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AMELIORATION OF CYCLOSPORIN-INDUCED NEPHROTOXICITY IN RATS BY INDUCTION OF HEPATIC DRUG METABOLISM

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Abstract—The aim of this study was to determine the effect of altered hepatic drug metabolism on the nephrotoxic and immunosuppressive properties of cyclosporin A (CsA) in the rat. From a consideration of the structures of those CsA metabolites identified so far, it seemed probable that the metabolism of CsA would occur at the hepatic cytochrome P-450 (cyt P-450) enzyme system. CsA (50 mg/kg/24 hr) administered orally for 14 days resulted in significant increases in both serum urea concentration and urinary N-acetyl-β-D-glucosaminidase activity, accompanied by renal proximal tubular vacuolation. The concomitant administration of either Aroclor 1254 (25 mg/kg/24 hr, i.p.) or phenobarbitone (PB) (40 mg/kg/24 hr, i.p.) but not 3-methylcholanthrene (3-MC) (15 mg/kg/72 hr, i.p.) resulted in abolition of the nephrotoxicity, assessed both biochemically and histologically, whilst the suppressive effect on the humoral response to SRBC was unaltered. Phenobarbitone also significantly decreased serum CsA concentrations. These results suggest that the PB-inducible set of cyt P-450 isoenzymes may be responsible or partly responsible for hepatic CsA metabolism.

The fungal metabolite cyclosporin A (CsA), which inhibits T lymphocyte activation [1–4], has been used successfully to prevent the rejection of a variety of organ grafts in many species, including man [5–8]. It has been reported, however, that patients receiving the drug often exhibit impaired renal function [9–11]. At doses higher than that required to attain immunosuppression, CsA has been reported to have similar but reversible effects on the kidneys of surgically-intact rats [12, 13]. The cells known to be affected in the rat are those of the straight segment of the proximal tubule [14].

There is evidence that CsA is extensively metabolized by hydroxylation and N-demethylation [15]. The structure of the metabolites suggests the involvement of the hepatic microsomal cytochrome P-450 (cyt P-450)-dependent mono-oxygenase system in the metabolism of CsA. In this study, known toxic doses of CsA were administered to rats in combination with various inducers of cyt P-450. Our aim was to determine the effect of possible alterations in CsA metabolism on the nephrotoxicity and immune suppression induced by the drug.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (250–300 g) were used throughout. They received Oxoid pasteurized rat and mouse breeding diet with tap water *ad libitum*, except as specified below.

Drugs. CsA (OL 27-400; Sandoz Ltd., Basle, Switzerland) was provided in powder form and dissolved initially at 20° in anhydrous ethanol. A 10% solution of the CsA-containing ethanol in olive oil (Boots Company Ltd, Nottingham, U.K.) was then prepared. This solution was administered to the conscious rat at a toxic dose of 50 mg/kg once daily by gastric intubation (p.o.) using a 4-fine gauge intravenous cannula (Portex Ltd., Hythe, U.K.). Aroclor 1254 (kindly supplied by Dr. C. R. Elcombe, CTL, ICI plc., Alderley Park, U.K.) was administered as a 4% (w/v) solution in olive oil and given intraperitoneally (i.p.) at a dose of 25 mg/kg once daily. 3-Methylcholanthrene (3-MC) (Fluka AG, Buchs, Switzerland) was dissolved in olive oil as a 1% (w/v) solution and administered i.p. at a dose of 15 mg/kg once every 3 days. Sodium phenobarbitone (PB) (BDH Chemicals Ltd., Poole, U.K.) was dissolved in physiological saline as a 4% (w/v) solution and administered i.p. at a dose of 40 mg/kg once daily.

Drug treatments. The following control treatments were administered in addition to those outlined below; CsA vehicle (p.o.), Aroclor 1254 vehicle (i.p.), Aroclor 1254 vehicle (i.p.) with CsA, CsA vehicle (p.o.) with Aroclor 1254, CsA vehicle (p.o.) with 3-MC and CsA vehicle (p.o.) with PB.

