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SERUM CYCLOSPORIN LEVELS, HEPATIC DRUG METABOLISM AND RENAL TUBULOTOXICITY

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Abstract—The present study was designed to examine inter-relationships between serum cyclosporin (CsA) levels, hepatic drug metabolising enzyme activity and CsA induced nephrotoxicity. CsA (25 mg/kg p.o.) was administered daily to male Sprague–Dawley rats: groups of animals were killed on days 0, 4, 7, 10 and 14 and thereafter at weekly intervals over the 7-week course of the experiment. Nephrotoxicity was evaluated by measuring tubular enzymuria and by light microscopy and serum CsA levels (parent drug plus certain metabolites) were determined by radioimmunoassay. The hepatic microsomal mono-oxygenase enzyme system was monitored by measurement of cytochrome P-450, aminopyrine *N*-demethylase and NADPH-cytochrome c reductase.

Nephrotoxicity appeared within 4 days of starting treatment and continued for 4 weeks. Between weeks 4 and 6 there was a period of complete remission followed by the return of renal damage. Aminopyrine *N*-demethylase activity fell during the first 4 weeks. During the period of remission, however, *N*-demethylase activity rose to a point significantly higher than pretreatment values and serum CsA levels fell to their lowest concentration. With relapse, hepatic *N*-demethylase activity again fell below normal and serum drug levels rose to their pre-remission values. From the third week onward, changes in NADPH-cytochrome c reductase activity paralleled those in *N*-demethylase activity. The hepatic microsomal concentration of cytochrome P-450 did not, however, change significantly during the 7-week period of CsA treatment.

Our results suggest that the spontaneous remission of CsA-induced nephrotoxicity is due to a reduction in circulating drug levels caused by increased hepatic CsA metabolism.

Nephrotoxicity is the most important side effect of cyclosporin (CsA) and has been well documented in patients [1–4] and experimental animals [5–7]. In a previous study, however, we have noted spontaneous improvement in renal function in rats during the continuous administration of a constant dose of CsA [8]. This period of remission was followed by a further episode of impaired renal function. The basis of this phenomenon is unclear, but may be related to circulating CsA levels, which we have suggested are affected by activity of the appropriate hepatic drug metabolising enzymes [9, 10]. In the present study, we have further investigated the inter-relationships between renal tubulotoxicity induced by CsA, trough serum drug levels and hepatic drug metabolism.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (250–300 g) bred in the University Animal Department, Foresterhill, Aberdeen, were used throughout. They were housed in a temperature-controlled environment and received Oxoid pasteurized rat and mouse breeding diet with tap water *ad libitum*, except as specified below.

Cyclosporin. CsA (OL 27–400; Sandoz Ltd., Basle, Switzerland) was provided in powder form and dissolved initially at 20° in absolute ethanol. A solution of 10% ethanol in olive oil B.P. (Boots Company Ltd.) was then prepared and 0.2 ml administered to the conscious rat by gastric intubation, using a 4-fine gauge intravenous cannula (Portex Ltd., Hythe, Kent, England).

Experimental protocol. Two groups of 40 rats each were treated with either CsA (25 mg/kg once daily) or vehicle. Urine was analysed immediately before the start of treatment, at 4, 7, 10 and 14 days and at weekly intervals thereafter until day 49. At each of these times, 4 rats from each group were killed by terminal ether anaesthesia and autopsies performed.

Blood and urine sampling. Whole blood samples were collected into plain tubes from the tail tips of rats under ether anaesthesia. Serum expressed from the clotted blood (2 hr at ambient room temperature) was stored at −20° until assayed.

Urine free of faecal contamination was collected overnight (16 hr) from animals placed in metabolic cages with access to water but without food throughout the collection period.

Radioimmunoassay of CsA. Estimations of trough CsA levels in serum samples obtained 16 hr after drug administration were performed using radioimmunoassay kits supplied by Sandoz Ltd. (Basle) as described by Donatsch *et al.* [11]. The immunoassay does not distinguish between the parent CsA molecule and certain of its metabolites.

