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ORIGINAL ARTICLE

The calcineurin activity profiles of cyclosporin and tacrolimus are different in stable renal transplant patients

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Summary

Cyclosporin and tacrolimus remain the cornerstone immunosuppressive drugs in organ transplantation. Dosing and monitoring these drugs is based on pharmacokinetic protocols, but measuring a pharmacodynamic parameter, calcineurin phosphatase (CaN) activity, could be a valuable supplement in determining optimal doses. Forty stable renal transplant patients were investigated three times in a 6-month period. Blood samples were drawn at 0, 1, 2, 3 and 4 h after oral intake of tacrolimus (FK) or cyclosporin at days 1 and 180. At day 90, one blood sample at trough level (FK) or C2 level (cyclosporin A, CsA) was drawn. CaN activity was determined in whole blood as the release of ^{32}P from a phosphorylated peptide. Activity of the ^{32}P was quantitated by liquid scintillation and results converted to Units CaN, utilizing a calibration curve with CaN. We demonstrated that calcineurin activity profiles at days 1 and 180 were the same for both drugs. Furthermore, we found that patients treated with tacrolimus or cyclosporin displayed different calcineurin activity profiles. We found that cyclosporin displayed greater calcineurin inhibition than tacrolimus. We have demonstrated that the two drugs exert significantly different effects on calcineurin activity in renal transplant patients with stable, well-functioning grafts and that tacrolimus-treated patients can maintain good, stable graft function with minimal CaN inhibition.

Introduction

The introduction of the calcineurin inhibitors cyclosporin and later tacrolimus to the immunosuppressive treatment of renal transplant patients has clearly resulted in prolonged graft survival [1], in particular reduction in the acute allograft rejections. The two calcineurin inhibitors exert their immunosuppressive action by inhibiting the enzyme calcineurin phosphatase (CaN), an intracellular calcium- and calmodulin-dependent phosphatase [2,3]. On inhibition, dephosphorylation of the nuclear factor of activated T cells (NFAT) and later initiation of transcription of certain cytokines in the T cell-mediated immune response are prevented [4,5]. The variable pharmacokinetics [6,7] and narrow therapeutic index of the drugs have made therapeutic drug monitoring (TDM) necessary.

Pharmacokinetic monitoring has been the method of choice in the transplantation units to the present, but pharmacodynamic monitoring of the calcineurin inhibitors as a sole monitoring tool or as a supplement could be helpful in TDM [8], hopefully resulting in prolonged graft survival. The pharmacodynamic assay used here is based on whole blood measurements, which have been shown to be superior to human peripheral blood mononuclear cells in a study by Caruso *et al.* [9].

Studies investigating CaN activity in relation to immunosuppressive treatment with calcineurin inhibitors have been published earlier. The majorities of these publica-

tions have involved CaN inhibition by cyclosporin. Halloran *et al.* [10] demonstrated that CaN activity in whole blood 1 h after drug intake was inhibited 71% compared with the predose level in pretransplant patients receiving their first dose of cyclosporin. They also investigated the CaN activity on days 3, 7 and 14 after renal transplantation and found maximal inhibition of CaN activity between 64% and 66% with no difference between inhibition on the days investigated.

Studies investigating CaN activity in tacrolimus-treated renal transplant patients are rare. We previously published a study with renal transplant patients on days 3 and 14 after transplantation [11]. We demonstrated a significant inhibition of calcineurin activity 2 h after oral ingestion of tacrolimus and a return of enzyme activity to predose levels after 4 h. Millan *et al.* [12] investigated both cyclosporin-treated and tacrolimus-treated renal transplant patients with stable renal graft function and found that both patient groups had lower CaN activity 2 h after oral intake of drugs compared to patients treated with MMF or healthy volunteers.

Characterization of the pattern of enzyme activity in patients with stable renal allograft function is very interesting and it is of great interest to investigate whether they display the same profile of enzyme activity in repeated measurements. To our knowledge no study has investigated the CaN activity in a group of stable renal transplant patients and followed them over time with repeated measurements of calcineurin activity.

