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Chronic Cyclosporin A Nephrotoxicity, P-Glycoprotein Overexpression, and Relationships with Intrarenal Angiotensin II Deposits

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P-glycoprotein (P-gp) expels hydrophobic substances from the cell, including chemotherapeutic agents and immunosuppressants such as cyclosporin A (CsA) and FK506. Exposure of cultured renal tubular cells to CsA induces P-gp overexpression in cell membranes. Angiotensin II has recently been implicated as the principal factor responsible for progression of interstitial fibrosis induced by CsA. To investigate the *in vivo* relationships between histological lesions, P-gp overexpression, and intrarenal angiotensin II deposits, we developed a model of chronic CsA toxicity in Sprague-Dawley rats treated with 25 mg/kg/day CsA for 28 and 56 days and fed either a standard maintenance diet or a low-salt diet. Immunohistochemical methods were used to study the expression of P-gp in renal tubular cells and the appearance of intrarenal angiotensin II deposits. Rats treated with CsA developed chronic nephrotoxicity lesions that were more evident in the group fed the low-salt diet. Treatment with CsA induced overexpression of P-gp in tubular cells of the kidney that increased with time. We found that immunohistochemical expression of P-gp was slightly more severe in rats fed a low-salt diet. Intrarenal deposits of angiotensin II were more evident in rats treated with CsA; these deposits also increased with time. This finding was also more relevant in rats given the low-salt diet. The up-regulation of P-gp was inversely related to the incidence of hyaline arteriopathy ($r = -0.65$; $P < 0.05$), periglomerular ($r = -0.58$; $P < 0.05$) and peritubular fibrosis ($r = -0.63$; $P < 0.05$), and intrarenal angiotensin II deposits in animals with severe signs of nephrotoxicity ($r = -0.65$; $P < 0.05$). These results support the hypothesis that the role of P-gp as a detoxicant in renal cells may be related to mechanisms that control the cytoplasmic removal of both toxic metabolites from CsA and those originating from the catabolism of signal transduction proteins (methylcysteine esters), which are produced as a result of *ras* activation in presence of angiotensin II. *(Am J Pathol 1997, 151:1705–1714)*

Cyclosporin A (CsA) induces immunosuppression by interfering with normal T-lymphocyte function.[1,2] In addition to its immunosuppressant activity, CsA reverts multidrug resistance (MDR) phenomena in tumor cells,[3] shows antiproliferative activity on epithelial cells,[4] inhibits elongation factor 2 of protein biosynthesis,[5] alters the expression of certain oncogenes such as c-*myc*, N-*ras*, and c-*fos*,[6] and induces transforming growth factor (TGF)-β expression.[7]

One of the main secondary effects of CsA treatment is nephrotoxicity, reported both in clinical[8] and experimental studies.[9,10] Morphological findings of CsA nephrotoxicity include vacuolar isometric degeneration of kidney tubules, hyaline arteriopathy, and stripped and diffuse interstitial fibrosis.[11,12]

Experimental models in rats have shown that low-sodium (LS) diets decrease renal blood flow and intensify histopathological lesions of nephrotoxicity, especially hyaline arteriopathy and stripped tubulointerstitial fibrosis.[13] Because the structural changes in this kind of model are related to the manipulation of salt depletion,[14] the role of the renin-angiotensin system (RAS) in chronic CsA nephrotoxicity needs to be considered. In this context, recent studies have shown that blockers of angiotensin II cellular receptor, as well as angiotensin II-converting enzyme inhibitors, prevent the development of interstitial fibrosis in experimental models of chronic CsA nephrotoxicity.[15,16]

The physiopathology of this process involves vascular, interstitial, and tubular factors; the role of P-glycoprotein (P-gp) has recently been identified in the latter two[17,18]

A preliminary report of these findings was presented in 1996 at the 2nd European Kidney Research Forum, Baveno (Lake Maggiore), Italy.

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(reviewed in Ref. 19). P-gp is a 170-kd molecule[20] coded by the *mdr*-1 gene in humans.[21] It belongs to the ATP-binding cassette (ABC) protein family, which includes the product of the cystic fibrosis gene.[22] In functional terms, P-gp acts according to a "hydrophobic vacuum cleaner" model,[23] expelling hydrophobic substances from the cell, including chemotherapeutic and antimitotic agents, local anesthetics, steroid hormones, diverse peptides, and immunosuppressants such as CsA and FK506.[24] Consequently, P-gp is present in tissues with secretory or molecular synthesis and transport functions such as the proximal tubules of the kidney.[17,25–27] Its overexpression has been demonstrated in association with increased deposits of CsA in post-transplant renal biopsies,[17] and it was recently reported that after CsA treatment P-gp is dramatically increased in the brush-border membranes (BBMs) of the renal tubules, intestine, and liver in a rat experimental model.[28] In addition, P-gp mediates the secretion of fluorescent analogues of cyclosporine in the renal proximal tubules of teleost fish.[29] It has also been demonstrated that P-gp is related to a volume-regulated chloride channel activity[30] and that its presence on the surface of cultured cells can be induced by saline hypertonic shock.[31]

The aims of the present study were to analyze the modifications induced by CsA in P-gp expression in kidney tubular cells of Sprague-Dawley rats and to characterize the relationship between these changes with the angiotensin II deposits and lesions of chronic CsA nephrotoxicity.

Materials and Methods

Animals

Eighty male Sprague-Dawley rats (Charles River, Saint Aubin les Elbeuf, France) with an initial body weight between 270 and 320 g were housed before and after treatment in metabolic cages in a temperature- and light-controlled environment. One-half received LS diet UAR 212 (0.05% Na) and the other half maintenance diet UAR A042 (Uar, Orge, France), with tap water *ad libitum*. All procedures were performed according to Spanish Government and European Union regulations (EEC Directive 86/609).

Drugs

Powdered CsA (Sandoz Pharmaceutical, Basel, Switzerland) was dissolved in propyleneglycol (PLG; Sigma Chemical Co., St. Louis, MO). Alzet 2ML4 osmotic pumps (Alza Corp, Palo Alto, CA) were filled with the CsA solution to obtain 25 mg/kg/day doses, delivered at a constant rate of 2.5 μl/hour. Control rats received PLG and 0.9% NaCl solution (SC), also via osmotic pumps.