(1) Aroclor 1254 plus CsA. Three groups of 6 rats each received one of the following drug treatments: either Aroclor 1254 or CsA, or CsA with Aroclor 1254. Each solution was given for 14 consecutive days beginning on day 0. Blood was taken for serum analyses on days 0 and 14. Urine was sampled on days −1, 6 and 13. The details of the method for blood and urine collection are described by Blair *et al.* [13].

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(2) 3-MC plus CsA. Two groups of 4 rats each received 3-MC and one of these groups also received CsA. 3-MC was given alone from day −6 and then with CsA, if required, from day 0 until the end of the experiment on day 14. Blood was taken for serum analyses on days −6, 0 and 14. Urine was sampled on days −7, −1, 6 and 13.

(3) PB plus CsA. Two groups of 6 rats each received PB and one of these groups also received CsA. As with 3-MC, PB treatment was begun on day −6 and continued until day 14. CsA treatment, where required, was begun on day 0. The urine and blood sampling schedule was identical to that employed for rats being treated with 3-MC.

On day 14 all animals were etherized for blood collection then killed by cervical dislocation.

Humoral response to sheep red blood cells (SRBC). SRBC were obtained from sheep blood in Alsever's solution (Difco Laboratories). The preparation and administration of these cells was performed after the method outlined by Whiting *et al.* [16]. Serum haemagglutinin titres were determined for each animal, on blood samples taken on day 14, in U-shaped microtitre plates (Nunc, Denmark) using Takátsy's microtechnique [17].

Blood and urine biochemical investigations. Serum urea and creatinine and the urine activity of N-acetyl-β-D-glucosaminidase (NAG) were measured as previously described [12].

Measurement of CsA in serum. Trough serum CsA levels were measured 24 hr after the final dose of CsA in samples collected immediately before the animals were killed. Prior to serum separation blood samples were left for 2 hr at ambient room temperature in order to standardize the redistribution of CsA between the various blood components. CsA concentrations were estimated by radioimmunoassay (RIA) [18] using kits kindly provided by Sandoz Ltd. The coefficient of variation for within batch analysis was 7%. It should be noted, however, that the anti-cyclosporin A antibody provided with the RIA kit cross reacts with certain CsA metabolites.

Preparation of tissue for microscopy. One kidney was removed when the rats were killed. Tissue blocks were fixed in 10% neutral buffered formalin and 5 μm thick paraffin sections were cut, then stained with haematoxylin and eosin (H & E).

Microsome preparation. Liver was removed after the rats were killed and used to prepare microsomes, as described elsewhere [19].

Determination of hepatic microsomal cyt P-450 concentration and mono-oxygenase activity. The concentration of cyt P-450 was determined by the method of Omura and Sato [20] from the reduced CO difference spectrum, using an extinction coefficient of 91 $mm^{-1}\,cm^{-1}$. All spectra were recorded using a Cary 219 spectrophotometer between 500 nm and 400 nm.

Aminopyrine N-demethylase (AD) activity was assayed using the Nash reaction, as described elsewhere [19]. Ethoxyresorufin O-deethylase (EROD) and pentyloxyphenoxasone O-dependentylase (PPOD) activities were assayed at 37° using the method of Burke and Mayer [21].

Protein was determined by the method of Lowry *et al.* [22] with bovine serum albumin as the standard.

Statistics. The significance of differences between two means was established by Student's *t* test for paired or unpaired samples. To test the significance of differences between each of the treatments simultaneously with the untreated control, one-way analysis of variance was used in conjunction with Dunnett's multi-comparison test [23].

