American Journal of Pathology, Vol. 146, No. 2, February 1995

Relationship between P-Glycoprotein Expression and Cyclosporin A in Kidney

An Immunohistological and Cell Culture Study

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P-glycoprotein (P-gp), encoded in humans by the* mdr-*1 gene, acts physiologically as an efflux pump to expel hydrophobic substances from cells. This glycoprotein is closely related to multidrug resistance in tumor cells and can be modulated by cyclosporin A (CsA). We investigated the relationship between CsA and P-gp in 52 renal allograft biopsies and in cultures of Madin-Darby canine kidney (MDCK) renal tubule cells to determine whether the intrarenal accumulation of CsA or chronic stimulation with the drug modified the expression of P-gp. Expression of P-gp and CsA was analyzed by immunohistochemistry. Immunostaining was evaluated semiquantitatively. Modulation of P-gp in MDCK cells after chronic stimulation with CsA for 7, 30, and 60 days was analyzed by flow cytometry. P-gp and CsA immunostaining in renal post-transplant biopsies showed considerable overlap in all cases (Spearman's test, r = 0.577, P < 0.001). After 7 days in vitro, the number of cells expressing P-gp increased progressively; a further increase in mean fluorescence was found after 60 days (P < 0.001, Student's t-test). Our findings suggest that in nonneoplastic cells, CsA may stimulate P-gp as a mechanism of detoxification. Individual differences in the adaptive responses to glycoprotein may be responsible for the appearance of nephrotoxicity or a CsA-resistant rejection reaction in cases of overexpression on lymphocytes and macrophages. (Am J Pathol 1995, 146:398–408)

Cyclosporin A (CsA) has proved to be a successful agent in the prevention and treatment of allograft rejection and in autoimmune diseases.[1,2] However, several lesions have been associated with this agent. Of these, chronic CsA nephropathy, characterized mainly by stripped or patchy fibrosis and hyaline arteriopathy, is particularly important in renal transplantation.[3,4] However, epithelial findings of CsA nephrotoxicity, including vacuolar isometric degeneration and the intracellular presence of mitochondria and/or hyaline droplets in renal tubules, suggest that CsA itself damages the epithelial cells of the kidney.[3–6] Nevertheless, these changes appear irregularly among transplant patients treated with CsA and may be related to individual differences in the response to treatment.[7] Despite these findings, the method of choice for regulating the administration of CsA in renal transplant patients is still periodic testing of serum drug concentrations.[8]

P-glycoprotein (P-gp), encoded in humans by the *mdr*-1 gene,[9,10] acts physiologically as an efflux pump to expel hydrophobic substances such as CsA from the cytoplasm.[11] This glycoprotein has also been shown to be closely related to multidrug resistance (MDR) in tumor cells[12–15] and can be modulated by CsA.[16–18]

The tissues richest in P-gp are the secretory type of epithelia, such as the proximal tubule of the kidney, the adrenal medulla, and the biliary ducts.[19–21] In addition, P-gp has been reported in several permanent

Supported by the Comisión Interministerial de Ciencia y Tecnologia through Grants CICYT FAR-529/93 and CICYT PETRI-0059/91 and by Sandoz Pharma SAE through contract 418/94 ATR University of Granada.

Accepted for publication October 25, 1994.

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A preliminary report of these findings was presented in 1992 at the Annual Meeting of the U.S. and Canadian Academy of Pathology in Atlanta, GA, and at the I Congreso Hispanoamericano de Nefrología in Madrid, Spain.

renal tubule cell lines such as Madin-Darby canine kidney (MDCK)[22] and in normal kidney primary cell cultures.[23]

The present study was designed to investigate whether the intrarenal accumulation of CsA induces alterations in P-gp expression by the renal parenchyma in post-transplant kidney biopsies and whether these alterations are reproducible *in vitro* in the MDCK cell line. To test these hypotheses, we used immunohistochemical techniques to determine the presence of intrarenal CsA deposits and P-gp in renal allograft biopsies with specific monoclonal antibodies (MAbs). In addition, we used immunostaining and flow cytometry to test P-gp expression in MDCK cells chronically stimulated with CsA.

Materials and Methods

Drugs

From a stock solution of CsA (base substance donated by Sandoz Pharma, Basel, Switzerland) at a concentration of 10 mg/ml in absolute ethanol (Merck, Darmstadt, Germany), we made successive dilutions with supplemented Eagle's minimal essential medium (Flow Laboratories, Irvine, CA) to obtain working concentrations from 0.5 to 10 μg/ml. The final concentration of ethanol did not exceed 0.1%.

Kidney Biopsies

The expression of P-gp and the intrarenal presence of CsA were analyzed in 52 renal post-transplant biopsies. All samples were obtained during episodes of renal allograft dysfunction within 1 to 31 weeks after transplantation (mean, 214.0 ± 63 days). The age range of the series was 22 to 63 years (mean, 43.0 ± 1.77), with a male:female ratio of 1.4:1.