The aim of this study was to measure CaN activity in a group of renal transplant patients with stable renal allograft function. Furthermore, to (i) investigate whether the CaN activity profile (0–4 h) is constant over 6 months for both cyclosporin and tacrolimus, (ii) describe the correlation between drug concentration and enzyme activity and (iii) study whether the calcineurin profile is the same for patients treated with either cyclosporin or tacrolimus.

Subjects and methods

Study design

The study was performed as a prospective clinical trial measuring CaN activity and drug concentrations in 40 renal transplant patients with stable renal allograft function. The study protocol was approved by the local ethics committee (Den videnskabsetiske Komité for Aarhus Amt, approval number 2002-0188). Informed consent was obtained from each participating individual.

Patients

Forty renal transplant patients with stable renal allograft function were included in the study. The patients were all outpatients from the Department of Renal Medicine C (Århus University Hospital, Skejby Sygehus, Denmark). All patients met the following inclusion criteria: stable renal allograft function S-creatinine <200 μM, variation in S-creatinine <20% for 6 months prior to inclusion]; renal transplantation more than 1 year before inclusion, receipt of graft from either cadaveric or living-related donor. We included patients with their first transplant and patients who previously had been transplanted. The following patients were excluded from the study: patients suspected of noncompliance, patients receiving medications known to interact with tacrolimus or cyclosporin pharmacokinetic and patients admitted to hospital with 20% or more rise in S-creatinine during the study period.

The study included three visits to the research laboratory, at days 1, 90 and 180 (±7 days). At the start of each visit, blood samples were collected for creatinine, urea, albumin, sodium, potassium, haemoglobin, blood sugar and leucocytes. At days 1 and 180 whole blood samples were drawn before and at 1, 2, 3 and 4 h after oral intake of either tacrolimus or cyclosporin in the patients' usual dosage. Whole blood samples were analysed for CaN activity and blood-drug concentration. At day 90 one blood sample was drawn to determine CaN activity and drug concentration at trough levels for the tacrolimus-treated patients and C2 level for the patients receiving cyclosporin. The reason for including a visit at day 90 was to ensure that the patients still fitted the inclusion criteria of stable allograft function. Whole blood samples for CaN activity were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were kept frozen at −80 °C until analysis, which was always within 2 weeks from collection. All other samples were collected according to routine procedures. Patients collected 24-h urine samples for determination of creatinine clearance. Of the initial 40 patients, one patient chose to end his participation after the first two visits, thus results were obtained for 39 patients. All patients were treated with either cyclosporin or tacrolimus and additionally with prednisolone. Six of the cyclosporin-treated patients and one tacrolimus-treated patient also received azathioprine as part of the immunosuppressive regimen. One patient in the tacrolimus-treated group was treated with mycophenolate mofetil. The demographics of the two patient groups are depicted in Table 1.

Calcineurin phosphatase assay

We used a method similar to that of Fruman *et al.* [13], and that published by our group earlier [14] and, briefly, did the following. CaN activity was measured as the release of radiolabelled phosphate from a phosphorylated 19 amino acid peptide (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-

Table 1. Patients demographics.

	Cyclosporin	Tacrolimus
Males:females	17:6	11:6
Age (years)	58 (27–68)	54 (30–63)
Transplant number 1:2:3	19:2:2	13:3:1
Cadaver:living-related donor	18:5	14:3
Time after transplantation (days)	2806 (524–6762)	1485 (421–2128)

Age and time after transplantation are reported as median (range).

Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu). Whole blood (40 μl) was lysed with a lysis buffer (150 μl) followed by three freeze/thaw cycles and then centrifuged at 4 °C, 12000 *g* for 10 min. A reaction mixture containing lysate (30 μl) and assay buffer (60 μl) including the radiolabelled peptide was prepared. The mixture incubated for 15 min at 30 °C, and the reaction was terminated by the addition of 5% trichloroacetic acid in a potassium phosphate buffer. Radioactivity was quantitated using liquid scintillation. 750 nM okadaic acid in the assay buffer was utilized to inhibit phosphatase PP1 and PP2A. To determine the activity of PP2C, a phosphatase resistant to both okadaic acid and CaN inhibitors cyclosporin/tacrolimus, each sample was compared with the same blood sample maximally suppressed with a surplus of cyclosporin (final concentration in the whole blood samples was 10 μM).