Osmotic Pump Implantation

Osmotic pumps were implanted into rats according to the instructions provided by Alza Corp. Rats were anesthetized with 40 mg/kg pentobarbitone sodium intraperitoneally. The back of each animal was cleaned with povidone-iodine, and a sagittal incision was made. The pump was placed beneath the skin and the incision was then closed by suture and treated with a plastic dressing spray. The pumps contained enough solution for 28 days, at which time they were replaced.

Experimental Design

The rats were randomly divided into the following groups of animals: 1) CsA group, 20 rats with CsA treatment (25 mg/kg/day); 2) LS+CsA group, 20 rats on a LS diet with CsA treatment (25 mg/kg/day); 3) PLG group, 10 rats receiving PLG; 4) LS+PLG group, 10 rats on a LS diet receiving PLG; 5) SC group, 10 rats receiving 0.9% SC; and 6) LS+SC group, 10 rats on a LS diet receiving 0.9% SC.

All rats were housed in metabolic cages for 1 week before treatment. After 28 and 56 days, 50% of the animals in each group were killed by heart puncture under light ether anesthesia, and whole blood was extracted to measure whole blood parameters and CsA levels. Both kidneys were removed and processed for histological, immunohistochemical, and Western immunoblotting analysis.

Blood Parameters Studies

Whole blood CsA levels and aldosterone were measured by radioimmunoassay (Cyclo-Trac SP, Incstar Corp., Stillwater, MN, and Aldo-CT, CIS Bio International, Gif-sur-Yvette, France).

Blood parameters (urea, creatinine, and serum Na^+) were measured using Hitachi 705 (Boehringer Mannheim, Mannheim, Germany) and Astra 4 (Beckman Instruments, Fullerton, CA) analyzers. Fractional excretion of sodium was calculated using standard formulas.

Renal Histopathology and Digital Image Quantification of Interstitial Fibrosis

For histological examinations, buffered 4% formaldehyde-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin, Masson-Goldner trichromic stain, and periodic acid-Schiff stain (PAS). Preparations of the renal cortex were examined by two experienced pathologists who evaluated the presence of isometric vacuolization in tubular cells. Lesions were scored on a five-point semiquantitative scale (1, absence; 2, lesions in fewer than 10% of the tubules; 3, lesions in 10 to 25% of the tubules; 4, lesions in 25 to 50% of the tubules; 5, lesions in more than 50% of the tubules). In PAS-stained sections, the presence of hyaline arteriopathy was also evaluated.

To quantify interstitial fibrosis, slides were examined automatically with digital image analysis. Because sclerosis in the renal cortex was distributed irregularly, images containing glomeruli (areas of periglomerular fibro-

sis) were quantified separately from images containing only tubules and interstitium. These images were selected mainly from the areas of the medullary rays, where the first signs of CsA-induced tubulointerstitial nephropathy usually appear.[13]

Image analysis was done in paraffin-embedded tissues stained with Sirius red. Peritubular and periglomerular areas were analyzed in 20 microscopic fields. The images were captured using a BCD-700 CCD Vidamax video camera connected to a BH-2 Olympus microscope (×200 magnification) and the 256-gray-level scale (black and white) was processed with the Visilog 4.1 image analysis program (Noesis S.A., Velizy, France) and the fibrosis HR package (Master Diagnóstica, Granada, Spain), both running under Windows (Microsoft, Redmond, WA). The fibrosis HR program automatically extracts basal membranes and connective tissue stained with Sirius red, eliminates the background of tubular cell nuclei, and extracts glomerular area according to an algorithm that identifies the glomerular uriniferous space. When there were zones of contact between the capillary area and the Bowman's capsule, it was occasionally necessary to discriminate them with the manual interaction option of the program. The results were expressed as the percentage of fibrosis relative to the other elements in the image after the glomerular area was subtracted. This method yielded accurate automatic quantifications of peritubular and periglomerular fibrosis.[32]

Primary Antibodies

Two murine monoclonal antibodies (MAbs) were used in a cocktail to detect P-gp: JSB-1 (IgG1 isotype, ascitic fluid; Sanbyo, Uden, The Netherlands) and P-glycoCHEK C-219 (IgG2 isotype, purified antibody; Centocor, Malvern, PA). Both MAbs, which react with a conserved cytoplasmic epitope of P-gp,[33,34] were used together at dilutions of 1:40 (JSB-1) and 1:10 (C-219) for immunohistochemistry.

Angiotensin II was detected with a specific polyclonal antibody at a dilution of 1:500 (Peninsula Laboratories, Belmont, CA).

Vimentin expression was assessed with a specific MAb (clone V9, IgG1 isotype; Dakopatts, Glostrup, Denmark) at a dilution of 1:50.

Immunohistochemical Methods

Immunostaining for P-gp was done on 4-μm cryostat sections with the alkaline phosphatase anti-alkaline phosphatase technique (APAAP) using the MAb cocktail directed against P-gp. After tissue was frozen in isopentane at −50°C and sectioned, the slides were air dried and fixed in acetone at 4°C for 5 minutes, allowed to dry, and post-fixed in chloroform for 30 minutes at room temperature. After two 3-minute washes in Tris-buffered saline (TBS), nonspecific reactivity was blocked with 0.3% casein and normal 20% rabbit serum. The sections were incubated with a mixture of the two diluted primary antibodies at 50% in a moist chamber at 4°C for 16 hours. Sections were then washed three times in TBS and incubated for 30 minutes at room temperature with rat absorbed anti-mouse rabbit immunoglobulin (Dakopatts) at 1:50 dilution in TBS. After careful washing, the slides were incubated with the mouse APAAP complex (Dakopatts) at 1:100 dilution for 30 minutes at room temperature and then washed and incubated in the chromogenic substrate fast red TR salt (Dakopatts) under microscopic control until the color signal appeared. Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma) at a concentration of 25 mg/ml. Samples were then washed in water, counterstained with Mayer's hematoxylin, and mounted with Aquatex (Merck, Darmstadt, Germany). Negative control slides were stained with an isotype-matched control cocktail instead of the primary anti-P-gp antibodies cocktail.

