX-12 (λ light chains, pan-B cells); ED1 (monocytes/macrophages); OX-39 (CD25; IL-2 receptor α chain gp 55) and OX-6 (MHC class II monomorphic). Lymphocyte analysis was performed using an EPICS C microcytofluorograph (Coulter Electronics, Luton, UK).

Hematological and biochemical determinations. Blood samples were collected under light ether anesthesia from cleaned tail tips, then processed for hematological and biochemical analyses as described previously (*17*). Urine free of fecal contamination was collected overnight from animals placed in individual metabolic cages and allowed free access to both food and water. Urine analysis was performed as described previously (*15*).

CsA immunoassay. Whole-blood CsA levels were measured using a Cyclo-trac SP radioimmunoassay kit (Incstar Corporation Stillwater, MN) which utilizes a monoclonal antibody specific to the parent CsA molecule.

Histology. Blocks of tissue were fixed in 10% neutral buffered formalin and processed to paraffin wax. Sections of 5 m were stained with hematoxylin and eosin.

Statistics. The significance of differences between means was determined by analysis of variance and by Student's *t* test for dependent or independent means, as appropriate.

RESULTS

Body weight. There was an increase in mean body weight of 32±8% in untreated control animals over the 14-day course of the experiment (Fig. 1). Similar increases were observed in both drug-vehicle-treated control groups. Weight gains in RAP- and CsA-treated rats were 18±4% and 27±8%, respectively (RAP group: $P<0.05$ compared with vehicle control on day 14). In contrast, animals treated with both drugs showed a 4±9% reduction in body weight ($P<0.001$ compared with either agent alone).

Hemopoietic system. There were no statistically significant differences in total WBC count or in absolute numbers of lymphocytes, monocytes, or neutrophils between treatment and control groups at any time point, except for a reduction (37–42%) in lymphocytes in the RAP/CsA group on days 4, 7, and 10 ($P<0.01$ compared with either agent alone). No significant

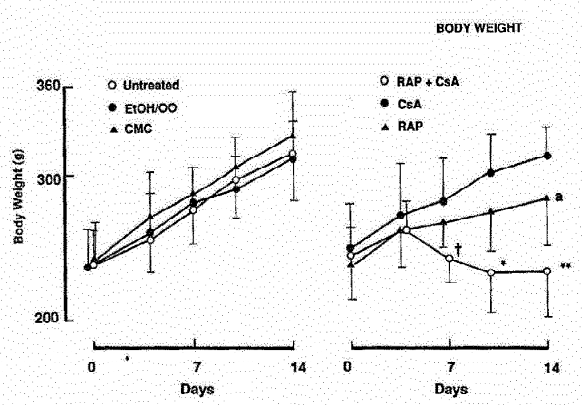

FIGURE 1. Body weights (mean ± SD) in untreated or drug-vehicle treated rats (left) and in animals given rapamycin (RAP), CsA, or a combination of both drugs (right): (a) significantly lower than vehicle control ($P<0.05$); (†) $P<0.05$; (*) $P<0.01$; (**) $P<0.001$ compared with either drug alone. EtOH/OO = ethanol/olive oil; CMC = carboxymethylcellulose vehicle. There were 5, 6, 4, and 4 animals in the untreated, RAP-alone, CsA-alone, and RAP + CsA groups, respectively.

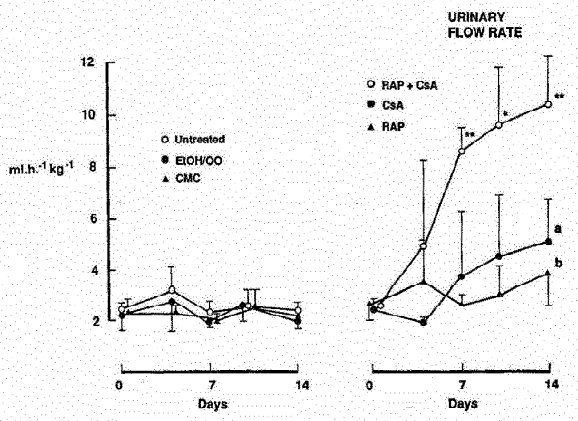

FIGURE 2. Urinary flow rate in untreated or drug-vehicle treated rats (left) and in animals given RAP, CsA, or a combination of both drugs (right). Results are means ± 1 SD obtained from groups of 4–6 animals: (a) $P<0.01$: (b) $P<0.05$ compared with vehicle control; (*) $P<0.01$; (**) $P<0.001$ compared with either drug alone. Notation as in Figure 1.

changes in the incidence of T cell subsets, B cells, or activation marker expression were found in drug-treated rats compared with controls. Histological examination of femoral bone marrow revealed no abnormalities in any of the drug-treated animals.