To convert activity measured in cpm to Units CaN we introduced daily calibration curves with bovine calcineurin using 0, 0.5, 1.0 and 2.0 Units CaN [14]. Calcineurin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and each bottle consisted of 86.95 Units CaN. The calcineurin was diluted with a 20 mM Tris/BSA buffer (pH 8.0). Each calibration curve consisted of 4 μl calmodulin (5 M), 6 μl Tris/BSA buffer and 20 μl of the appropriate concentration of calcineurin. The purified bovine calmodulin used in preparing the calibration curve was purchased from BIOMOL Research Labs., Inc. (Plymouth Meeting, PA, USA). About 60 μl assay buffer was added and the calibration curve samples were assayed according to standard assay procedures.

Blood cyclosporin and blood tacrolimus concentrations

Blood tacrolimus concentrations (ng/ml) were determined using an EMIT 2000 tacrolimus assay on a Cobas Mira analyzer (Triolab, ABX, Mont Pellier, France) [15], and the cyclosporin concentrations (ng/ml) were determined with a Dade Behring EMIT assay (Syva, Cupertino, CA, USA) using a Cobas Mira analyzer [16].

Statistical analysis

All CaN activity data and blood-drug concentrations were subjected to logarithmic transformation to assume normal distribution of the data. A multivariate general linear model (SPSS 10.0) was applied to test the hypotheses that: (i) the CaN activity profile was unchanged between days 1 and 180, (ii) the absorption profiles of both tacrolimus and cyclosporin were unchanged between days 1 and 180, (iii) the CaN activity profile was the same regardless of whether the patients received cyclosporin or tacrolimus, (iv) the values at each time point (0, 1, 2, 3 and 4) were the same despite immunosuppressive treatment and (v) single measurements of CaN activity (C2 for cyclosporin and C0 for tacrolimus) were the same between days 1, 90 and 180. A paired Student's *t*-test was utilized to compare the biochemical findings in Table 2. Area under the calcineurin activity curve (AUC_{CaN}; 0–4 h) was determined utilizing the trapezoidal rule and comparisons carried out by unpaired, double sided *t*-test.

Each drug was analysed by a random coefficient linear regression model with ln (enzyme activity) as the response and ln (drug) as the explaining variable. In the model, each patient had individual intercept and slope parameters (random coefficients). This implies that the degrees of freedom for testing the hypothesis of no association between enzyme activity and drug concentration (e.g. $\beta = 0$) reflect the number of patients in the

Table 2. Biochemical parameters and dosage of calcineurin inhibitors at inclusion and after 6 months.

	Cyclosporin			Tacrolimus		
	Day 1	Day 180	*P*-value	Day 1	Day 180	*P*-value
Creatinin clearance (ml/min)	67 ± 21.7	64 ± 19.5	0.43	59 ± 18.8	62 ± 19.6	0.48
Serum-creatinine (μM)	126 ± 31	130 ± 29.7	0.17	133 ± 29.2	132 ± 27.3	0.85
Urea (mM)	9 ± 2.5	10 ± 2.3	0.07	10 ± 3.1	9 ± 2.9	0.29
Haemoglobin (mM)	9 ± 0.8	8 ± 0.9	0.15	9 ± 0.6	9 ± 0.7	0.90
Albumin (μM)	580 ± 52.7	581 ± 56.6	0.90	587 ± 56.1	576 ± 49.6	0.23
Dosage (mg/day)	223 ± 65	215 ± 64	0.03	5.4 ± 1.8	5.1 ± 2.0	0.11

Values are expressed as mean ± SD. Paired *t*-test was used for the comparisons.

sample and not the total number of observations. The model was fitted by PROC MIXED, SAS v. 9.1.3.

