Pharmacodynamic Monitoring of Cyclosporine A in Renal Allograft Recipients Shows a Quantitative Relationship Between Immunosuppression and the Occurrence of Recurrent Infections and Malignancies

Claudia Sommerer,[1,5] Mathias Konstandin,[2] Thomas Dengler,[2] Jan Schmidt,[3] Stefan Meuer,[4] Martin Zeier,[1] and Thomas Giese[4]

Background. At present it is unclear which dose and consecutive blood levels of cyclosporine A (CsA) are optimal with respect to immunosuppressive efficacy and drug specific side effects at the level of individual patients. Several pharmacodynamic measures of CsA effects have been proposed, but have not become clinical routine yet. Besides the lack of practicability, the biological relevance of these assays has not been determined so far.
Methods. Residual expression of nuclear factor of activated T-cells (NFAT)-regulated genes two hours after drug intake was used as molecular pharmacodynamic marker to assess CsA effects on lymphocytes and correlated with the frequency of recurrent infections and malignancies in patients with five or more years of follow-up posttransplantation.
Results. Recurrent infectious complications were observed in 44% and malignancies in 20% of the 133 patients studied. Patients with a strong suppression of NFAT-regulated genes by CsA—as judged by a residual level of transcription of less than 15% after drug intake— develop more frequent infections (53% vs. 29%; $P=0.005$) and malignancies (22% vs. 4%; $P=0.002$). The lack of correlation between the incidence of these complications and CsA blood concentration might point to the interindividual differences in the sensitivity towards calcineurin inhibition.
Conclusion. The data presented here reveal a clear relation between the frequency of infectious and malignant complications and the degree of suppression of NFAT-regulated genes by CsA in transplanted patients. Therefore, pharmacodynamic monitoring of CsA efficacy in transplanted patients might be a useful tool to adjust immunosuppressive therapy in individual patients.

Keywords: Monitoring of immunosuppression, Renal transplantation, Cyclosporine therapy.

(*Transplantation* 2006;82: 1280–1285)

Cyclosporine A (CsA) is the most commonly used immunosuppressive drug in renal transplantation. With the introduction of calcineurin inhibitors, long-term allograft survival has significantly improved (*1*), primarily by reduction of acute rejection episodes. However, rational means to balance both the benefits and side effects of CsA treatment are not sufficiently established yet (*2*, *3*).

One reason for that is a significant inter- and intraindividual variation of CsA absorption and its narrow therapeutic window (*4*). Assessment of CsA dosing is primarily performed by blood trough levels, pharmacokinetics such as measurement of two-hour peak levels, or by various area under the curve assessments (AUC, 4 to 12 hr). For the majority of patients, trough level drug monitoring seem to be adequate, but it remains unclear at which dose an optimal degree of immunosuppression is achieved in individual patients. For example, CsA trough levels poorly correlate with the frequency of acute rejection episodes (*5*). Some recent studies showed a better correlation of CsA peak levels (as a marker of total CsA exposure) with adequate immunosuppression (*6*, *7*); in other studies, CsA two-hour levels did not predict rejection or toxicity (*8*). Measurement of the total drug exposure as AUC for four hours after CsA uptake was suggested to be the best method for assessing the variation of CsA absorption and risk of acute rejection or drug toxicity (*9*). However, all these mentioned CsA measurements are unable to predict and monitor the biological effects of CsA. Several approaches have been undertaken to measure the biological consequences of CsA-based immunosuppression (*10*). Dosing of CsA according to the molecular effect of the drug on its target

C.S. and M.K. contributed equally to this work.
[1] Department of Nephrology, University of Heidelberg, Heidelberg, Germany.
[2] Department of Cardiology, University of Heidelberg, Heidelberg, Germany.
[3] Department of Surgery, University of Heidelberg, Heidelberg, Germany.
[4] Department of Immunology, University of Heidelberg, Heidelberg, Germany.
[5] Address correspondence to: Claudia Sommerer M.D., Department of Nephrology, University of Heidelberg, Im Neuenheimer Feld 162, 69120 Heidelberg, Germany.
E-mail: claudia.sommerer@med.uni-heidelberg.de
Received 3 July 2006. Revision requested 1 August 2006.
Accepted 9 August 2006.