During the first 12 postoperative days, all patients were treated with CsA (8 mg/kg/day), prednisone (0.25 mg/kg/day), and anti-lymphocytic globulin on alternate days, at a dose of 10 mg/kg/day. During the subsequent 6 months a maintenance regime was established, with administration of CsA adjusted to keep stable serum levels at 80 to 200 ng/ml, and a progressive reduction of prednisone to 5 mg/day. The patients did not receive any of the drugs known to induce MDR phenomena, although 25% of the patients received antihypertensive medication.

Serum levels of CsA were determined by standard radioimmunoassay techniques with anti-CsA MAb (Incstar, Stillwater, MN).

Ten renal samples from normal kidneys and ten from patients with glomerulonephritis were analyzed as controls. Tissues from patients with glomerulonephritis were used only as negative controls for CsA immunostaining.

Cell Line and Culture Techniques

The MDCK cell line, purchased from the American Type Culture Collection (Rockville, MO), was routinely grown in minimal essential medium (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), penicillin (100 U/ml, Sigma Chemical Co, St. Louis, MO), and streptomycin (50 μg/ml, (Sigma) at 37 ± 0.5 C with 5% CO_2. Under normal conditions, MDCK cells constitutively express moderate amounts of P-gp, which is polarized on the cell surface.[22] Before each assay, individual cell viability was tested by propidium iodide exclusion (0.1 μg/ml, Sigma).

Cytotoxicity and Growth Inhibition Assays

Cytotoxicity was tested by the MTT calorimetric method (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, thiazolyl blue, 5 mg of MTT per ml of PBS, pH 7.2, Sigma) according to a previously described method.[24] The initial inoculum consisted of 5×10^3 cells/well in 96-well microtiter plates (Costar, Cambridge, UK); CsA was added the next day at a range of concentrations from 0.5 to 10 μg/ml (five replicates per concentration).

Percent survival (% T/C) after 6 days of culture was calculated by dividing mean absorbance in CsA-treated cells (T) by mean absorbance in control cultures (C), and multiplying the quotient by 100. Cytotoxicity assays were done in triplicate. Cell numbers were calculated from standard curves constructed from the test absorbance values.

Antiproliferative activity induced by CsA in MDCK cells was determined as tritiated thymidine uptake in cell nuclei. Uptake of the radioactive precursor in CsA-treated and control cells incubated with concentrations of ethanol ranging from 0 to 10% was evaluated after 7 (short-term) and 60 (long-term) days, according to the method described by Daidone et al.[25] The initial inoculum (2×10^6 cells) was seeded in a culture flask (Costar), and CsA at a concentration of 1 μg/ml was added the next day. This concentration was used because, according to the dose-response curve in MDCK cells, it had no cytotoxic or antiproliferative effects after 7 days of culture. The supplemented culture medium (including the drug) was replaced every 48 hours. At the end of each incubation

period, the cells were trypsinized with a solution of 0.02% EDTA and 0.05% trypsin (Sigma), and 4×10^4 cells/well were seeded in 96-well microtiter plates (Costar), with five replicates per treatment. After incubation with tritiated thymidine at a final concentration of 1 μCi/ml (specific activity, 25 Ci/mmol, Amersham, Little Chalfont, UK) for 6 hours at 37 ± 0.5 C with 5% CO_2 and a stop medium (supplemented minimal essential medium with 2 mg/ml nonradioactive thymidine (Sigma) for 24 hours, cell suspensions were passed through Whatman glass fiber GF/C filters and harvested. Finally, 2 ml of OptiPhase HiSafe II scintillation fluid was added (LKB, Loughborough, UK) and the vials were read (1 minute/vial) with an LS2800 gamma counter (Beckman, Fullerton, CA). The results were recorded as counts per minute (cpm).

Primary Antibodies

Two murine MAbs were used 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). For immunohistochemical studies, both MAbs were used together at dilutions of 1:40 (JSB-1) and 1:10 (C-219) as a 1:1 cocktail. Both antibodies react with a conserved cytoplasmic epitope of the plasma membrane-associated 170- to 180-kd glycoprotein present on both human and animal MDR tumor cells.[26,27] The MAb C-219 also recognizes proteins P210 and P180, which can be induced together with P-gp in the course of the MDR phenomenon[28] and which show some degree of cross-reaction with certain forms of myosin in skeletal muscle.[29]

In flow cytometric analyses, 0.5 μg of JSB-1 and C-219 MAbs were used. As a negative internal control we used a cocktail of MAbs that specifically detect a broad spectrum of keratins (IgG isotype; Immunostain, Whitney, UK), including both high and low molecular weight cytokeratins. The anti-CsA MAb, provided by Sandoz Pharma, corresponds to an IgG1 isotype. It shows 100% cross-reactivity for CsA; cross-reactivities for different metabolites are 34% (met 1), 50% (met 16), 76% (met 17), and 55% (met 26), with much lower figures for all other metabolites.

Immunohistochemical Methods

Immunostaining was done with the phosphatase-antiphosphatase technique (APAAP) with MAbs directed against P-gp and CsA.