Results

In this study our aim was to investigate a group of renal transplant patients with stable renal allograft function. To certify that our patients had stable renal allograft function throughout the entire observational period of 6 months, we compared some key biochemical results, and the results are depicted in Table 2. We compared creatinin clearance, S-creatinine, urea, haemoglobin and albumin, and none of the parameters tested was significantly different between days 1 and 180. As seen in Table 2, there were minor dose adjustments during the 6-month study period.

Figure 1 shows the relation between blood tacrolimus concentration and CaN activity. Because the profiles of both CaN activity and blood tacrolimus concentrations were not found to be statistically different between the two visits, we included in the figure the mean of measurements from days 1 and 180. The CaN activity curve shows that there is a slight but significant inhibition of the enzyme activity 2 h after oral intake of tacrolimus compared with time 1- and 3-h. The corresponding tacrolimus concentration displays a rapid increase, reaching C_{max} 1 h after intake of drug. There is clearly a delay of CaN inhibition after drug intake.

Figure 2 depicts the relation between CaN activity measurements and blood cyclosporin concentrations. The CaN activity displays a rapid decrease and reaches minimal activity after 1 h, followed by a gradual increase in enzyme activity thereafter. The minimal CaN activity reflects the C_{max} for cyclosporin, which is seen 1 h after drug intake. There is a clear inverse proportionate relation between CaN activity and blood cyclosporin concentration. The peak in drug concentration is followed by a rapid decrease in blood-drug concentration.

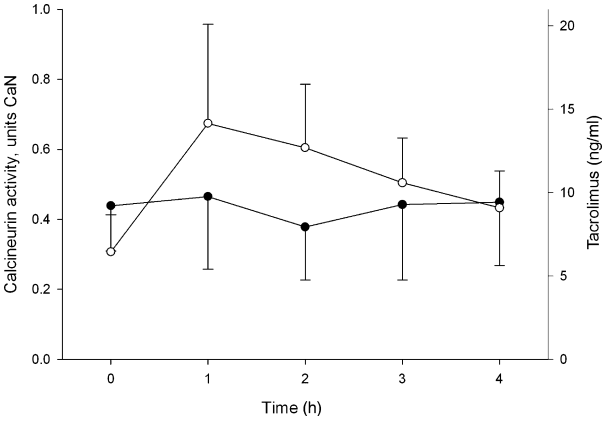

Figure 1 Blood tacrolimus concentrations (ng/ml) and calcineurin activity (Units CaN) from 17 tacrolimus-treated renal transplant patients with stable allograft function. The graph includes both results from days 1 and 180. Whole blood samples were drawn before and at 1, 2, 3 and 4 h after oral intake of tacrolimus. Closed circles represent calcineurin activity measurements and open circles represent blood-drug measurements. Results are represented as mean ± SD.

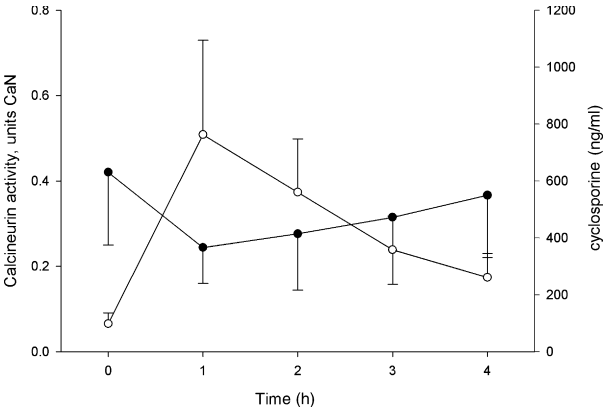

Figure 2 Blood cyclosporin concentrations (ng/ml) and calcineurin activity (Units CaN) from 23 cyclosporin-treated renal transplant patients with stable allograft function. The graph includes both results from days 1 and 180. Whole blood samples were drawn before and at 1, 2, 3 and 4 h after oral intake of cyclosporin. Closed circles represent calcineurin activity measurements and open circles represent blood-drug measurements. Results are represented as mean ± SD.