Renal function. No significant change in urinary flow rate (UFR) was observed in either control group or in animals receiving RAP alone (except for a minor increase on day 14) over the experimental period. Rats treated with CsA, however, demonstrated a progressive increase in UFR from day 7 onward ($P<0.01$ on day 14; Fig. 2). An earlier and more striking progressive increase in UFR was observed in animals given both drugs. By day 14, a 4-fold increase in UFR, compared with pretreatment values, was recorded in the drug combination

group, compared with a 2-fold increase in animals given CsA alone.

The only statistically significant renal function abnormality observed in RAP-only-treated rats was a relatively minor increase in plasma creatinine (Tables 1 and 2). CsA-only-treated animals, however, demonstrated statistically significant elevations in plasma urea concentration (days 7 and 14) and creatinine concentration (day 14), with a significant 25% decrease in creatinine clearance rate noted on day 7. The corresponding 20% decrease in clearance rate in RAP-treated rats was not statistically significant. Moreover, CsA-treated rats showed a two-fold increase in N-acetyl-β-D-glucosaminidase enzymuria (Table 2). These CsA-induced abnormalities were exacerbated by combination of RAP and CsA. By day 14, the extent of the changes in each parameter were greater than those observed with either drug alone or when these individual drug effects were combined. An elevation in fractional excretion of sodium was observed only in the drug combination group (Table 2)—treatment with either drug alone or their respective vehicles being without significant effect.

GFR, estimated by measures of the clearance of ^{51}Cr EDTA, was not significantly effected by treatment with either drug vehicle alone or with RAP alone (Table 3). However, GFR was reduced by 28% and 61%, respectively, in animals treated with either CsA alone or in combination with RAP by day 13.

Liver function. The results of liver function tests are shown in Table 4. While no change in plasma bilirubin levels was observed in RAP-treated animals, hyperbilirubinemia of similar magnitude was noted in animals receiving CsA either alone or in combination with RAP. Significant reductions in AST and alkaline phosphatase activities were observed in CsA- and RAP-treated animals, respectively. No significant changes in enzyme activities, however, were found in the drug combination group.

Plasma glucose levels. Progressive increases of similar magnitude in plasma glucose levels were observed in the RAP and CsA groups, while more marked increases (approximately equivalent to the additive effects of RAP and CsA) were seen in rats given both drugs (Table 5). Urinary glucose concentrations were markedly elevated in each experimental group, particularly in animals given both drugs.

CsA blood levels. Although there was a wide spread of results, no significant effect of RAP on whole-blood CsA levels was observed (Table 6).

Histology. No significant histological abnormalities were observed in any of the untreated or vehicle control animals. Rats treated with RAP, however, demonstrated thymic medullary atrophy (all animals) and moderate (1 rat), mild (4 rats), or severe (1 rat) focal myocardial necrosis (Fig. 3). Skeletal muscle was unaffected. In one animal, hepatitis and focal hepatocyte necrosis was also observed. The kidneys and all other tissues examined appeared normal. In CsA-treated rats, thymic medullary atrophy and phagocytic activity were more pronounced than seen following RAP administration; hearts and livers appeared normal, although kidneys in all animals displayed mild focal acute tubular necrosis. In the drug combination group, the changes in the thymus were similar to those seen with CsA alone; focal myocardial necrosis was observed in all

TABLE 1. Effects of RAP and CsA on renal function (results, expressed as the mean ± SD, with the number of animals given in parentheses, were compared with day 0 values)