Briefly, 4-μ cryostat sections were air dried and fixed in acetone at 4 C for 5 minutes, allowed to dry, and postfixed in chloroform for 30 minutes at room temperature. After two 3-minute washes in Tris-buffered saline, nonspecific reactivity was blocked with normal rabbit serum at 20%. The sections were incubated with the cocktail of antibodies against P-gp, and the anti-CsA MAb, in a moist chamber at 4 C for 16 hours. Subsequently, sections were washed three times in Tris-buffered saline and incubated for 30 minutes at room temperature with anti-mouse rabbit immunoglobin (Dakopatts, Glostrup, Denmark) at a 1:50 dilution in Tris-buffered saline. After extensive washes, slides were incubated with the APAAP complex at 1:100 dilution (Dakopatts) for 30 minutes at room temperature, 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). A negative control was run without the primary anti-P-gp and CsA antibodies.

Immunostaining for the MAb against CsA and its metabolites, and for P-gp, was evaluated semiquantitatively and independently by two experienced pathologists who were unaware of the clinical or histological diagnosis. Staining was scored as 1 (negative or normal pattern), 2 (mild), 3 (moderate), or 4 (intense). Minor differences between the two observers were resolved by conference. Histopathological examination of all cases was done on B-5-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin, Masson's trichromic stain, and periodic acid-Schiff.

Immunofluorescence Staining and Flow Cytometry

The induction of P-gp expression by CsA in MDCK cells was evaluated sequentially after 7, 30, and 60 days of culture. An indirect method of immunofluorescence staining with fluorescein isothiocyanate-labeled IgG was used in all assays. Initially, 5×10^6 cells/ml were fixed with formaldehyde at 2% (Merck) in Dulbecco's phosphate-buffered saline PBS medium (DPBS, Seromed, Berlin, Germany), pH 7.3, supplemented with 0.1% bovine serum albumin (Sigma) and permeabilized with 0.1% Triton X-100 (Merck) in DPBS. Before the addition of MAbs (JSB-1 and C-219), nonspecific activity was blocked with

20% normal goat serum (Dakopatts) in DPBS. The cells were then incubated with the MAb at 4 C for 60 minutes, washed twice with 0.1% DPBS-bovine serum albumin, and immunostained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (H and L chain-specific; Ortho, Raritan, NJ) diluted to 1:80 in DPBS supplemented with 20% (vol/vol) normal goat serum. Cells incubated with anti-CD3 primary antibody and then exposed to the secondary antibody served as the negative control. For each sample, at least 5000 cells were harvested.

Specific mean fluorescence uptake of MAbs was defined by the difference in mean channel fluorescence between total and nonspecific uptake histo-