Urinary NAG activity. Determinations of urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity were conducted as previously described [5]. NAG levels were expressed as IU per mg creatinine in the urine.

Preparation of tissue for microscopy. For light

microscopy, tissue (kidney, liver, thymus, spleen, lumbar lymph nodes and bone marrow) was fixed in 10% neutral buffered formalin and processed to acrylic resin: 2 μm sections were stained with haematoxylin and eosin. For electron microscopy, 1 mm^3 kidney blocks were fixed in 4% formaldehyde and 1% glutaraldehyde, then processed to epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination in a Jeol 100S transmission electron microscope.

Histological assessment of tubular cell damage. Sections of renal tissue were examined "blind" by an experienced renal histopathologist. The following scoring system was adopted to indicate the incidence of proximal straight tubular profiles affected: (0), normal; (1), <5%; (2), >5%; (3), >50%; (4), >90%.

Microsome preparation. After tissue sampling for microscopic examination the remainder of the liver was used to prepare microsomes as described by Burke *et al.* [12].

Determination of cytochrome P-450 concentration and aminopyrine N-demethylase and NADPH-cyt c reductase activities. The concentration of hepatic microsomal cytochrome P-450 (cyt P-450) was measured by the technique of Omura and Sato [13] from the CO-reduced difference spectrum, using a Cary 219 spectrophotometer and an extinction coefficient of 91 $mm^{-1}.cm^{-1}$.

Aminopyrine *N*-demethylase (AD) activity was measured by the method of Nash, as described previously [12]. NADPH-cytochrome c reductase (cyt c reductase) was measured by direct spectrophotometry at 37°, using the method of Phillips and Langdon [14] and an extinction coefficient for reduced cytochrome c of 21.1 $mm^{-1}.cm^{-1}$. Protein levels were determined by the method of Lowry *et al.* [15] with bovine serum albumin as standard.

Statistics. The significance of differences between means was determined using Student's *t*-test for independent samples. P values <0.05 were considered significant.

RESULTS

In this and in previous studies we observed no significant pathological effect attributable to the CsA vehicle (ethanol/olive oil).

Body weight changes and effects on tubular cells. The effects of CsA on body weight, urinary NAG activity and the extent of damage inflicted on renal tubular cells are shown in Table 1. During the 10 days following the start of CsA treatment there was a 10% decrease in body weight, accompanied by progressive NAG enzymuria. Over the same period, a steady increase in the degree of proximal straight tubular cell injury was observed. Histologically, this damage was already apparent by day 4 and consisted of varying degrees of cytoplasmic vacuolation, affecting an increasing proportion of tubular cell profiles as time progressed. At the ultrastructural level, these changes could be attributed to dilatation of the endoplasmic reticulum, with concomitant increases in lysosome number and size.

From days 10 to 35, the animals showed progressive weight gain. During the same period, there was a gradual reduction in urinary NAG activity which had returned to normal by day 28. Unfortunately, we were unable to estimate NAG levels on day 35. Between days 10 and 28, the kidneys from the four animals killed at each time point showed similar degrees of proximal straight tubular damage histologically (Fig. 1a). In contrast, three of the four animals killed at day 35 had histologically normal kidneys (Fig. 1b), the fourth exhibiting only minimal (<5%) tubular damage. Between days 35 and 42, a fall in body weight was again observed and there was striking biochemical and microscopic evidence (Fig. 1c) of tubular cell damage. Indeed, the structural changes on day 42 were the most pronounced observed during the study.