Immunostaining for vimentin and angiotensin II was done in 4-μm paraffin-embedded kidney sections using standard multilink streptavidin-biotin-peroxidase and alkaline phosphatase kits, respectively (Master Diagnóstica). For vimentin, deparaffinized sections were treated with 0.3% H_2O_2 in methanol for 12 minutes and were then autoclaved for 10 minutes in 0.1 mol/L citrate buffer (pH 6.0) for antigen retrieval before immunostaining.

Immunostaining for the neoexpression of tubular vimentin, P-gp, and angiotensin II was evaluated semiquantitatively by two experienced pathologists and scored for tubular vimentin as follows: 1, negative pattern; 2, fewer than 10% of tubules stained; 3, 10 to 25% of tubules stained; 4, 25 to 50% of tubules stained; and 5, more than 50% of tubules stained. P-gp and angiotensin II were scored as follows: 1, normal pattern; 2, mild overexpression; and 3, moderately intense overexpression (see Figures 1, C–E, and 3, B–D).

Western Immunoblot Analysis

P-gp was analyzed in crude membrane fractions using MAbs C-219 (Centocor) and JSB-1 (Sanbyo) as described below. For the preparation of the membranes, rat kidneys were homogenized in 1 mmol/L Tris/HCl (pH 7.5), 5 mmol/L EDTA, containing the protease inhibitor phenylmethylsulfonyl fluoride at 0.5 mmol/L in a dounce homogenizer. Nuclei and cell debris were pelleted at 600 × *g* for 10 minutes at 4°C, and the supernatant was centrifuged at 100,000 × *g* for 45 minutes at 4°C. The resulting crude membrane pellets were resuspended in lysis buffer (10 mmol/L Tris/HCl, pH 7.5, 5 mmol/L EDTA, 5 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride) and stored at −80°C. Proteins were quantitated by the Lowry method, and 100 μg were loaded onto duplicate 7.5% acrylamide gels for analysis. One gel was subsequently stained with Coomassie blue, and the other was electroblotted to nitrocellulose at 200 mA for 16 hours at 4°C. P-pg was detected with the MAbs C-219 and JSB-1. The filter was incubated overnight at 4°C with a mixture of both MAbs at dilutions of 1:1000 (JSB-1) and 1:500 (C-219) in phosphate-buffered saline with 0.1% bovine serum albumin and 0.1% Tween 20. The signal was detected by incubating with a rabbit anti-mouse secondary

Table 1. Analytical, Histological, and Immunohistochemical Results after 28 and 56 Days in Different Treatment Groups Given a Normal Diet

	28 Days			56 Days			Statistical significance (ANOVA2)			
Group	CsA	PLG	SC	CsA	PLG	SC	SBG	SBD	SIBG	NKT
FW	332 ± 41	363 ± 20	368 ± 58	401 ± 9.0	406 ± 39.0	409 ± 25.0	NS	NS	$P < 0.01$	CsA *vs* AlIs
WC	76 ± 36	88 ± 16	97 ± 66	139 ± 13	134 ± 34.0	146 ± 28.0	NS	$P < 0.001$	No	No
Cr	0.60 ± 0.14	0.59 ± 0.1	0.51 ± 0.1	0.69 ± 0.1	0.62 ± 0.05	0.60 ± 0.1	NS	NS	No	No
Urea	73 ± 45	52 ± 24	38 ± 5.0	92 ± 53	50 ± 12.0	52 ± 9.0	$P < 0.05$	NS	No	CsA *vs* AlIs
Na^+	143 ± 60	148 ± 9.0	142 ± 4.0	146 ± 4.0	147 ± 7.0	150 ± 5.0	NS	NS	No	PLG *vs* CsA
ALD	258 ± 323	362 ± 408	111 ± 35	305 ± 431	133 ± 125	136 ± 96.0	NS	NS	No	No
FeNa	0.13 ± 0.07	0.11 ± 0.05	0.14 ± 0.04	0.09 ± 0.03	0.11 ± 0.03	0.15 ± 0.04	NS	NS	No	SC *vs* AlIs
PTF	10.27 ± 2.23	8.32 ± 0.97	8.19 ± 1.02	13.64 ± 5.27	8.26 ± 1.68	8.52 ± 0.59	$P < 0.001$	NS	No	CsA *vs* AlIs
PGF	9.44 ± 1.34	8.24 ± 0.49	6.85 ± 1.31	12.41 ± 3.94	7.67 ± 1.77	7.84 ± 0.67	$P < 0.01$	NS	No	CsA *vs* AlIs
ITV	1.37 ± 0.04	1.2 ± 0.5	1.0 ± 0.0	2.0 ± 0.7	2.0 ± 0.8	1.4 ± 0.2	NS	NS	No	No
PHA	16.3%	0%	0%	34.3%	0%	0%	$P < 0.01$*	NS*		
T P-gp	2.25 ± 0.67	1.0 ± 0.0	1.05 ± 0.37	2.55 ± 0.6	2.02 ± 1.12	1.5 ± 0.15	$P < 0.001$	$P < 0.01$	No	CsA *vs* AlIs
AGII	1.51 ± 0.75	1.26 ± 0.43	1.0 ± 0.0	2.55 ± 0.76	2.0 ± 1.11	1.5 ± 0.57	NS	$P < 0.05$	No	CsA *vs* AlIs
VIM	3.0 ± 1.2	1.6 ± 0.08	1.0 ± 0.0	3.7 ± 1.2	1.6 ± 0.9	1.2 ± 0.3	$P < 0.01$	NS	No	CsA *vs* AlIs

Values are the mean ± SD. FW, final weight; WC, weight change (final weight − initial weight); Cr, serum creatinine; Na^+, serum sodium; ALD, plasma aldosterone; FeNa, fractional excretion of sodium; PTF, percentage peritubular fibrosis; PGF, percentage periglomerular fibrosis; ITV, isometric tubular vacuolization; PHA, percentage hyaline arteriopathy; T P-gp, tubular P-gp expression; AGII, angiotensin II intrarenal deposits; VIM, tubular vimentin; CsA, cyclosporin A group; PLG, propyleneglycol group; SC, sodium chloride group; SBG, significance between groups; SBD, significance between 28 and 56 days; SIBG, statistical interaction between groups and time variables; NKT, Newman-Keuls test; NS, not significant.
*Percentage comparison test.

antibody (Dakopatts) followed by a mouse APAAP complex (Dakopatts) and then visualized with 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium reagents (Master Diagnóstica).