RESULTS

The effect of CsA and inducing agents on renal function and morphology

The 6-day pretreatment of animals with 3-MC or PB had no significant effect on the serum urea concentration or the urinary NAG activity measured on day 0. CsA significantly increased the serum urea

Table 1. The effect of CsA, inducing agents or CsA plus inducing agents on serum urea concentration

Treatment	Serum urea (mmole/l)			
	Day 0		Day 14	
Untreated control (6)		7.7 ± 0.4		6.0 ± 1.1
CsA (6)		5.7 ± 0.5		14.6 ± 5.2***
Aroclor 1254 (6)	NS	6.5 ± 1.2	NS	7.3 ± 2.5
Aroclor 1254 + CsA (6)		6.8 ± 0.8		7.1 ± 1.3
3-MC (4)	NS	8.2 ± 1.0	††	10.3 ± 1.5**
3-MC + CsA (4)		6.5 ± 1.0		15.3 ± 1.2**
PB (6)	NS	7.2 ± 0.7	NS	7.6 ± 0.5
PB + CsA (6)		6.1 ± 0.7		9.4 ± 0.7**

Results are means ± S.D., with the numbers of rats shown in parentheses after the treatment designation

Significant differences (by Student *t*-test for paired samples) between day 0 and day 14 values are shown thus: **P < 0.01; ***P < 0.001.

Significant differences (by Student *t*-test for independent samples), between values on either day 0 or day 14, for inducer plus CsA compared to value for that inducer alone are shown thus: ††P < 0.01; NS, not significant.

Table 2. The effect of CsA, inducing agents or CsA plus inducing agents on urinary NAG activity

Treatment		Urinary NAG activity (IU/mg) Day 0		Day 7		Day 14
Untreated control (6)		705 ± 99		743 ± 136		641 ± 155
CsA (6)		571 ± 164		1095 ± 474**		1321 ± 394***
Aroclor 1254 (6)	NS	676 ± 269	NS	976 ± 200	NS	775 ± 161
Aroclor 1254 + CsA (6)		630 ± 113		1183 ± 369**		1010 ± 305**
3-MC (4)	NS	1052 ± 255	†	1100 ± 178	NS	1139 ± 175
3-MC + CsA (4)		911 ± 106		1774 ± 391*		1249 ± 464
PB (6)	NS	1249 ± 234	NS	1474 ± 684	NS	1129 ± 255
PB + CsA (6)		1281 ± 81		1053 ± 171		1178 ± 117

Results are means ± S.D., with the number of rats shown in parentheses after the treatment designation.

Significant differences (by Student *t*-test for paired samples) between day 0 and day 7 and 14 values are shown thus: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Significant differences (by Student *t*-test for independent samples) between values on either day 0, 7 or 14, for inducer plus CsA compared to value for that inducer alone are shown thus: †$P < 0.05$; NS, not significant.

concentration over the two week course of its administration, either when used as the sole treatment or when given in combination with 3-MC or PB (Table 1: comparing day 0 and day 14 values for the same group of rats). There was no significant urea increase over the 14 days, however, when CsA was administered with Aroclor 1254. Of the inducers themselves, only 3-MC produced an increase in serum urea at day 14 compared to day 0. When day 14 urea levels, after combined treatment of inducer with CsA, were compared with day 14 urea levels after treatment with the same inducer alone, both Aroclor 1254 and PB (but not 3-MC) prevented a significant rise due to CsA.

Administration of CsA, either alone or in combination with 3-MC or Aroclor 1254, over 14 days produced a significant rise in urinary NAG activity (Table 2: comparing day 0 with day 7 and/or day 14 values for each group of rats). Co-treatment with CsA and PB, however, prevented the 14-day rise in NAG activity. No inducing agent on its own caused any significant change in NAG activity. When day 14 NAG activities for each combination of inducer plus CsA were compared with the same inducer alone, there were no significant differences. At day 7, however, the co-administration of 3-MC and CsA was associated with a rise in NAG activity.

Vehicle control treatments had no effect on serum urea concentrations or urinary NAG activities (data not shown).

Table 3. The effect of inducing agents on trough serum CsA concentration at day 14

Treatment	CsA (μg/ml)	Range (μg/ml)
CsA (6)	3.98 ± 2.45	1.22–7.02
Aroclor 1254 + CsA (6)	1.76 ± 1.22	0.94–3.88
3-MC + CsA (4)	3.08 ± 0.63	2.64–3.80
PB + CsA (6)	0.67 ± 0.52†	0.18–1.28

Results are means ± S.D. (number of rats shown in parentheses under 'treatment') and compared to those of the CsA group by Dunnett's test for multi-comparisons: †$P < 0.05$.