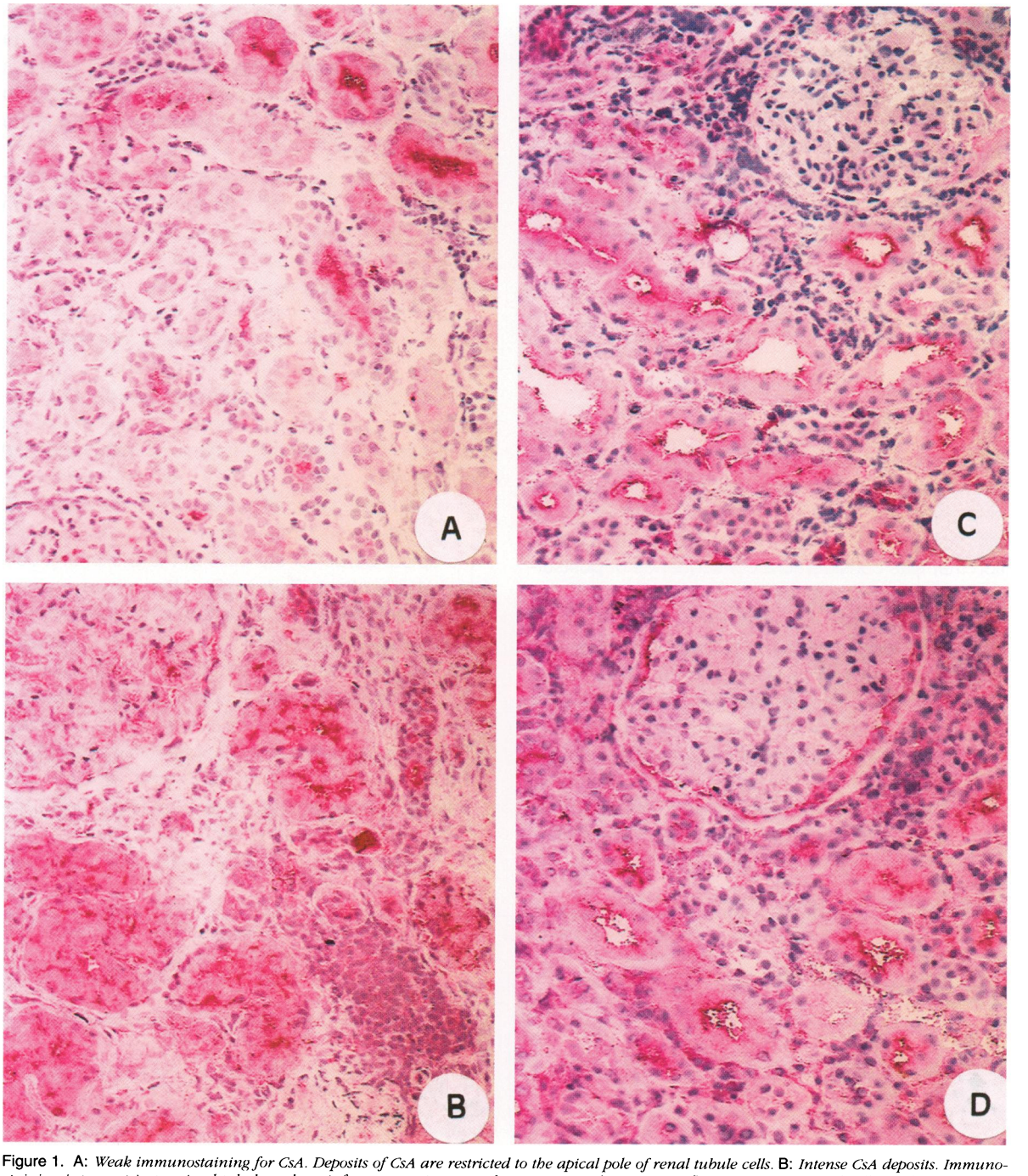

Figure 1. A: *Weak immunostaining for CsA. Deposits of CsA are restricted to the apical pole of renal tubule cells.* B: *Intense CsA deposits. Immunostaining is present in proximal tubules, where it forms gross intracytoplasmic accumulations, in inflammatory interstitial cells, and in glomeruli.* C: *Normal expression of P-gp. Immunostaining is present along the apical border of proximal tubule cells and Henle's loop.* D: *Intense overexpression of P-gp. Positivity is observed along the apical border, in the interstitium, and in Bowman's glomerular capsule. APAAP; magnification, ×200.*

grams of the MDCK cell line.[30] The samples were analyzed on a Cytoron Absolute flow cytometer and the results were analyzed with the ARS software package (both from Ortho).

Statistical Methods

Statistical correlations between the intracellular presence of P-gp and CsA were analyzed with Spearman's rank correlation tests for paired variables. The distribution of intrarenal CsA deposits in different types of rejection, and their association with CsA nephrotoxicity lesions, were tested with the χ^2 method. Relations between continuous quantitative variables (eg, serum concentrations of CsA, patient age, and percent immunostaining in cytometric analyses) were studied with one-way analysis of variance and Student's *t*-test.

Results

Immunohistochemistry and Histopathology

Histopathological examination of the series disclosed 31 cases of acute interstitial rejection, 8 of acute vascular rejection, 10 of chronic rejection, 2 of recidivant glomerulonephritis, and 1 case of allograft dysfunction that was considered a consequence of postsurgical renal arterial stenosis.

Studies of frozen sections for intrarenal CsA revealed four distinct patterns of immunostaining. Absent or faint staining (9 cases and 20 controls) was identified when immunostaining was nonexistent (controls) or when the apical border and lumen were colored in <15% of the renal tubules of the sample. Mild staining (18 cases) was restricted to the apical pole of the renal tubule cells (Figure 1A) and to inflammatory cells in the glomerulus and interstitium. Moderate positivity (14 cases) was recorded when deposits of CsA formed gross intracytoplasmic accumulations of the drug (intracellular aggregates) in the proximal tubule of the kidney, accompanied by mild diffuse interstitial deposits. Intense staining (11 cases) consisted of the same features as above together with intraglomerular deposits (Figure 1B).

Table 1A summarizes the distribution of the lesions associated with CsA nephrotoxicity and the relation between these lesions and intrarenal levels of the drug in the 52 samples we analyzed. Larger amounts of CsA in kidney tissue were clearly associated with an increased incidence of hyaline arteriopathy in the biopsy material ($P < 0.05$, χ^2 test).

Significantly, we found no correlation between serum levels of CsA (mean, 105 ng/ml; range, 42 to 145 ng/ml), time elapsed after transplantation (mean, 263 ± 413 days; range, 3 to 1825 days), and the intensity of intrarenal deposits of CsA.

In patients with interstitial rejection, intrarenal deposits of CsA were mild in 19 cases and moderate or intense in 12 cases. Two of the cases with vascular rejection presented mild deposits, and 6 had moderate or intense deposits. These differences were not statistically significant. Four types of immunostaining of cryostatic sections for P-gp with the MAbs were identified. In the normal pattern (21 cases and the 10 normal controls), immunostaining was present only in the apical border of the cells of the proximal tubule and Henle's loop (Figure 1C). Mild immunostaining (11 cases) was similar in pattern but appeared in all renal tubules. Moderate overexpression (13 cases) was identified as the appearance of aggregates of P-gp, particularly in apical regions of the tubules and interstitial inflammatory infiltrates. Intense overexpression (7 cases) was recorded when interstitial and tubular deposits of P-gp appeared together with staining in Bowman's glomerular capsule, which normally does not express P-gp (Figure 1D).