Statistical Analysis

Data are expressed as the mean ± SD. The normality of distribution of the values was assessed with the Kolmogorov-Smirnov test. Two-way analysis of variance (ANOVA2) was used to compare P-gp, angiotensin II and vimentin expression, renal function, and histopathological values and their interactions with two points in time (28 and 56 days) in the different treatment groups. The specific statistical weight of the mean in each group in the ANOVA2 test was determined with the Newman-Keuls test. Differences between individual quantitative or semiquantitative variables was assessed with Student's *t*-test and Mann-Whitney *U* test.

The relationships between parametric variables were evaluated with Pearson's test and those between nonparametric variables with Spearman's test. χ^2 and percentage comparison tests were used to compare qualitative variables. Differences at the 0.05 level of probability were accepted as significant.

Results

Functional and Histological results

The administration of CsA for 28 and 56 days at a dose of 25 mg/kg/day increased serum creatinine and urea in the CsA-treated groups (Tables 1 and 2); the difference between treated and control groups was significant for urea (ANOVA2 test). Although the CsA-treated group fed the LS diet had increased analytical changes compared with animals given the normal diet, the differences did not reach

Table 2. Analytical, Histological, and Immunohistochemical Results after 28 and 56 Days in Different Treatment Groups Given a Low-Salt Diet

	28 Days			56 Days			Statistical significance (ANOVA2)			
Group	CsA	PLG	SC	CsA	PLG	SC	SBG	SBD	SIBG	NKT
FW	298 ± 36.0	345 ± 34.0	351 ± 52.0	251 ± 28.0	343 ± 22.0	328 ± 25.0	$P < 0.001$	$P < 0.1$	No	CsA *vs* AlIs
WC	34 ± 46.0	77 ± 37.0	89 ± 42.0	−4.0 ± 25.0	61 ± 16.0	68 ± 29.0	$P < 0.01$	$P < 0.1$	No	CsA *vs* AlIs
Cr	0.69 ± 0.13	0.60 ± 0.1	0.62 ± 0.1	0.89 ± 0.38	0.66 ± 0.16	0.68 ± 0.1	NS	NS	No	CsA *vs* AlIs
Urea	104 ± 52.0	44 ± 7.0	47 ± 8.0	161 ± 146	54 ± 18.0	51 ± 6.0	$P < 0.01$	NS	No	CsA *vs* AlIs
Na^+	131 ± 4.0	139 ± 5.0	140 ± 8.0	135 ± 10.0	137 ± 7.0	128 ± 4.0	NS	NS	No	ALLS *vs* AlIs
ALD	2468 ± 1861	1803 ± 718	1782 ± 138	2961 ± 1301	2732 ± 1005	2503 ± 193	NS	NS	No	No
FeNa	0.11 ± 0.17	0.06 ± 0.06	0.14 ± 0.08	0.11 ± 0.08	0.08 ± 0.06	0.08 ± 0.06	NS	NS	No	No
PTF	13.5 ± 3.4	9.5 ± 0.7	7.8 ± 1.4	14.1 ± 3.5	11.4 ± 4.2	9.1 ± 0.2	$P < 0.001$	NS	No	SC *vs* AlIs
PGF	11.9 ± 3.0	8.1 ± 0.3	7.7 ± 1.3	12.9 ± 4.6	9.9 ± 4.1	7.6 ± 0.7	$P < 0.001$	NS	No	SC *vs* AlIs
ITV	1.75 ± 0.7	1.2 ± 0.4	1.0 ± 0.0	3.2 ± 1.0	2.4 ± 1.0	1.3 ± 0.2	$P < 0.01$	$P < 0.1$	No	CsA *vs* AlIs
PHA	30.5%	0%	0%	61.5%	0%	0%	$P < 0.01$*	$P < 0.05$*		
T P-gp	2.47 ± 0.6	1.65 ± 0.82	1.0 ± 0.0	2.75 ± 0.6	2.1 ± 0.75	1.27 ± 0.45	$P < 0.01$	NS	No	AlIs *vs* AlIs
AGII	1.61 ± 0.55	1.25 ± 0.50	1.6 ± 0.57	2.74 ± 0.75	1.87 ± 0.77	1.8 ± 0.51	NS	$P < 0.05$	No	CsA *vs* AlIs
VIM	3.1 ± 1.7	2.0 ± 0.8	1.6 ± 0.9	3.9 ± 1.4	2.6 ± 1.5	1.5 ± 0.6	$P < 0.01$	NS	No	CsA *vs* AlIs

Values are the mean ± SD. FW, final weight; WC, weight change (final weight − initial weight); Cr, serum creatinine; Na^+, serum sodium; ALD, plasma aldosterone; FeNa, fractional excretion of sodium; PTF, percentage peritubular fibrosis; PGF, percentage periglomerular fibrosis; ITV, isometric tubular vacuolization; PHA, percentage hyaline arteriopathy; T P-gp, tubular P-gp expression; AGII, angiotensin II intrarenal deposits; VIM, tubular vimentin; CsA, cyclosporin A group; PLG, propyleneglycol group; SC, sodium chloride group; SBG, significance between groups; SBD, significance between 28 and 56 days; SIBG, statistical interaction between groups and time variables; NKT, Newman-Keuls test; NS, not significant.
*Percentage comparison test.