The treatment of rats with CsA resulted in histological damage to the kidneys, which took the form of vacuolation of the straight segment of the proximal tubule (Fig. 1A). In contrast, kidneys from rats treated jointly with CsA and PB or CsA and Aroclor 1254 were normal (Fig. 1B). Co-treatment with 3-MC, however, failed to prevent the renal damage caused by CsA.

Serum CsA levels

The combination of either Aroclor 1254 or PB resulted in a decrease in mean trough serum CsA concentrations compared to that obtained when CsA was given alone (Table 3). Due to the large inter-animal variation in serum CsA concentrations, however, only with PB was the change significant. Co-treatment with 3-MC had no effect on the serum CsA concentration.

The effect of inducing agents on the efficacy of CsA. In all the rats receiving CsA there was suppression of the humoral immune response to SRBC (Table 4). The co-administration of inducing agents had no effect on the immunosuppressive activity of CsA.

Cyt P-450 concentrations and related activities

The results in Table 5 confirmed that Aroclor

Table 4. The effect of CsA, inducing agents or CsA plus inducing agents on haemagglutination titres at day 14

Treatment	Titre ($-\log_2$)
Untreated control (6)	5.0 ± 1.2
CsA (6)	0.3 ± 0.5††
Aroclor 1254 (6)	6.3 ± 1.9
Aroclor 1254 + CsA (6)	1.3 ± 1.5††
3-MC (4)	5.6 ± 0.4
3-MC + CsA (4)	< 1††
PB (6)	6.5 ± 0.6
PB + CsA (6)	< 1††

Results are means ± S.D. (number of rats shown in parentheses under 'treatment') and compared to those of the untreated control by Dunnett's test for multi-comparisons: ††$P < 0.01$.