Table 1. The effects of CsA on body weight, NAG enzymuria and renal histology

Day	Weight (g)	NAG IU/mg creatinine	Tubular cell damage
0	339 ± 13	864 ± 95	0 ± 0
4	338 ± 13	945 ± 540	0.5 ± 0.6
7	335 ± 6	1157 ± 646	1.5 ± 0.3
10	303 ± 8**	1755 ± 304*	2.3 ± 1.0*
14	325 ± 16	1501 ± 517	2.0 ± 1.2†
21	339 ± 41	1263 ± 196†	1.8 ± 1.0†
28	373 ± 26*	621 ± 127†§	2.5 ± 1.3*
35	394 ± 20**	ND	0.3 ± 0.5‖
42	348 ± 29	1663 ± 107**	3.0 ± 0.8**§
49	352 ± 42	1502 ± 335*	2.8 ± 0.8**

Results are means ±S.D. Four animals per group.
Results compared to day 0 value †$P < 0.05$; *$P < 0.01$; **$P < 0.001$ and to previous value §$P < 0.01$; ‖$P < 0.001$ by Student's *t* test for independent samples.
ND = not determined.

At no time point in the study was there any evidence of glomerular damage, as observed by light or electron microscopy.

Histological changes in other tissues. In comparison to the kidney, sections of the liver, thymus, spleen, lymph nodes and bone marrow showed structural damage which was progressive over the entire experimental period. In the liver the changes consisted of moderate predominantly centrilobular fatty change, whereas lymphocytic depletion was observed in the lymphoid organs. Bone marrow showed hypoplasia and increased fat space. A full account of the histological changes observed in rats after 7 week's treatment with 25 mg/kg/day CsA has been published elsewhere [8].

Effects of CsA on drug-metabolising enzyme activity. To ascertain the influence of CsA on the hepatic mono-oxygenase enzyme system, microsomal cyt P-450 concentrations and AD and cyt c reductase activities were quantified throughout the study (Table 2). Although there was no significant change in cyt P-450 levels, a highly significant fall in AD activity had occurred by day 14. Between days 14 and 35, by which latter time the tubular cell injury was minimal, a steady increase in AD activity was recorded. Indeed, at day 35 AD activity was 33% above that prior to the start of CsA administration.