Because Figs 1 and 2 displayed different CaN activity, although the drug levels obtained ensured good stable graft function, we investigated whether the profiles were different. We found that the CaN activity profiles between the two drugs were different for both results from days 1 and 180 ($P = 0.017$ and $P < 0.0001$). We compared the $AUC_{CaN0–4}$ for cyclosporin-treated patients and tacrolimus-treated patients, and the comparisons demonstrated that cyclosporin treatment resulted in much lower AUC_{CaN} (1.74 units·h) compared with tacrolimus treatment (1.18 units·h) ($P < 0.0001$).

Figures 3 and 4 depict logarithmic-transformed drug concentrations and enzyme activities. In Fig. 3, there is a significant correlation between cyclosporin concentration and CaN activity ($P < 0.0001$), and the regression line is characterized by the following: $\ln(\text{CaN}) = -0.2295 \ln(\text{cyclosporin}) + 0.1003$. The 95% confidence interval for the intercept is 0.1003 ± 0.4210 and for the slope is -0.2295 ± 0.0677. Figure 4 shows the CaN activity measurements plotted against blood tacrolimus concentrations, and it is obvious that there is no correlation between the two parameters ($P = 0.27$).

We investigated whether the profiles of CaN activity (0–4 h) were the same at days 1 and 180 and found the profiles to be identical for both patient groups [cyclosporin-treated patients ($P = 0.87$) and tacrolimus-treated

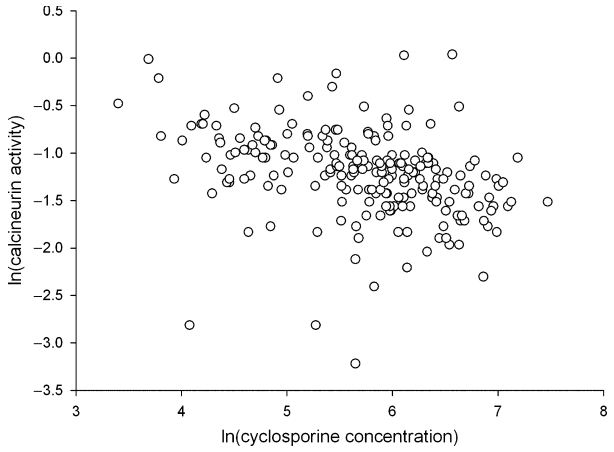

Figure 3 The graph depicts the correlation between blood cyclosporin concentration and calcineurin activity. The regression line is characterized by the following: ln(CaN) = −0.2295 ln(CsA) + 0.1003. The 95% confidence interval for the intercept is 0.1003 ± 0.4210 and for the slope −0.2295 ± 0.0677.

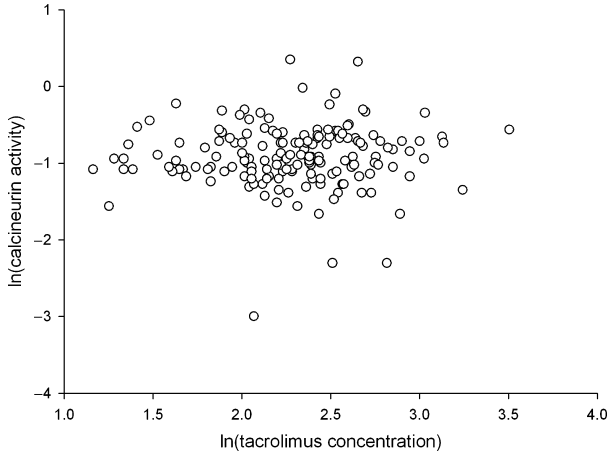

Figure 4 The graph depicts the correlation between blood tacrolimus concentration and calcineurin activity. The regression line is characterized by the following: ln(CaN) = −0.0852 ln(FK) − 0.7126. The 95% confidence interval for the intercept is −0.7126 ± 0.3745 and for the slope −0.0852 ± 0.1458.

patients ($P = 0.41$)]. Furthermore, we investigated whether the blood-drug absorption profile (0–4 h) was the same between days 1 and 180 for both cyclosporin and tacrolimus. We found that the blood-drug absorption profile did not change during the 6-month period [cyclosporin-treated patients ($P = 0.12$) and tacrolimus-treated patients ($P = 0.41$)]. Because we studied the patients three times during a 6-month period, we also made calculations including the visit after 3 months. For cyclosporin-treated patients, the C2 value for CaN activity of all three visits was compared, and we found no significant difference between the three measurements ($P = 0.86$). A similar conclusion was reached for the tacrolimus-treated patients comparing the trough level measurements at all three visits ($P = 0.99$).