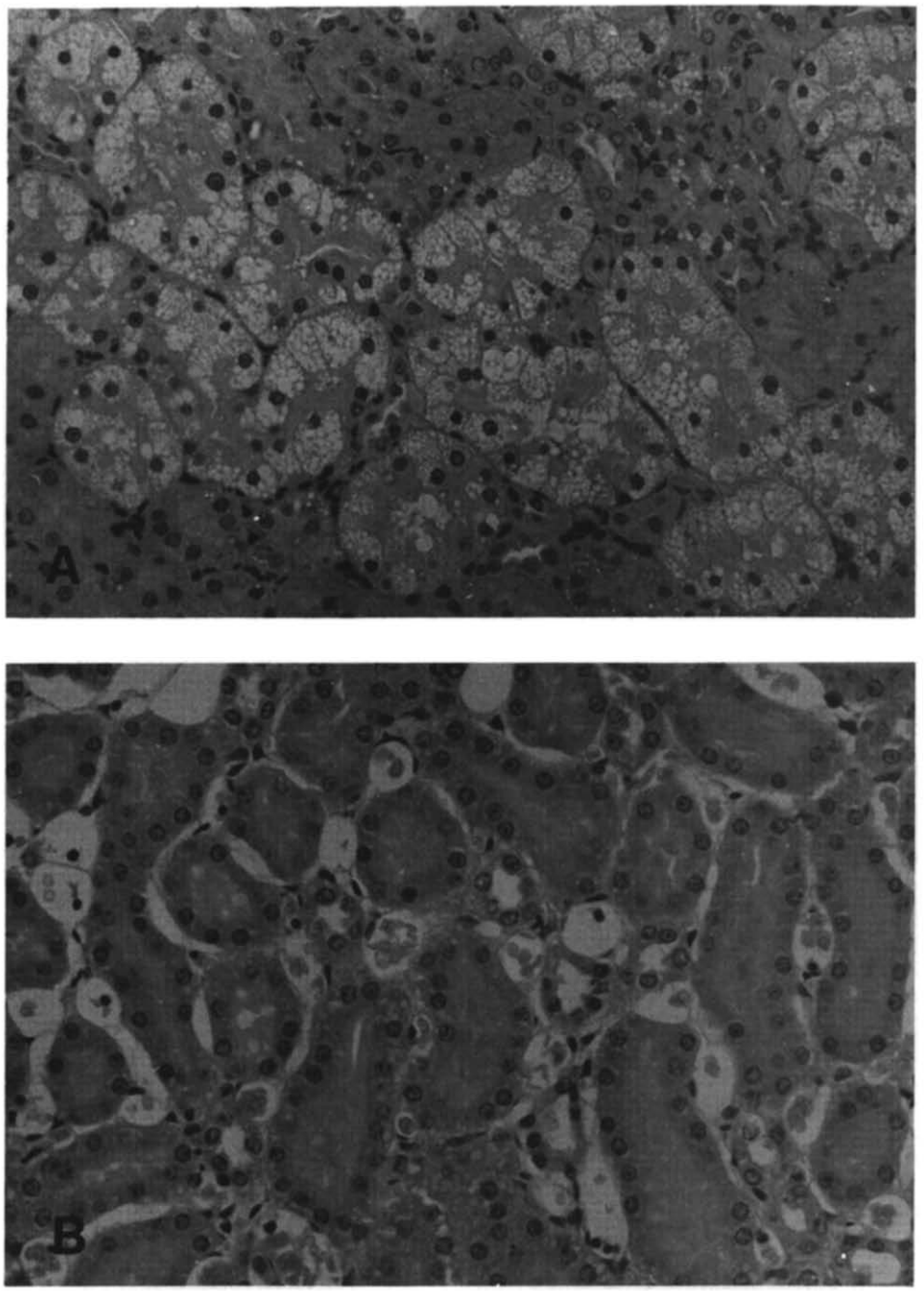

Fig. 1. Renal proximal straight tubules (A) after CsA alone and (B) following CsA and Aroclor 1254 H & E (× 350).

1254, 3-MC and PB, each either alone or in combination with CsA, induced cyt P-450 and/or the appropriate mono-oxygenase activities. CsA when given on its own caused a small induction of EROD, but had not effect on the other parameters. (When, however, CsA-treated rats were compared with rats treated with CsA vehicle alone (data not shown), there was a significant difference ($P < 0.05$) between the means of cyt P-450 concentration.) Co-administration of CsA had no significant effect on the induction caused by Aroclor 1254 or 3-MC, but it did significantly reduce the degree of induction of cyt P-450 and AD by PB. Vehicle treatments alone did not effect P-450 concentration or its mono-oxygenase activities (data not shown).

DISCUSSION

The administration of CsA (50 mg/kg) to normal rats once daily for 14 days resulted in significant renal toxicity, evidenced by increases in the serum urea concentration and urinary NAG activity and by vacuolation of the straight segment of the renal proximal tubules. This confirms previous studies both here

Table 5. The effect of CsA, inducing agents or CsA plus inducing agents on cyt P-450 concentration and related activities

Treatment		Cyt P-450[a]		AD[b]		EROD[b]		PPOD[b]
Untreated control (6)		1.1 ± 0.3		7.1 ± 1.5		0.18 ± 0.01		0.05 ± 0
CsA (6)		0.7 ± 0.1		5.8 ± 1.0		2.64 ± 0.88		0.16 ± 0.04
Aroclor 1254 (6)	NS	2.5 ± 0.1**	NS	12.8 ± 1.3**		ND		ND
Aroclor 1254 − CsA (6)		2.7 ± 0.3**		11.8 ± 4.1**		ND		ND
3-MC (4)	NS	1.4 ± 0.1	NS	8.0 ± 1.0	NS	16.9 ± 6.89	NS	0.27 ± 0.05
3-MC + CsA (4)		1.6 ± 0.2*		6.9 ± 1.1		15.30 ± 5.2**		0.30 ± 0.08
PB (6)	†††	1.8 ± 0.2**	†††	19.2 ± 2.1**	†	0.51 ± 0.12	NS	3.44 ± 2.22**
PB + CsA (6)		1.1 ± 0.2		13.1 ± 1.2**		0.31 ± 0.13		1.87 ± 0.49**

ND = not determined.