Table 1. *Distribution of CsA-Induced Lesions*

	A. According to intrarenal concentrations of the drug				B. According to intensity of P-gp expression in kidney tissue			
Lesions	Faint	Mild	Moderate	Intense	Normal	Mild	Moderate	Intense
SF	4/9 (44.4)	10/18 (55.6)	5/14 (35.7)	6/11 (54.5)	9/21 (42.6)	6/11 (54.5)	7/13 (53.8)	3/7 (42.9)
HA*	1/9 (11.1)	6/18 (33.3)	6/14 (42.9)	8/11 (72.7)	7/21 (33.3)	5/11 (45.6)	7/13 (53.8)	2/7 (28.6)
CF	2/9 (22.2)	1/18 (5.6)	2/14 (14.3)	2/11 (18.2)	2/21 (9.5)	2/11 (18.2)	1/13 (7.7)	1/7 (14.3)
MG/HD*	3/9 (33.3)	8/18 (44.4)	10/14 (71.4)	4/11 (36.4)	7/21 (33.3)	9/11 (81.8)	5/13 (38.5)	2/7 (28.6)
PCC	3/9 (33.3)	4/18 (22.2)	4/14 (28.6)	1/11 (9.1)	5/21 (23.8)	1/11 (9.1)	5/13 (38.5)	1/7 (14.3)
MA	2/9 (22.2)	4/18 (22.2)	3/14 (21.4)	2/11 (18.2)	5/21 (23.8)	2/11 (18.2)	3/13 (23.1)	1/7 (14.3)
DET	260 ± 425	162 ± 276	360 ± 415	318 ± 582	301 ± 423	127 ± 108	223 ± 345	432 ± 719

SF, stripped fibrosis; HA, hyaline arteriopathy; CF, calcification MG/HD, megamitochondria/hyaline droplets; PCC, peritubular capillary congestion; MA, mixoid arteriopathy; DET, days elapsed from transplant (mean ± SD). Numbers in parentheses are percentage of positive cases.

*$P < 0.05$, χ^2 test.

Table 1B shows the distribution of lesions associated with CsA nephrotoxicity and the relation between the presence of these lesions and P-gp expression in the 52 samples we analyzed. Normal or mild levels of expression were associated with higher numbers of cases with megamitochondria and hyaline granules in the cytoplasm of tubule cells, in contrast with cases that showed moderate or intense overexpression ($P < 0.05$, χ^2 test).

The correlations between immunostaining with anti-P-gp and anti-CsA in renal tissue are shown in Figure 2. There was considerable overlap between the graphs of CsA and P-gp immunostaining in the majority of cases (Spearman's correlation test: $r = 0.577$, $P < 0.001$).

Table 2. *Mean Absorbance (A_{540}) and Percent Cell Survival Obtained from the Dose-Response Curves of MDCK Cells Treated for 7 Days with Cyclosporin A*

CsA (μg/ml)	A_{540} mean ± SD*	% T/C†
0	0.759 ± 0.097	100
0.5	0.774 ± 0.177	102
1	0.683 ± 0.205	90
2	0.504 ± 0.207‡	66
5	0.117 ± 0.036‡	15
7	0.083 ± 0.046‡	11
10	0.004 ± 0.010‡	1

*All values are the arithmetic mean ± standard deviation of individual values from three independent experiments, with five replications of each.
†Percent survival with respect to control cultures.
‡$P < 0.001$, analysis of variance.

Cell Culture Results

Table 2 shows mean absorbance values and cell survival, calculated from the dose-response curves in MDCK cells treated with CsA. Exposure of MDCK cells to different concentrations of CsA led to a dose-dependent decrease in cell growth.

Evaluations of cell viability with the MTT method (see Materials and Methods) showed that CsA had no cytotoxic or antiproliferative effect, even at concentrations within the pharmacologically active range (0.5 to 1.0 μg/ml). At 1.0 μg/ml, survival was 90% of the control value. When cells were incubated with CsA at 2 to 10 μg/ml, mean absorbance decreased markedly in comparison with that in untreated cells ($P < 0.001$, analysis of variance; Table 2).

Tritiated thymidine uptake after 7 days of culture did not differ between untreated cells (668 ± 124 cpm) and cells incubated with 1.0 μg/ml CsA (589 ± 38 cpm). However, after 60 days, this dose of CsA decreased cell growth in comparison with controls (1276 ± 108 cpm *versus* 1529 ± 119 cpm, $P < 0.01$, Student's *t*-test).