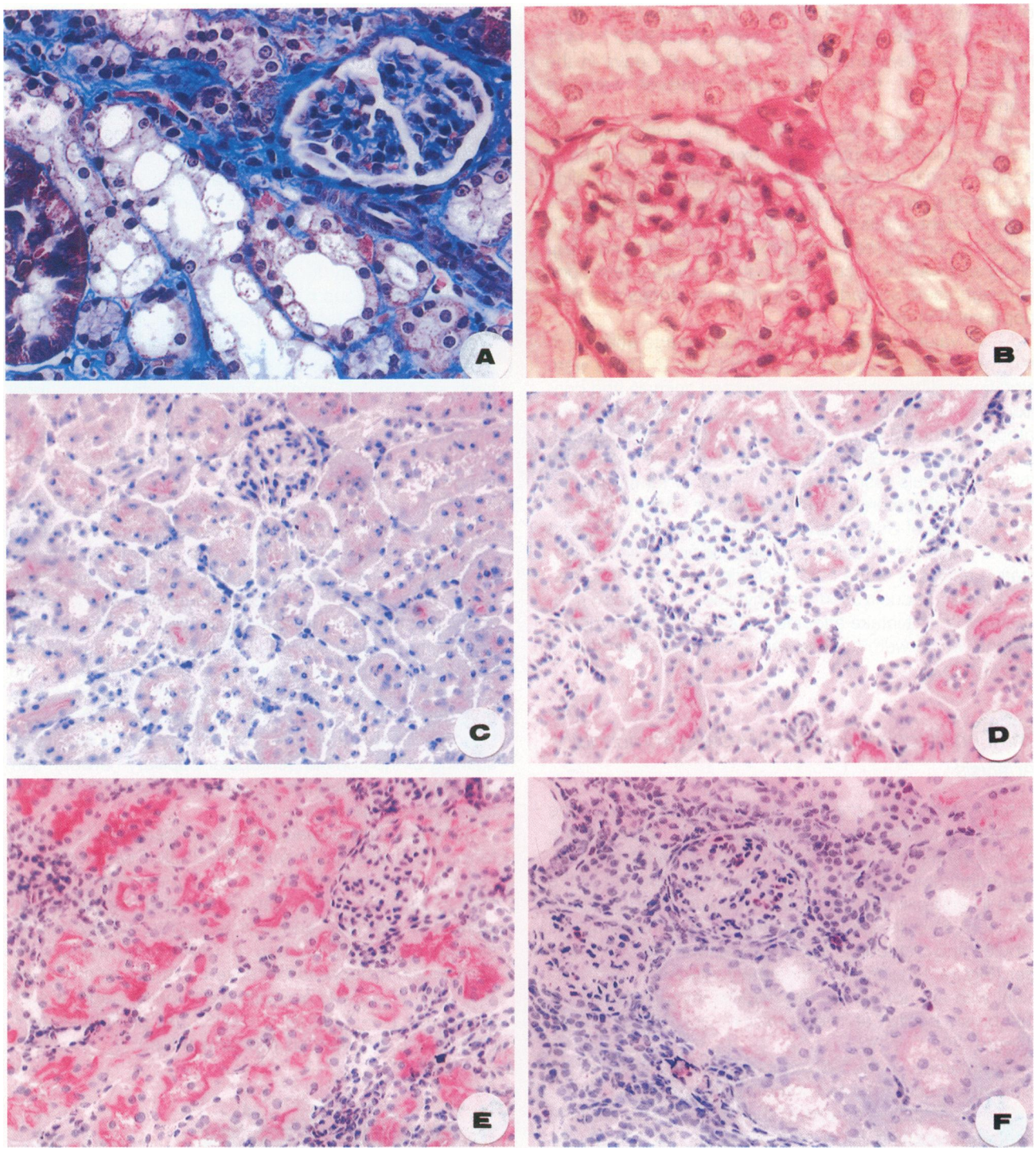

Figure 1. A: Isometric tubular vacuolization and moderate periglomerular fibrosis in CsA nephrotoxicity. Masson-Goldner trichromic stain; magnification, ×200. **B:** PAS-positive hyaline arteriopathy in a rat given the LS diet. PAS stain; magnification, ×400. **C:** Normal expression of P-gp in approximately 50% of the proximal cortical tubules in a rat kidney. APAAP; magnification, ×200. **D:** Mild overexpression of P-gp in proximal tubules of the kidney. Note the absence of stain in distal tubules near the glomerulus. APAAP; magnification, ×200. **E:** Moderately intense overexpression of P-gp. Strong positivity is observed in proximal and distal tubules on the apical brush border. APAAP; magnification, ×200. **F:** Tubular collapse and periglomerular fibrosis in the kidney of a rat given the LS diet. Expression of P-gp is weak in the proximal tubules, in concordance with clinical and histological signs of nephrotoxicity. APAAP; magnification, ×200.

statistical significance. The weight increase was smallest in group LS+CSA, and by the end of the experiment mean body weight in this group had decreased with respect to the initial mean body weight (day 56, Table 2).

As shown in Tables 1 and 2, there were significant differences in serum sodium concentration and plasma aldosterone levels between groups with normal and LS intake ($P < 0.05$, Student's t-test). The mean serum level

of CsA was 1980 ± 989 ng/ml at 28 days and 2651.6 ± 1532 ng/ml at 56 days in the CsA group and 2222 ± 1417 ng/ml at 28 days and 2345 ± 1154 at 56 days in the LS+CsA group; these differences, however, were not significant.

Histopathological examination of the kidney disclosed lesions typical of chronic CsA toxicity in both groups of treated animals. However, the lesions (especially hyaline arteriopathy and isometric tubular vacuolization) were more evident in rats fed the LS diet (Figure 1, A and B; Tables 1 and 2). Interstitial fibrosis was distributed irregularly and was more common in the medullary rays zone although some spread to periglomerular zones was also apparent (Figure 1A).

Mean values obtained by automatic image analysis in the two zones are given in Tables 1 and 2. There were significant differences between treated animals and controls that received PLG solvent or saline solution. In the group fed the LS diet (Table 2), the differences between animals given PLG or salt solution were also significant (Newman-Keuls test), although in overall terms the degree of periglomerular and peritubular fibrosis after 28 and 56 days of CsA treatment did not appear to depend on the presence of salt in the diet. However, semiquantitative analyses of the other histological lesions (isometric tubular vacuolization and hyaline arteriopathy) revealed that salt intake affected the extent of both types of lesions, which were more severe in animals given the LS diet (Table 2).

Immunohistochemical and Western Immunoblot Analysis

Immunostaining of cryostat sections for P-gp with the cocktail of MAbs revealed three different patterns: 1) a normal pattern equivalent to that in untreated controls, with mild immunostaining only in the brush border of the cells in approximately 50% of the proximal tubules (Figure 1C), 2) mild immunostaining in all renal proximal tubules and distributed homogeneously in the brush border (Figure 1D), and 3) moderately intense overexpression, with the appearance of marked immunostaining of the brush border of both proximal and distal tubules (Figure 1E).