[a] nmole/mg protein.

[b] nmole/min/mg protein; AD, aminopyrine N-demethylase; EROD, ethoxyresorufin O-deethylase; PPOD, pentyloxyphenoxasone O-depentylase.

Results are means ± S.D. (number of rats shown in parentheses under 'treatment') and compared to those of the untreated controls by Dunnett's test for multi-comparisons: *$P < 0.05$; **$P < 0.01$.

Significant difference (by Student *t*-test for independent samples) for inducer plus CsA compared to those for that inducer alone are shown thus: †$P < 0.05$; †††$P < 0.001$; NS, not significant.

and elsewhere [12–14]. Co-treatment of CsA with either PB or Aroclor 1254, however, prevented this nephrotoxicity. Although we do not know the reason for this effect of PB or Aroclor 1254, a strong possibility must be that it was due to their induction of the cyt P-450-mediated metabolism of CsA, either in the liver or the kidney. There is evidence in support of this. In patients, it appears that nephrotoxicity occurs only when an individually determined threshold concentration of CsA is exceeded. In our experiments, prevention of CsA nephrotoxicity by PB or Aroclor 1254 was accompanied by a decrease in trough CsA serum concentration. The inference is that the induction of CsA metabolism resulted in the lowering of the CsA serum concentration to below the toxic threshold.

CsA is extensively metabolized in animals and man and the structures of the metabolites so far identified [15] are appropriate to a primary involvement of the cyt P-450-dependent mono-oxygenase enzyme system. PB and Aroclor 1254 however, induce UDP-glucuronyl transferase in addition to cyt P-450 and an induction of CsA conjugation, presumably following oxygenation, is an alternative explanation for our results, although Maurer *et al.* have reported an absence of conjugated metabolites in rat urine [15]. Experiments are currently in progress to resolve this question.

PB and 3-MC induce different sets of cyt P-450 isozymes, with differing substrate and reaction specificities [24]. Aroclor 1254 combines the cyt P-450 inducing properties of PB and 3-MC [25]. Our results indicate that it is the PB-inducible cyt P-450 isozymes that may detoxify CsA. The effects of PB and Aroclor 1254 on the parameters of CsA nephrotoxicity, however, were not identical. Both PB and Aroclor 1254 prevented the proximal tubular vacuolation. The rise in serum urea, however, was prevented by Aroclor 1254 but only lessened by PB. In contrast, the increase in urinary NAG was prevented by PB but not Aroclor 1254.

An important observation in this study was that the prevention of CsA nephrotoxicity by PB or Aroclor 1254 was achieved without any alteration to the observed immunosuppressive effect of CsA on the T lymphocyte dependent haemagglutinin response to SRBC.

There have been several recent clinical reports which can be related to the findings described here. Both Morgenstern *et al.* [26] and Ferguson *et al.* [27] have described interactions between CsA and the antifungal agent ketoconazole resulting in high trough serum CsA concentrations and White *et al.* [28] have confirmed these observations in experimental animals. Ketoconazole is a known inhibitor of cyt P-450-catalysed metabolism *in vivo* [29], although Daneshmend [30], having shown in healthy volunteers that ketoconazole had no apparent effect on cyt P-450-catalysed metabolism as assessed by the antipyrine half-life test, has suggested that the ketoconazole-CsA interaction may be due to changes in protein binding or the volume of distribution of CsA. A further case report from Langhoff and Madsen [31] showed that concomitant administration of CsA and the antituberculous agent rifampicin results in a decrease of blood CsA levels below measurable limits. Rifampicin is a known inducer of cyt P-450 [32]. These observations [26–28] support the findings of the present study and indicate that serum CsA concentrations need to be carefully monitored when the drug is given in combination with others.

The present study might be taken as an indication of the possibility of modifying the circulating trough levels of CsA in human patients, to reduce the nephrotoxic properties whilst retaining immunosuppression. A more practical solution to the problem of nephrotoxicity might be the use of a non-toxic, but still immunosuppressive metabolite of the parent CsA molecule: to date, this has proved elusive.

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