Parameter	Day	Treatment			
		Untreated (5)	RAP (6)	CsA (4)	RAP + CsA (4)
Plasma urea (mmol/L)	0	5.3±0.4	4.5±0.2	4.7±0.2	4.4±0.8
	7	4.8±0.3	5.6±0.5	5.8±0.6#	11.2±4.2**c
	14	5.1±0.6	5.7±0.7	7.2±0.6**	12.9±3.1**c
Plasma creatinine (μmol/L)	0	48±6	43±4	46±4	43±7
	7	50±8	57±7*	53±5	71±17**c
	14	53±2	50±3#	60±9#	82±13**c
Creatinine clearance (ml/hr/kg)	0	320±33	315±45	285±36	362±74
	7	348±78	250±53	215±76*	173±9**c
	14	302±50	254±56	250±35	113±21**c

# $P < 0.05$; * $P < 0.01$; ** $P < 0.001$—RAP or CsA groups compared with RAP + CsA group: c $P < 0.001$. Treatment with either drug vehicle alone was without significant effect on any of the above three parameters.

TABLE 2. Effects of RAP and CsA on NAG enzymuria and the fractional excretion of sodium (FENa) (results, expressed as the mean ± SD, with the number of animals given in parentheses, were compared with day 0 values)

Parameter	Day	Treatment			
		Untreated (5)	RAP (6)	CsA (4)	RAP + CsA (4)
NAG (U/mmol urinary creatinine)	0	72±26	89±29	76±10	76±25
	7	81±20	51±17	152±84**c	143±25*c
	14	70±24	80±33	152±53**	236±129**c
FENa (%)	0	1.13±0.10	1.03±0.27	1.09±0.09	0.95±0.24
	7	0.93±0.22	1.05±0.33	1.00±0.36	1.17±0.44
	14	1.04±0.08	0.93±0.34	0.87±0.26d	2.55±0.64**c

* $P < 0.01$; ** $P < 0.001$—RAP + CsA compared with RAP; c $P < 0.001$ or with CsA values: d $P < 0.001$. Treatment with either drug vehicle alone was without significant effect on either of the above parameters.

4 animals (2 moderate, 2 more severe than with RAP alone). Livers showed normal histological appearances. No abnormalities were detected in the bone marrow, pancreases or any other organs of drug-treated animals.

DISCUSSION

Previous studies concerning the immunosuppressive activity of RAP in rodents have shown that doses similar to that used in this study (1.5 mg/kg/day) markedly prolong allograft survival in rats when administered intramuscularly (*4*), or in mice, when given intraperitoneally (*5*). Despite these similarities, however, we were unable to investigate the bioavailability of RAP due to the absence of a satisfactory analytical technique. Oral administration of 2 mg/kg/day to pigs has been reported to suppress renal allograft rejection, with prolonged graft survival following drug withdrawal in some animals (*20*). Doses of 2.5–10 mg/kg per os have also been shown to inhibit antibody (IgE) production and the development of experimental autoimmune disorders in the rat (*21*). Although one previous short report suggests that, in rats given cardiac allografts, RAP administered systemically (0.5–50 mg/kg) exhibits only mild toxicity, short courses of 2 mg/kg/day per os in dogs are severely toxic (*4*). Like FK506, RAP produces vascular lesions in this species, although it has been reported that these are less widespread than has been experienced in dogs treated with FK506 (*4*). Clearly, detailed investigations of the pathological properties of RAP in various nonhuman species are essential.

We have found, in this study of normal rats, that RAP at the dose selected, caused little or no impairment of either renal or hepatic function. Similar observations were made with FK506 (1 mg/kg im) over a 7-day period (*16*). In contrast, our parallel comparative investigations in CsA-treated animals revealed significant impairment of kidney function, including GFR, evidence of renal tubular cell damage, and minor liver functional abnormalities. The dose of CsA selected was within the therapeutic range for oral administration in rats (*22*).