The animals of the SC group taking the normal or LS diets never scored above 2 for P-gp expression.

Semiquantitative analyses showed that chronic CsA treatment induced P-gp overexpression in the tubules (Tables 1 and 2) of rats fed the normal diet and in animals given the LS diet (ANOVA2 test). Overexpression tended to progress with time. The increase in P-gp expression was slightly greater in the group of rats given the LS diet. The solvent PLG also induced P-gp, in contrast to the absence of expression in the group that received saline solution (Newman-Keuls test). In the group fed the LS diet, the differences between animals given PLG or salt solution were also significant (Newman-Keuls test).

When only CsA-treated groups after 56 days were considered, P-gp overexpression in the renal tubules was inversely related to the presence of hyaline arteriopathy ($r = -0.67$; $P < 0.05$), peritubular ($r = -0.64$; $P < 0.05$), and periglomerular ($r = -0.54$; $P < 0.05$) fibrosis (Spearman test; Figure 1F). In contrast, we found no significant relation between serum CsA levels and P-gp overexpression in the renal tubules.

To demonstrate the specificity of the MAbs JSB-1 and C-219 used for immunohistochemical studies, we performed a Western blot control analysis with protein extracts obtained from four kidney samples (two rats from the SC control group and two from the CsA group; Figure 2). The two control animals showed a mild immunohistochemical reactivity for P-gp whereas the rats selected from the CsA group had an intense staining. The immunohistochemical features parallel the intensity of the Western blot bands (Figure 2).

Analyses of vimentin expression in paraffin-embedded sections revealed variable immunostaining of the renal tubule cells, ranging from no positivity in the control groups and in animals fed the normal diet to intense positivity (>50%) in tubules with signs of chronic lesions in fibrotic areas (Figure 3A).

Immunostaining of paraffin sections for angiotensin II demonstrated three different patterns: 1) a normal pattern equivalent to that found in untreated controls, with immunostaining of the capillary network and peritubular connective tissue of the inner medulla as well as mild focal staining of the outer medulla (Figure 3B), 2) a mild pattern consisting of features as in the first pattern plus diffuse immunostaining of peritubular connective tissue in the outer medulla, with some focal spread to the medullary rays (Figure 3C), and 3) a moderate/severe pattern consisting of features as in the second pattern plus focal staining of the cortical peritubular capillary network, surrounding connective tissue, and cytoplasm of some cortical tubular cells (Figure 3D).

Semiquantitative analysis of immunostaining for angiotensin II showed that, in rats treated with CsA, the deposits were more evident than in control animals (Tables 1 and 2; Newman-Keuls test). Angiotensin accumulation increased with time and was related to dietary salt restriction (data not included). In CsA-treated animals the angiotensin II deposits correlated with serum creatinine ($r = 0.47$; $P < 0.01$) and with mean percentage of periglomerular ($r = 0.52$; $P < 0.001$) and peritubular ($r = 0.54$; $P < 0.001$) fibrosis (Spearman test).

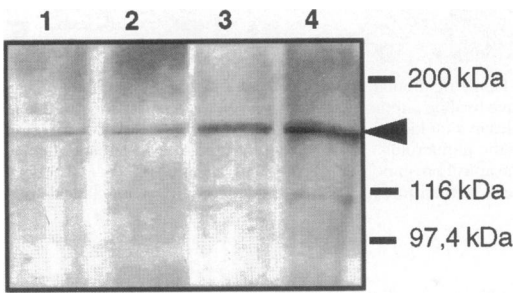

Figure 2. Western blot analysis of P-gp expression in rats. Total membrane proteins were obtained from kidneys of SC control group (**lanes 1** and **2**) and CsA group (**lanes 3** and **4**) 56 days after CsA treatment. The **arrow** indicates a strong band of 170 kd recognized specifically by the mixture of MAbs C-219 and JSB-1. There is also a weak band in the 116-kd range.