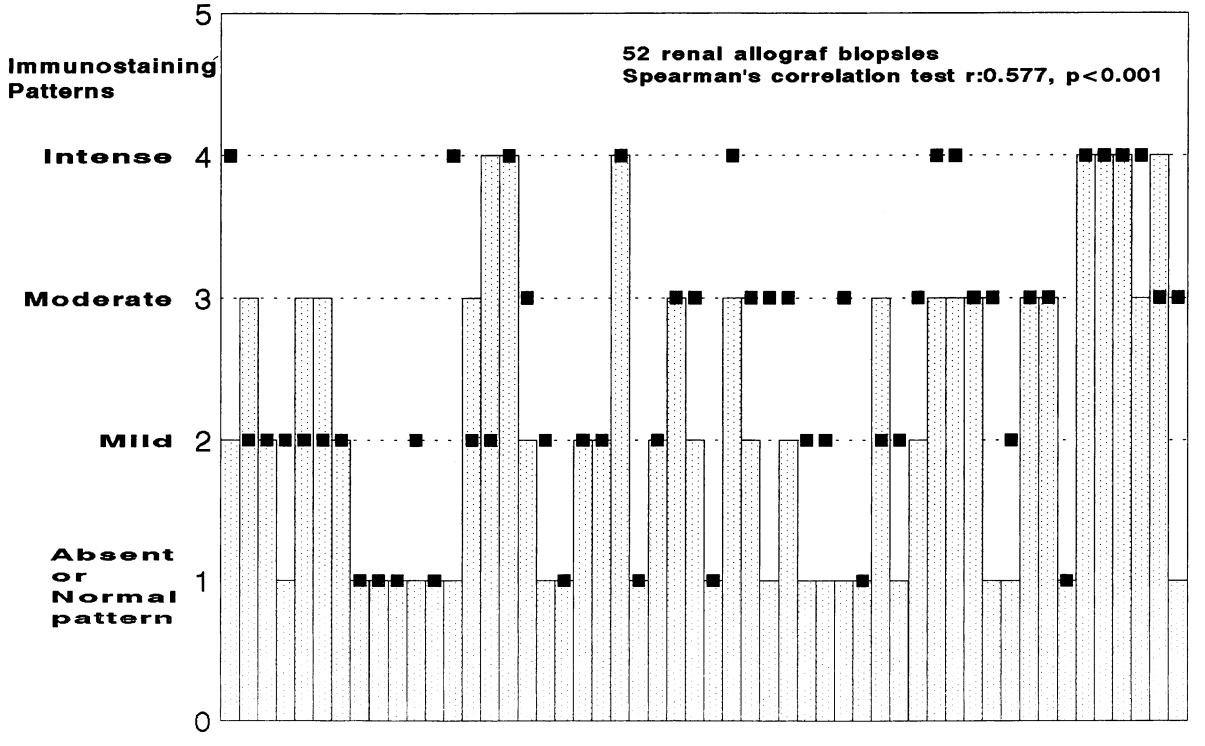

Figure 2. *Diagram comparing immunohistochemical results obtained in 52 post-transplant renal biopsies. Bars, P-gp; ■, CsA.*

Under standard culture conditions, MDCK renal tubule cells constitutively express P-gp, and similar percentages of positive cells were observed with both MAbs (Table 3). Treatment with CsA at 1 μg/ml led to a steady increase with time in the number of cells expressing P-gp (Table 3); expression was greatest at the end of the culture period (Table 3 and Figure 3). Mean fluorescence intensity (mean channel), which is directly related with antigen expression per cell, remained near the control value until day 30 of incubation. After 60 days of treatment, mean fluorescence intensity was significantly greater in CsA-treated cells (102 ± 4.1) than in control cells (61.8 ± 7.1, $P < 0.001$, Student's *t*-test) when MAb JSB-1 was used (Table 3).

Discussion

Our findings show that P-gp is overexpressed in post-transplant human kidney biopsies in the presence of large intracellular deposits of CsA and in the MDCK cell line after 60 days of exposure to this drug when as many as 92.9% of the cell population was positive for P-gp. Moreover, in cultured MDCK cells, mean channel fluorescence had increased significantly after 8 weeks of exposure to the drug.

Three crucial aspects of the methods we used in this study are the dose of drug chosen for *in vitro* assays of chronic stimulation with CsA of the MDCK renal tubule cell line, the antibodies used to detect P-gp, and the use of flow cytometry as an objective measure of the modifications in the MDR phenotype induced in the MDCK cell line.

Earlier studies of primary cultures of renal tubule cells found that the cytotoxic effect of CsA was time and dose dependent at doses >50 ng/ml[31] which is much lower than the dose we used in the present study. However, the cells used by Wilson and Hartz[31] were developed in primary cultures of renal tubule cells, whereas the MDCK cell line comprises cells that turned immortal spontaneously. This distinguishes MDCK from normal cells and is probably associated with a greater resistance to drugs with cytotoxic effects. In this connection, cell line LCC-PK_1, derived from the proximal tubule of the pig kidney, shows no appreciable morphological changes or growth until CsA is supplied at a dose of 2 μg/ml or greater.[32]

Because proteins P210 and P180 are almost always induced together with P-gp in the course of the MDR phenomenon[28] and renal tubule cells lack myosin, the cross-reaction that MAb C-219 shows with these proteins[28,29] should have no relevant effect on our results.