Discussion

We have demonstrated that the inhibitory profile of CaN activity is different in renal transplant patients with stable renal allograft function treated with tacrolimus compared to those treated with cyclosporin. Furthermore, we have demonstrated that the patients with stable graft function can be assumed to have the same CaN activity profile over time. Finally, we have shown that there is a correlation between blood cyclosporin concentration and CaN activity, but this could not be shown for blood tacrolimus concentration and CaN activity under these conditions.

To our knowledge, it is the first time results have been presented of pharmacodynamic monitoring from a study of renal transplant patients followed over time period. We conclude that in renal-transplanted patients with stable renal allograft function, the CaN activity profile is constant over time. Additionally, we found that the CaN activity profiles are different in the two patient groups with regard to, the degree of inhibition as well as the time needed to reach maximal enzyme inhibition. For the cyclosporin-treated patients, we found a significant inhibition of enzyme activity with maximal inhibition occurring 1 h after oral intake of drug. This finding was reported earlier by Halloran *et al.* [10]. They found the maximal inhibition of CaN activity to occur after 1–2 h. The tacrolimus-treated patients showed a small but significant inhibition, reaching a maximum 2 h after drug intake. CaN inhibition by cyclosporin over the 4-h period was much greater than for tacrolimus, as illustrated in Figs 1 and 2 and from the AUC_{CaN} comparisons. To our knowledge, this has not been reported earlier. Millan *et al.* [12] reported a significant inhibition of CaN activity in 10 tacrolimus-treated renal transplant patients with stable renal allograft function compared with healthy volunteers. Because the author measured enzyme activity before drug intake and after 2 h, it is not possible to compare the time to reach maximal inhibition. Previously published results from our group of calcineurin activity measurements from recently transplanted patients [11] demonstrated a significant inhibition of the enzyme activity that was maximal after 2 h. It seems to have been confirmed that the time to reach maximal CaN inhibition in patients treated with tacrolimus is 2 h, regardless of whether the patients were recently transplanted or on maintenance immunosuppressive therapy.

Taking a closer look at the data from the tacrolimus-treated patients reveals that the inhibition of CaN by tacro-

limus in these stable patients is just barely significant. In fact, we did not find any inhibition in enzyme activity at days 1 ($P = 0.255$) and 180 ($P = 0.213$), but there were a just significant difference in the combined data ($P = 0.027$). It is intriguing that these tacrolimus-treated patients maintain good, stable renal allograft function with almost no CaN inhibition. A possible explanation for the differences found in patients early after transplantation as we have reported earlier [11] and in patients on maintenance immunosuppressive therapy could be related to dosing differences. Early after transplantation the patients receive much larger doses of calcineurin inhibitors. It cannot be ruled out that CaN can be upregulated either by the treatment with CaN-inhibiting drugs (as a simple positive feedback mechanism) or by the transplantation itself. This theory needs further studies, including focusing on protein and mRNA determinations. It could also be speculated if tacrolimus utilizes CaN-independent pathways to exert its immunosuppressive effect [17,18].

The overall CaN activity profiles were found to be different for the two drugs investigated and this could partially be explained by binding properties for the two drugs. The underlying mechanisms for the different inhibition profiles of tacrolimus and cyclosporin are basically unknown, but focusing on the molecular level could give a possible explanation. Recently, Huai *et al.* [19] published a study with crystal structures of cyclosporin–CyP (cyclophilin)–CaN and tacrolimus–FKBP12 (FK-binding protein)–CaN complexes and they reported that although the majority of the CaN residues involved in binding are common for the two complexes, a significant number are unique for each drug–immunophilin complex. Hydrogen binding between two amino acids of CyP and CaN has a direct effect on the catalytic activity of CaN. In contrast, the nearest FKBP12 binding is so far away that it cannot exert the same effect. Huai *et al.* [19] suggest that this hydrogen bonding might imply a different mechanism regarding regulation of CaN catalysis by the cyclosporin–CyP complex.