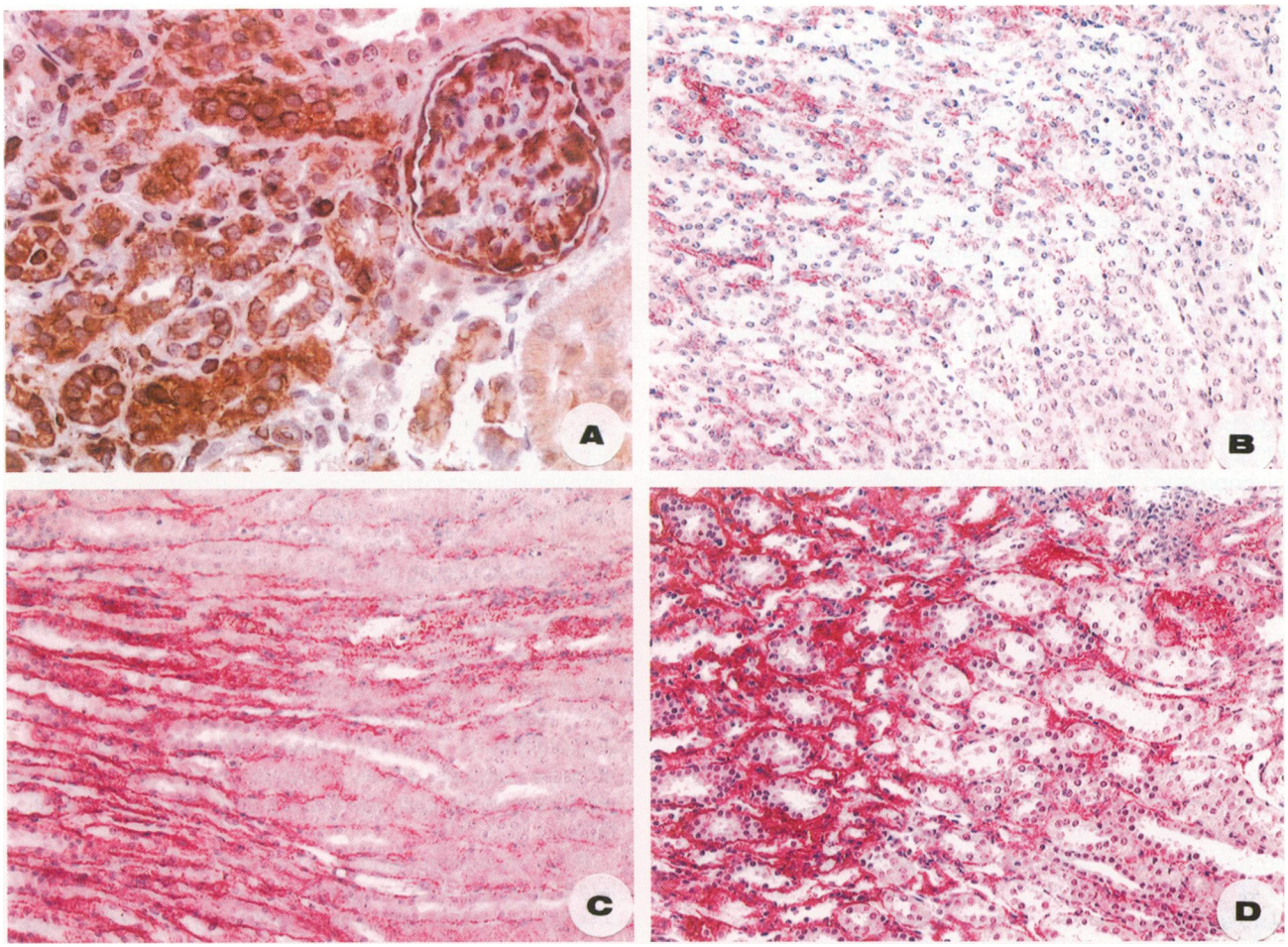

Figure 3. Neoexpression of vimentin in tubular cells and Bowman's capsule in severe tubular damage in CsA-treated rats. Streptavidin-biotin-peroxidase stain; magnification, ×200. **B:** Normal immunostaining in the inner medulla of a rat kidney. Note the slight focal spread to the outer medulla. Streptavidin-biotin-alkaline phosphatase stain (SBAP); magnification, ×100. **C:** Deposits of angiotensin II in the outer medulla of the kidney with some spread to the medullary rays. SBAP; magnification, ×100. **D:** Severe impregnation of the outer medulla and focal staining of the cortical peritubular capillary network in a rat with histological lesions of nephrotoxicity. SBAP; magnification, ×100.

When all animals were considered, regardless of the type of diet or whether CsA treatment was given, we found no significant correlation between intrarenal angiotensin II accumulation and P-gp overexpression. However, when we compared mean accumulation of angiotensin II in CsA-treated rats in which P-gp expression was normal or elevated, we found a small but significant difference (2.08 ± 1.2 *versus* 2.8 ± 1.4; $P < 0.05$, Mann-Whitney test). In animals with the most severe nephrotoxicity lesions (interstitial fibrosis > 12% and presence of hyaline arteriopathy), angiotensin II deposition correlated inversely with P-gp overexpression ($r = -0.65$; $P < 0.05$, Spearman test).

The difference in vimentin immunostaining between treatment groups was highly significant (ANOVA2, Newman-Keuls test; Tables 1 and 2).

Discussion

Our study analyzes P-gp expression in kidney tubular cells in an experimental *in vivo* model of chronic CsA nephrotoxicity and examines the relationship between intrarenal angiotensin II deposits and P-gp overexpression.

The possible role of P-gp overexpression in controlling CsA nephrotoxicity was initially proposed by our group[17] on the basis of 1) immunohistochemical evidence for P-gp overexpression in kidney biopsies from patients with kidney graft dysfunction and 2) the relationship between its overexpression and the intrarenal accumulation of CsA. The present immunohistochemical results demonstrate that chronic CsA treatment in rats induces progressive overexpression of P-gp reduced to BBMs on tubular cells, a fact also demonstrated by photoaffinity labeling methods.[28] The immunostaining specificity of P-gp restricted to BBMs in kidney tubules has been confirmed by Western immunoblotting using an equivalent MAbs cocktail (Figure 2). This is the first study that characterizes the absence of relevant cross-reactivity of JSB-1 and C-219 MAbs in the kidney. It showed a strong 170-kd band related to P-gp as well as a weak 116-kd one. The presence of this second band can be explained by either background reaction or by cross-reactivity with an uncharacterized protein. Jette et al[35] described the

C-219 antibody producing cross-reactivity with a 120-kd protein in the mouse brain capillaries and that this protein does not belong to the P-gp family. To clarify the significance of this protein, a complementary study may be necessary, although we consider that this is not relevant assuming that our immunohistochemical and Western blot results for P-gp are parallel.

It has been recently suggested that CsA may act as a P-gp blocker in the renal tubule[18] via a mechanism of competitive inhibition similar to that demonstrated in neoplastic cells.[3] This inhibition may lead to the accumulation of an endogenous toxin or of toxic CsA metabolites in tubular cells.[18]

In neoplasms and experimental *in vitro* studies, CsA has been used as a P-gp blocker[3] on the basis of its function as a specific substrate with high affinity for this glycoprotein[24,36] and its ability to displace other substrates through competitive inhibition.[37] However, many studies in cell cultures, including tubular kidney cells such as MDCK, have shown that exposure of this cell line to CsA increases the expression of P-gp.[17]

In our *in vivo* model of P-gp induction, CsA was continuously infused at a high dose, taking into consideration that rats have an innate resistance to the drug.[38] In place of the solvent used commercially for CsA (Cremophor EL, Sandoz) and known to interact with P-gp,[39] we used PLG. The expression of P-gp was greater in animals that received PLG than in controls given saline solution. This result was associated with a greater incidence of isometric tubular vacuolization and periglomerular and peritubular fibrosis, particularly in animals given the LS diet (Table 2) and after 56 days of treatment.

In neoplastic cells, it has been argued that the mechanism by which CsA induces P-gp overexpression may be related to the presence of a repressor and *trans*-type element that undergoes degradation due to the inhibition of protein synthesis, thus allowing the expression of *mdr* genes and increasing its mRNA.[40] The inhibition of protein synthesis by CsA may accelerate the degradation of this *trans* factor and eventually increase P-gp levels.