Flow cytometry has several advantages over conventional immunocytochemical methods in evaluating the induction of the MDR phenotype in the MDCK cell line.[30] Cytometry not only reliably detects changes in the number of cells that contain P-gp on their membrane but also reveals subtle increases in the number of P-gp molecules on individual cells, as shown by the steady increase in mean channel fluorescence with duration of CsA treatment (Table 3 and Figure 3).

The P-gp-linked acquisition or amplification of the MDR phenotype during or after chemotherapy has been well documented both *in vitro*[33,34] and in clinical studies.[12,35] This phenomenon has also been observed after CsA treatment in neoplastic cells with and without amplification of the MDR phenotype.[36,37] Although several *in vitro* studies have shown that tumor cells exposed to pharmacological doses of antineoplastic drugs increase their expression of P-gp, causing drug resistance,[34,35] CsA and other agents, including calcium channel blockers, are able to reverse P-gp-mediated drug resistance.[16,38,39]

CsA blocks P-gp expression in neoplastic cells,[40] interfering with the cytoplasmic system of cyclophilin[41–43] by 1), reducing the expression of the

Table 3. *Percentage of Cells Positive for Glycoprotein P-170, Detected with mAbs JSB-1 and C-219, in the MDCK Renal Tubule Cell Line after 7, 30, and 60 days of Pharmacological Induction with 1 μg/ml Cyclosporin A*

Time (days)		JSB-1		C-219	
		% Positive cells*	Mean channel fluorescence*	% Positive cells*	Mean channel fluorescence*
7	Control	43.3 ± 7.3	51.8 ± 10.4	47.0 ± 4.6	72.8 ± 12
	CsA	63.7 ± 13 (10)†	77.3 ± 17.4	71.2 ± 8.2 (3)†	69.0 ± 10
30	Control	55.1 ± 6.4	56.5 ± 10	54.6 ± 11.6	64.5 ± 11.2
	CsA	75.6 ± 6.8 (6)	78.3 ± 8.2	76.3 ± 10.6 (3)†	77.0 ± 14.7
60	Control	42.1 ± 12.3	61.8 ± 7.1	68.8 ± 10.8	59.5 ± 14.7
	CsA	76.6 ± 4.4 (6)†	102 ± 4†	92.9 ± 6.4 (3)†	82.9 ± 10.4

*All values are the arithmetic mean ± standard deviation of individual values from independent assays.
†Significant difference ($P < 0.001$, Student's *t*-test) in comparison with controls. Numbers in parentheses are the number of determinations in independent experiments.