Another explanation for the difference found in CaN inhibition profiles could, at least to some degree, be the very different pharmacokinetic properties of the two drugs. The peak concentration of cyclosporin is relatively much higher than tacrolimus as demonstrated by a C_{max}/trough ratio that was 3.6 times higher in this study. The trough levels of CaN activity were not different in the cyclosporin and the tacrolimus groups and were not different from the activity levels we found in healthy volunteers (unpublished results). It therefore seems that the pharmacokinetic properties of the tacrolimus formulation (Prograf®, Fujisawa, Osaka, Japan) allow stable graft function at a lower total calcineurin inhibition than the cyclosporin formulation (Sandimmun Neoral®, Novartis, Basel, Switzerland).

The temporal profile of CaN inhibition is also dependent on the time needed to penetrate cells (binding of immunophilin to drug followed by binding to calcineurin), which could explain the hysteresis we find in the tacrolimus-treated patients. It has been proposed by Kung and Halloran [20] that FKBP12 (tacrolimus-binding protein) is more limiting compared with CyP (cyclophilin) leading to a lesser degree of CaN inhibition by tacrolimus compared with cyclosporin.

The fact that the patients investigated in this study all had S-creatinine below 200 μM and demonstrated little variation in their biochemical parameters and that we found the tacrolimus-treated patients to have no or very little inhibition of CaN raises the question whether the CaN inhibition is necessary to maintain graft function in renal-transplanted patients on maintenance immunosuppressive therapy. The scenario could be that CaN is upregulated to a higher activity level and therefore is inhibited in our patients. The inhibition we have observed in the cyclosporin-treated patients might not be obligatory for avoidance of allograft rejection. If this were the case, it could be speculated that CaN inhibition by cyclosporin primarily causes nephrotoxic side effects.

The clinical importance of the fact that these two immunosuppressive drugs display different CaN-inhibitory profile is not known at this moment. In a recent publication Kramer *et al.* [21] document that tacrolimus is a safe and efficient drug. They found significantly fewer episodes of graft loss and biopsy-proven acute allograft rejection in patients treated with tacrolimus than patients treated with cyclosporin microemulsion during a 24-month follow up. Furthermore, the patients on tacrolimus had significantly better renal graft function (S-creatinine) than the cyclosporin-treated patients. This could mean that, though the two drugs have been considered to be similar, they may also display differences. The less immunosuppression because of calcineurin inhibition could favour graft survival and minimize side effects, but further studies are needed to elucidate this very important issue. Determining CaN activity profiles in renal transplant patients could be a valuable supplement to classical pharmacokinetic drug monitoring. Patients may display subjective and objective signs of both overdosing and underdosing, even though blood-drug concentration are within the therapeutic interval. Therefore, measuring the patient's CaN profile could help adjusting the dosage thereby avoiding side effects without increasing the risk of acute rejection.

In conclusion, this study has demonstrated that the CaN inhibition profiles for both CsA and FK are stable over a period of 6 months. Furthermore, we found that the two drugs possess different CaN inhibition profiles, as

the CsA-treated patients showed significant CaN inhibition at 1 h postdose corresponding nicely to peak CsA blood concentration. Different results were found in the group of FK-treated patients, where we found no significant CaN inhibition on neither days 1 or 180 despite relevant blood FK concentrations. It therefore seems that in FK-treated patients stable graft function can be maintained despite very limited CaN inhibition. Additionally, we demonstrated a correlation between CaN activity and blood cyclosporin concentration, which was not found between CaN activity and blood FK concentration. There still remain a lot of unanswered questions in the field of CaN inhibition in transplant patients for future research to attend to. Hopefully, our observations can add to the already growing knowledge about the mechanism of action of these clinically important immunosuppressive drugs and contribute to the development of the most optimal dosing of the drugs.

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