In experimental models with rats, LS diets worsen CsA-induced nephrotoxicity,[41] a result initially ascribed to functional prerenal ischemia secondary to the volumetric depletion these animals were subjected to.[42] However, in these experimental models the predominant lesion, together with interstitial fibrosis, is hyaline arteriopathy,[43] thought to be the most relevant lesion in renal biopsies from CsA-treated patients.[12] This lesion has been linked with an increased activity of the RAS.[44] The important role of angiotensin in triggering the fibroblastic activation leading to interstitial fibrosis was recently reported in CsA- and FK506-induced nephrotoxicity.[16,18,45]

In the present study, we found that hyaline arteriopathy was increased in animals fed a LS diet compared with those given a normal diet. In both groups, this increase was time dependent. However, we were unable to demonstrate unequivocal differences in the amount of periglomerular and peritubular fibrosis between rats on a normal diet and those fed a LS diet. One possible explanation for this finding is the longer duration of our experiments (8 weeks) than in previous ones, which lasted 4 to 6 weeks.[15,16,43] Some of these studies reported that intrarenal activation of the RAS was accompanied by the presence of a marked macrophage infiltrate. These cells were initially located in medullary rays but eventually spread, after 25 days or more after treatment, to periglomerular areas.[46] This phenomenon also occurred in the model of obstructive nephropathy.[47]

We evaluated the lesions after 4 and 8 weeks of CsA treatment and found that they had progressed to periglomerular areas (as demonstrated by morphometry), probably due to a mechanism related to increased secretion of TGF-β by the macrophage infiltrate.[48,49] In the normal kidney, TGF-β is localized preferentially in the perivascular and periglomerular connective tissue, which are areas subjected to the greatest physiological interstitial remodeling and which are also the sites where the progressive fibrosis characteristic of experimental models of obstructive nephropathy first appears.[47]

Shihab et al[50] have recently reported that, in salt-depleted rats, treatment with CsA for 28 days induces an increase in TGF-β mRNA, which accumulated mainly in the medullary portion of the kidney. In our experimental model, the intrarenal accumulation of angiotensin II predominates in the outer medulla and medullary rays, that is, in exactly those sites where the expression of type I angiotensin II receptors was greatest.[51] This finding strongly supports a likely relationship between these two mediators in the pathogenesis of CsA-induced interstitial fibrosis.[18,44,46,50]

P-gp overexpression in CsA-treated animals correlated inversely with periglomerular and interstitial fibrosis and the development of hyaline arteriopathy in the outer medulla and medullary rays. This supports a hypothetical role for P-gp as a detoxicant in the regulation of pharmacological nephrotoxicity.[17–19,28,52] Angiotensin II was recently shown to induce *ras* activation via type I angiotensin II receptors in vascular smooth muscle cells.[53] However, this finding remains controversial as other authors[54] have suggested that angiotensin-II-induced cellular activation takes place via *raf*-1. In either case, P-gp activation by angiotensin II may occur via *ras*[55] or via *raf*-1 with subsequent activation of the nuclear transcription factor AP-1.[56]

For tubular and mesangial renal cells, it has been proposed[19] that competitive P-gp blocking induced by CsA may elicit an accumulation of highly cytotoxic prenylcysteine methyl esters that are produced during catabolism of ras and G proteins.[57] It can be hypothesized that angiotensin-II-overstimulated arteriolar smooth muscle cells could die by a similar mechanism and eventually be replaced by hyaline substance configuring the characteristic CsA-induced hyaline arteriopathy. This notion may be supported by 1) the inverse statistical relationship between P-gp immunostaining intensity in BBMs and the hyaline arteriopathy present in CsA-treated rats (predominantly in those with a LS diet) and 2) the recent description by Sugawara et al[27] of P-gp expression in smooth muscle cells.[27]

When all animals were analyzed, we found no relationship between P-gp overexpression and angiotensin II deposits; this unexpected result merits examination. Our

previous immunohistochemical findings in kidney transplant biopsies have shown a high degree of individual variability in P-gp overexpression. Patients with no evidence of positive induction of the MDR phenotype nonetheless had the highest incidence of nephrotoxic lesions.[17] Moreover, a recent study of 38 patients with renal carcinoma showed that, in comparison with the surrounding normal kidney tissue as an internal control, the amounts of *mdr*-1 mRNA in both normal and tumor samples varied up to sevenfold in some patients.[58] The results of this study were similar; P-gp expression, determined immunohistochemically, varied widely in controls, some of which had values in the range of mild overexpression. This individual variability may be partly responsible for the lack of correlation between P-gp overexpression and angiotensin II deposition when the results were considered globally. When only those animals with the greatest histological degree of CsA nephrotoxicity were considered (mean interstitial fibrosis > 12% plus presence of hyaline arteriopathy), P-gp overexpression showed a significant inverse correlation with angiotensin II deposits ($r = -0.65$; $P < 0.05$, Spearman test).

The most evident physical and analytical differences between the LS+CsA group and rats fed the normal diet were the lower serum sodium, the relatively higher serum concentrations of creatinine and urea, and the lower final weight in the first group (Tables 1 and 2).[43,50]

Plasma aldosterone concentrations in all LS groups were 10-fold higher than in animals given the normal diet. This finding may account for the small, nonsignificant differences in the fractional excretion of sodium between the different groups (Tables 1 and 2).

Finally, a particularly interesting feature in the kidney is the possible relationship between P-gp and the protein kinase C (PKC) system. The mechanism by which PKC regulates P-gp overexpression in neoplastic cells is linked with activation of the *ras* family of oncogenes. Chin et al[59] found that the *mdr*-1 promoter is a target sequence for the c-Ha-*ras*−1 oncogene. The pathway of *ras* activation via PKC is one of the mechanisms by which the angiotensin II type 1 receptor transduces signals to the cell nucleus.[53]

In summary, we show that chronic CsA treatment leads to overexpression of P-gp in renal tubules of the kidney and deposits of angiotensin II in the outer medulla and medullary rays with focal extension to the cortical peritubular capillary network. Both phenomena are probably related to the interaction between the ras protein system and the PKC system. For this reason, P-gp overexpression may help prevent tubular lesions as well as the hyaline arteriopathy and interstitial fibrosis that characterize chronic CsA-induced nephrotoxicity.

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