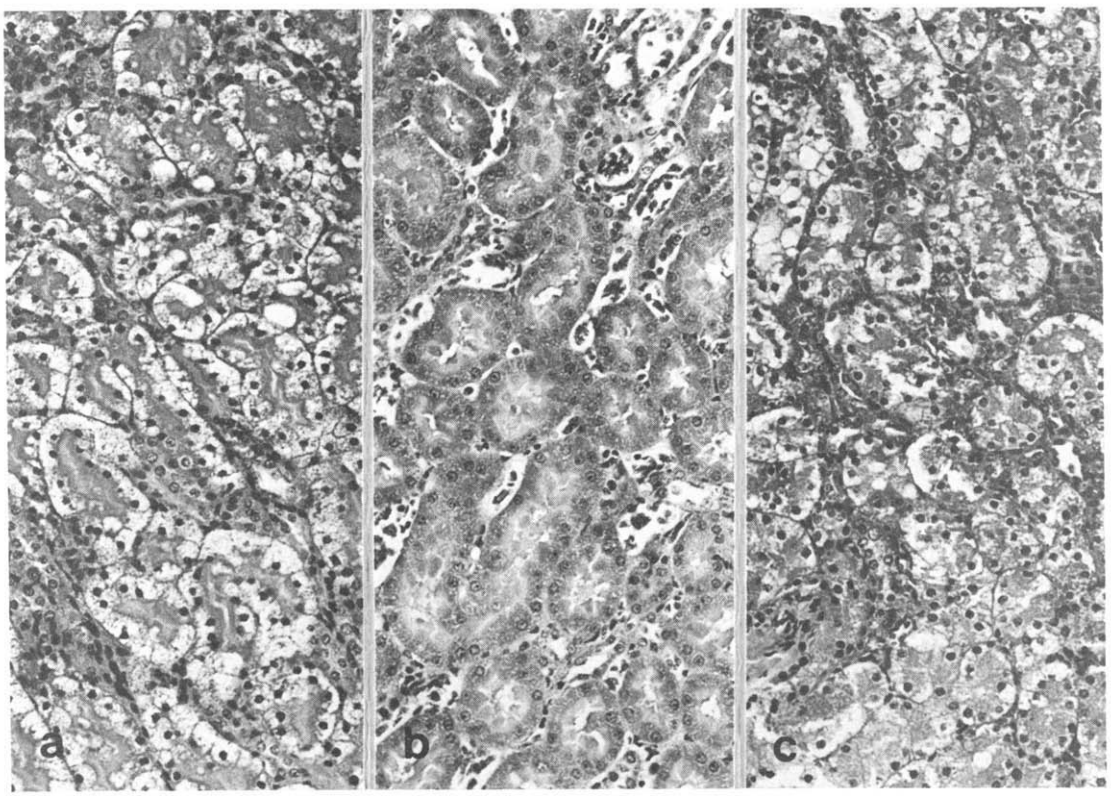

Fig. 1. Spontaneous remission of CsA-induced nephrotoxicity. (a) Renal cortex after CsA treatment for 28 days, showing gross proximal straight tubular cell vacuolation; (b) at 35 days, the histological appearance is normal; (c) by 42 days, structural damage is again pronounced. Haematoxylin and eosin ×170.

Thereafter, however, there was a dramatic fall in AD activity (40% decline between days 35 and 42), which accompanied the second phase of tubular cell injury. Cyt c reductase activity was measured on day 0 and from the third week of CsA treatment onward. The pattern of changes in reductase activity mirrored the changes in AD. On day 21, the reductase activity was significantly lower than on day 0, followed by a steady increase up to day 35, after which it declined again. From days 21 to 49 reductase activity was always significantly lower than pretreatment values.

Serum CsA levels. CsA levels were measured on days 28–49, the period during which first remission then re-emergence of tubular cell damage occurred, accompanied by first augmentation then loss of mono-oxygenase enzyme system activity. As can be seen in Table 3, there was a highly significant (70%) fall in serum drug levels between days 28 and 35. This was followed by a progressive increase in CsA concentrations to pre-remission levels on day 49.

Table 2. The effects of CsA on hepatic drug metabolising enzyme activity

Day	Cyt P-450 (nmole/mg protein)	AD (nmole formaldehyde/min/mg protein)	Cyt c reductase (nmole/min/mg protein)
0	0.94 ± 0.04	6.58 ± 0.34	382.2 ± 27.5
4	0.96 ± 0.04	5.32 ± 0.78†	ND
7	0.93 ± 0.02	6.29 ± 0.59	ND
10	0.95 ± 0.04	4.68 ± 0.84*§	ND
14	0.96 ± 0.04	4.18 ± 0.46**	ND
21	0.94 ± 0.12	5.35 ± 0.45*§	74.8 ± 14.6**‖
28	0.83 ± 0.13	5.74 ± 0.78	124.9 ± 17.4**‖
35	0.92 ± 0.15	8.79 ± 0.45**‖	246.1 ± 38.0**‖
42	0.86 ± 0.16	3.56 ± 0.79**‖	147.6 ± 57.9**§
49	0.82 ± 0.19	4.05 ± 0.87*	114.3 ± 7.3**

Results are means ± S.D. Four animals per group.

Results compared to day 0 value †$P < 0.05$; *$P < 0.01$; ** < 0.001 and to previous value §$P < 0.05$; ‖$P < 0.001$ by Student's *t*-test for independent samples.

AD = Aminopyrine *N*-demethylase. ND = Not determined.

Table 3. Trough serum CsA levels

Day	CsA (μg/ml)
28	6.45 ± 1.82
35	1.95 ± 1.05*
42	4.66 ± 0.88*
49	9.45 ± 4.72

Results are means ±S.D. Four animals per group.
Results compared to previous result by Student's *t*-test for independent samples *$P < 0.01$.