In this study, both RAP and CsA caused elevations in both blood and urinary glucose concentrations. We and others have previously reported abnormalities of glucose homeostasis in CsA-treated rats (*23, 24*) associated with damage to pancreatic β cells (*25, 26*). Although in a previous investigation, we found that FK506 (1 mg/kg) did not affect glucose levels (*16*), hyperglycemia has been reported in rats given higher doses (2 or 4 mg/kg/day) of the drug (*27*). The diabetogenic potential of

TABLE 3. Influence of RAP and CsA on GFR: ^{51}Cr EDTA clearance (ml/hr/kg body weight)[a]

Untreated (A)	RAP (B)	CsA (C)	RAP + CsA (D)
481.5±66.7 (5)	367.5±128.6 (6)	346.9±73.0 (4)	141.8±37.0 (4)

[a] Results, obtained on day 13 of treatment, are expressed as the mean ± SD, with the number of animals given in parentheses. Results compared by Student's *t* test for independent samples: (A) vs. (C), $P < 0.05$; (A) vs. (D), $P < 0.001$; (C) vs. (D), $P < 0.001$; (A) vs. (B), not significant. Treatment with either drug vehicle alone had no significant effect on GFR.

TABLE 4. Effects of RAP and CsA on plasma estimates of liver function (results, expressed as the mean ± SD, with the number of animals given in parentheses, were compared with day 0 values)

Parameter	Day	Treatment group: Untreated (5)	RAP (6)	CsA (4)	RAP + CsA (4)
Bilirubin (μmol/L)	0	3±1	2±1	2±1	2±1
	7	2±1	3±1	4±2[#]	6±3*
	14	2±1	2±1	5±2*	5±2*[a]
AST (IU/L)	0	76±6	71±6	75±8	68±5
	7	77±2	79±25	54±5*	121±68
	14	91±14	85±9	66±5[f]	90±18
Alk. phos. (IU/L)	0	323±57	285±36	305±50	287±62
	7	312±31	243±18[#]	318±36	263±61
	14	304±81	226±1**	314±95	262±53

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$—RAP results compared to RAP + CsA: **** $P < 0.05$, and results from days 7 and 14 compared: ***** $P < 0.05$. Treatment with either drug vehicle alone was without significant effect on the above parameters.

TABLE 5. Effects of RAP and CsA on plasma and urine glucose levels (results, expressed as the mean ± SD, with the number of animals given in parentheses, were compared with day 0 values)

Parameter	Day	Treatment: Untreated (5)	RAP (6)	CsA (4)	RAP + CsA (4)
Plasma glucose (mmol/L)	0	11.2±0.9	11.8±0.5	12.1±0.6	11.4±0.9
	7	11.2±1.4	15.2±3.5[#]	16.6±2.5[#]	24.8±7.6**[b]
	14	12.1±2.0	19.7±5.8**	19.3±2.6**	27.3±8.9**
Urinary glucose (mmol/mmol urine creatinine)	0	0.5±0.1	0.7±0.1	0.7±0.1	0.8±0.2
	7	0.5±0.1	10.5±17.6[#b]	47.3±65.7[#]	159.7±80.1**
	14	0.5±0.1	88.2±86.1*	129.2±75.4**	363.4±213.8**

[#] $P < 0.05$; * $P < 0.01$; ** $P < 0.001$—RAP and CsA results compared with RAP + CsA results: [b] $P < 0.01$. On day 14 both plasma and urine glucose results obtained from RAP, CsA, and RAP + CsA groups were significantly different from the untreated group ($P < 0.001$). Treatment with either drug vehicle was without significant effect on the above parameters.

TABLE 6. Influence of RAP on CsA levels[a]

Day	Treatment	
	CsA (4)	RAP + CsA (4)
7	2937±1393	2850±331
14	2303±296	1682±674

[a] Results (mean ± SD, with the number of animals given in parentheses) are expressed as $ng \cdot ml^{-1}$ and were obtained using whole-blood samples and a monoclonal antibody specific to the native CsA molecule.