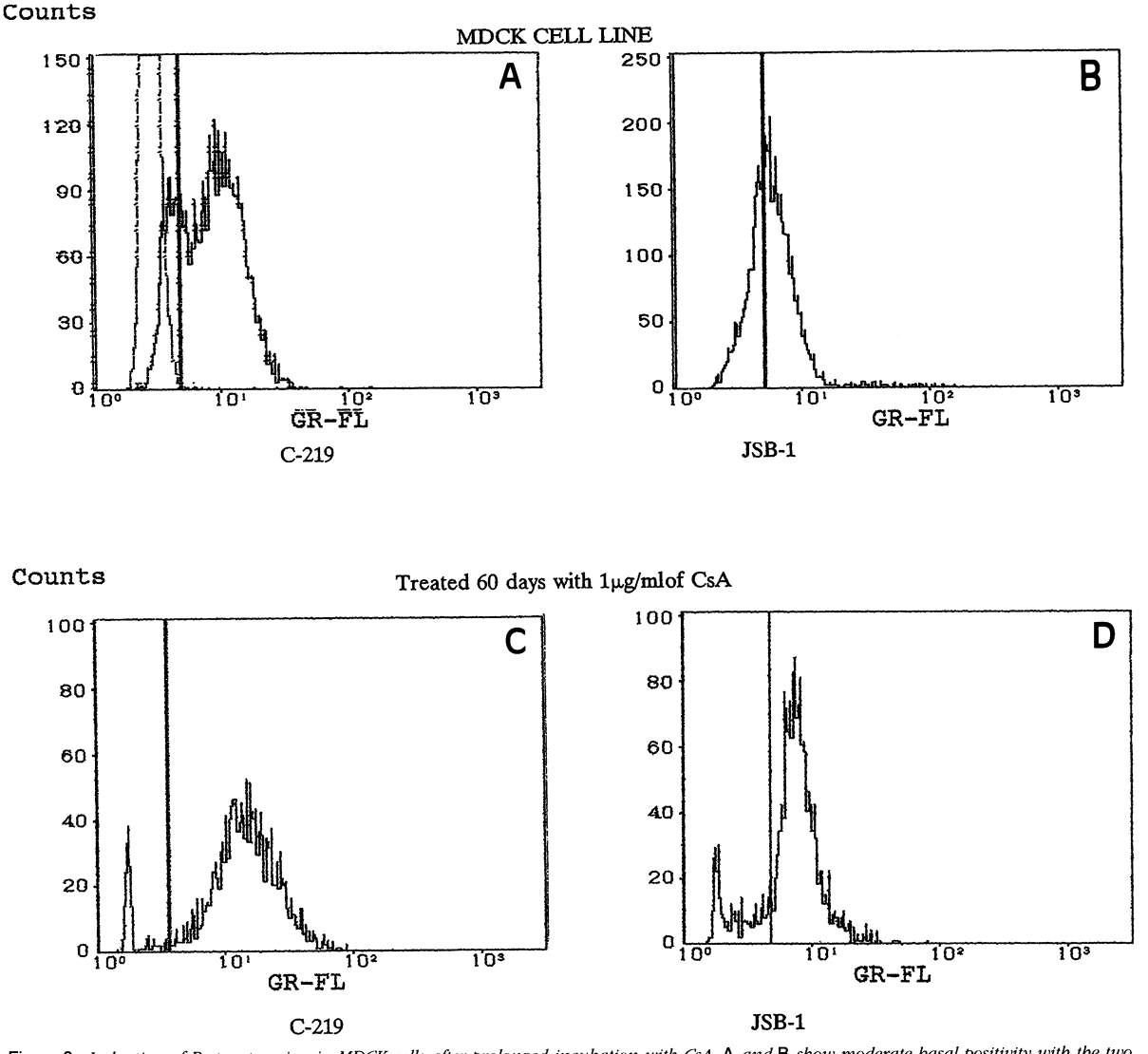

Figure 3. *Induction of P-gp expression in MDCK cells after prolonged incubation with CsA.* A *and* B *show moderate basal positivity with the two MAbs against P-gp. After 60 days of incubation with CsA* (C *and* D), *the proportion of positive cells increased, as did the mean channel fluorescence, shown logarithmically on the abscissa.*

mdr-1 gene;[16,17,33] 2), altering protein synthesis by blocking the isomerase activity attributed to this immunophilin;[44,45] and 3), reducing proliferative capacity by interfering with the expression of the c-*myc* and r-*fos* oncogenes.[46] As an alternative to the effects on neoplastic cells described above, evidence has been reported of a stereoisomeric competitive bond between CsA and P-gp, which blocks the ATPase activity dependent on this glycoprotein.[40]

It has been postulated that in normal cells, P-gp acts by expelling CsA from the cellular cytoplasm,[11] as occurs with other hydrophobic molecules in the phenomenon of hydrophobic vacuum described by Gottesman et al.[47] In cells of normal genetic content not subjected to processes of oncogenic amplification, the continuous action of a hydrophobic substance such as CsA should have some stimulatory effect on the P-gp detoxifying system.[48,49] The significantly lower numbers of cases with megamitochondria and hyaline droplets in patients in whom overexpression of P-gp was most intense supports the hypothesis that in normal cells prolonged toxic insult such as that caused by CsA induces the overexpression of P-gp as a mechanism of detoxification. Moreover, the frequency of tubular atrophy in CsA-induced lesions and the rarity of inclusion bodies and

tubular vacuolization[50] suggest that the latter two changes are early, transient histological signs of tubular atrophy. The fact that patients in the present study had not received treatment with other drugs known to induce MDR is additional evidence of the association between CsA deposits and immunohistochemical evidence of P-gp in the proximal tubule.

The lack of correlation between the intensity of intracellular deposits and the serum levels of CsA or post-transplantation time may be related with the existence of individual types of response to this agent; these responses are probably more dependent on cellular mechanisms of transport and accumulation[7] than on the pharmacological dose, as intrarenal CsA deposits were significantly associated with an increased incidence of hyaline arteriopathy that could not be attributed exclusively to immunological causes. According to one attractive hypothesis, different patterns of P-gp overexpression may account for individual variations in susceptibility to CsA nephrotoxicity[7,51] and to the irreversible rejection reactions that occur in the presence of large intrarenal CsA deposits.[52] In patients prone to develop CsA nephrotoxicity, the normal P-gp response may be defective, leading to the retention of excess amounts of the drug in the cells. Conversely, an excessive or prolonged response may lead to CsA-resistant rejection crises in some patients[52] in whom CD4+ lymphocytes might expel CsA from the cytoplasm, as occurs with antineoplastic drugs in tumoral cells.[11,47] In this connection, there is initial evidence of amplification of P-gp expression, which tends to increase with time, in circulating lymphocytes of heart transplant patients under treatment with CsA.[53] In addition, many cells containing large amounts of P-gp on their membranes have been found in the inflammatory infiltrate of lung transplant patients with steroid-resistant rejection crisis.[54] Nevertheless, additional studies in larger series are required to confirm this hypothesis as well as to explain the different responses of neoplastic and nonneoplastic cells to CsA.

We therefore conclude that the immunohistochemical detection of CsA and P-gp together with the surveillance of serum levels may be of value in the follow-up of CsA-treated transplant patients for the early detection of CsA nephrotoxicity and possibly CsA-resistant rejection crisis.

Acknowledgments

We thank Karen Shashok for translating parts of the original manuscript into English and Ms. F. Sáez, Ms. M. D. Rodríguez, Ms. M. J. De Federico, and Ms. P. Alfaro for technical assistance.

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