DISCUSSION

We have studied the renal tubulotoxicity of CsA in relation to hepatic drug metabolising enzyme activity and serum drug levels for 7 weeks. Damage to the proximal renal tubule, consisting of cytoplasmic vacuolation and an increase in the incidence of lysosomes, was cyclical in nature, with the initial structural changes being progressive over the first 10–28 days. This was followed by reversion to an almost normal histological appearance on day 35, with a second episode of damage evident on days 42 and 49. The changes in body weight of the animals were inversely related to the degree of renal tubular damage. Tubular cell injury was chronologically related both to loss of hepatic AD and cyt c reductase activities and to trough serum CsA levels. Conversely, remission coincided with comparatively high AD activity, a return of reductase activity towards control levels and low serum CsA levels.

The mono-oxygenase system incorporates both cyt P-450 and cyt c reductase. Since there was no significant change in cyt P-450 levels during the study, it is possible that the changes in AD activity were due to comparable changes seen in cyt c reductase activity. AD activity has been used here as an index of mono-oxygenase activity and the question of whether mono-oxygenase activities with other substrates are also affected by CsA is currently under investigation. The cyclical nature of the changes in hepatic mono-oxygenase activity suggests that the decrease in activity was caused by a mono-oxygenase-generated metabolite of CsA perhaps in a fashion analogous to that proposed for either parathione [16], troleandomycin [17] or allyl-sopropylacetamide [18] (which illustrate respectively three different mechanisms of decreased cyt P-450 activity due to drug metabolites). The reduction in mono-oxygenase activity so produced would result eventually in decreased levels of active metabolite, with consequent restoration of drug metabolism. In due course however, re-initiation of the "suicide inactivation" process would occur.

The structure of CsA metabolites is consistent with the involvement of cyt P-450-dependent drug metabolism [19]. Moreover, use of Aroclor 1254 to induce both the cytochrome and its associated AD activity has been shown to reduce the nephrotoxicity of CsA, without affecting its immunosuppressive capacity [10]. Although the radioimmunoassay method cannot distinguish between parent CsA and certain of its metabolites, our findings are most consistent with cyclical changes in trough serum CsA concentrations being determined largely by similar cyclical changes in hepatic microsomal mono-oxygenase system activity. Moreover, our results suggest that the renal tubular damage is due to the parent CsA molecule (rather than a metabolite) exceeding a threshold toxic level.

CsA exerts its nephrotoxic effect selectively on the proximal straight tubule and previous studies [7, 20] have shown that NAG enzymuria correlates well with this structural damage. Similar histological and ultrastructural changes to epithelial cells lining the rat proximal renal tubule have been demonstrated at the same CsA dose by Farthing *et al.* [21] and Ryffel [22].

We have previously reported the cyclical nature of the tubulotoxicity of CsA with spontaneous improvement in renal function in normal [8] and uninephrectomised animals [Gavin *et al.*, submitted for publication], even though the dose of drug administered was not altered during the treatment period. This and our earlier study [8] showed that the temporary reduction in tubular toxicity (as evidenced by NAG-enzymuria and microscopy) occurred around day 35.

The cyclical nature of the tubulotoxicity was not observed in the liver, lymphoid tissue or bone marrow, in all of which structural damage was progressive over the experimental period. Only in the case of the kidney could serum CsA levels be related to the degree of structural abnormality. This indicates the further possibility that different agents (i.e. metabolites of CsA) may be responsible for structural changes in other tissues.

The findings of the present study, i.e. that cyt P-450-related drug metabolising enzyme activity can be temporally related to both trough serum CsA levels and renal tubular damage, indicate the need for caution in the choice of other therapeutic agents for use in combination with CsA. Indeed, there is already evidence from human patients that cimetidine [23] and the anti-fungal agent ketoconazole [24] may potentiate CsA nephrotoxicity by inhibiting metabolism of the drug, whilst rifampicin may render CsA ineffective by inducing its metabolism [25].

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