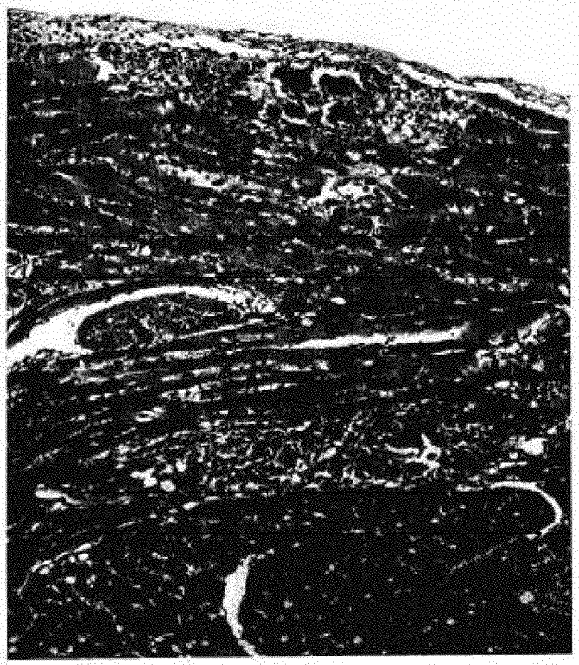

FIGURE 3. Focal myocardial necrosis in a RAP-treated rat 14 days after the start of drug administration (1.5 mg/kg/day i.m.). Similar abnormalities were observed in animals given CsA+RAP, but not in rats receiving CsA alone or either drug vehicle. (original magnification: ×120).

both RAP and FK506 in rats may therefore be dose-related, with relatively minor differences in tolerance threshold between the two macrolides. In the present study, no histological abnormalities attributable to either RAP or CsA were detected in the pancreases of any of the drug-treated animals. However, the doses of both agents may have been below the threshold level at which structural damage to the pancreatic islet β cells appeared, as glucose intolerance was present at CsA doses of <20 mg/kg for 14 days, also in the absence of histological abnormalities(*24*).

A notable feature of this study was the potentiation of CsA nephrotoxicity in the presence of RAP. GFR reduction in the drug combination group was more than double than observed in the animals treated with CsA alone, and RAP was without significant effect on renal clearance rates. In addition, increased values of the fractional excretion of sodium, suggestive of renal parenchymal damage, were only observed in the drug combination group, the other treatments being without significant effect. The drug interaction was not seen in relation to liver function and could not be ascribed to elevation of CsA blood levels in the presence of RAP, although sample timing may not have been entirely appropriate to demonstrate such an interaction. However, similar interactive nephrotoxicity between another macrolide antibiotic, erythromycin, and CsA has been reported, accompanied by increased circulating CsA levels (*28*). Moreover, FK506 has been shown to inhibit the human hepatic metabolism of CsA in vitro (*29*). Although the mechanisms resulting in the exacerbation of CsA-induced renal dysfunction by RAP are not clear, no histological evidence of interactive renal toxicity between RAP and CsA was observed. Earlier studies have demonstrated that the combination of an aminoglycoside antibiotic (gentamicin) and CsA in the rat produces distinct zones of damage within the proximal tubule (*30*). The influence of higher doses of RAP alone on renal function and structure in this species should provide insight into possible mechanisms underlying the interactive toxicity between RAP and CsA. In clinical investigations, gentamicin (*31*), erythromycin (*32*), and FK506 (*2*) have been reported to exacerbate CsA nephrotoxicity.

The only significant histological abnormalities consistently observed in rats given RAP alone were thymic medullary atrophy, which has also been observed in CsA-treated (*15*) or FK506-treated animals (*16*), and focal myocardial necrosis. It is probable that the RAP-induced changes in the thymus are, as with CsA or FK506, attributable to damage inflicted by the drug on medullary epithelial cells, with consequent disruption of the microenvironment within which thymocytes differentiate and mature, under the influence of epithelial cell-derived thymic hormones (*33, 34*). To our knowledge, myocardial necrosis has not previously been reported in RAP-treated animals, including mice given 24 mg/kg/day in CMC i.p. for 14 days (R. E. Morris, Stanford University School of Medicine; personal communication). Thiru et al. (*35*), however, observed this lesion in baboons given similar doses of FK506 intramuscularly. Cardiomyopathy has also been reported in FK506-treated cynomolgus monkeys (*36*). While vasculitis has been documented in RAP-treated dogs (*4*), there was no evidence of vascular abnormalities in the heart, gastrointestinal tract, or any other organ examined in the present study. The myocardial necrosis may be attributable to a directly injurious effect of RAP; the increased incidence of severity in animals also given CsA may, as with renal function, be a direct consequence of drug interaction. Further studies are clearly required to determine whether this lesion is reproduced in larger species, including higher primates.

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