ISSN 0041-1337/06/8210-1280
DOI: 10.1097/01.tp.0000243358.75863.57

cells could improve allograft survival and at the same time increase drug safety.

For this purpose, both the inhibition of the primary target of CsA—the phosphatase calcineurin—and its consequence the inhibition of nuclear factor of activated T-cells (NFAT)-regulated cytokines such as interleukin-2 (IL-2) were evaluated as pharmacodynamic measures for the effects of CsA (*11–13*).

Recently we described a reliable, precise, and robust whole blood assay based on the measurement of the expression of three NFAT-regulated genes (IL-2, interferon [IFN]-γ, and granulocyte-macrophage colony stimulating factor [GM-CSF]) in phorbol myristate acetate (PMA)/ionomycin stimulated lymphocytes before and 2 hr after CsA intake (*14*, *15*). The inhibition of genes in this assay reflects calcineurin inhibition and is independent from other commonly used immunosuppressive drugs. In order to validate this assay as a new molecular marker for the degree of immunosuppression in individual patients, we correlated the residual expression of NFAT-regulated genes with the frequency of recurrent infections and malignancies in patients with five or more years of follow-up posttransplantation.

PATIENTS AND METHODS

Patient's Characteristics

The study was approved by the local ethical committee and informed consent was obtained from all patients. All stable renal transplant patients on CsA therapy of the renal transplant outpatient clinic of the Department of Nephrology of the University of Heidelberg, Germany, who met the following inclusion criteria, were included from August 2002 to December 2005. Inclusion criteria were: stable renal transplant function with a creatinine <4 mg/dl (creatinine clearance calculated with Modification of Diet in Renal Disease formula >20 ml/min), change of creatinine in the last year <0.5 mg/dl, no change of immunosuppressive therapy in the last three years, no apparent acute infection, and at least 60 months after renal transplantation. We studied 133 stable renal allograft recipients (58 female/75 male), mean time of 130 months (60–306) after renal transplantation. Mean age of all patients was 56 years (20–78), mean body mass index 24 kg/m^2 (18–41). Immunosuppressive therapy consisted mainly of dual immunosuppression with low-dose steroids (2–8 mg/d) and CsA; in 21.8% of patients a triple immunosuppression with additional mycophenolic acid (22/133; 16.5%) and azathioprine (7/133; 5.3%) was used. None of the 133 patients had received induction therapy or antibody treatment for previous rejections.

Blood samples were drawn predose and two hr after oral intake of CsA at time of inclusion. Acute infection was excluded in all patients by the absence of clinical symptoms, negative urinary sediment and culture, normal high sensitive C-reactive protein and no detectable cytomegalovirus pp65 early antigen. Patients were monitored with respect to malignancy, previous recurrent viral and bacterial infections, and opportunistic infections occurring in the stable period at least 12 month after transplantation. Clinical data were obtained from patient's records.

Cyclosporine A Determination

All CsA blood levels were measured by enzyme multiplication immunoassay technique (Date-Behring) in the same laboratory.

Sample Preparation

Heparinized peripheral blood was stimulated with 1 ml of complete RPMI 1640 containing 100 ng/ml PMA and 5 μg/ml ionomycin (Sigma) for three hr at 37°C. After red cell lysis with ACK buffer (0.15M ND4CI, 1.0 mM $KHCO_3$), leukocytes were lysed with 300 μL of MagNA-Pure lysis buffer supplemented with additional 1% (w/v) dithiothreitol (RAS, Mannheim), and the sample was frozen at −70°C. After thawing, mRNA was isolated with the MagNA-Pure-LC device using the mRNA standard protocol for cells. The elution volume was set to 50 μl. One aliquot of 8.2-μl RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (AMV-RT) and oligo- (dT) as a primer (First Strand cDNA synthesis kit; Roche) according to the manufacturer's protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 μl and stored at −20°C until polymerase chain reaction (PCR) analysis.

Quantitative Analysis of Gene Expression

The NFAT-regulated genes IL-2, IFN-γ, and GM-CSF have been identified as suitable genes for this assay in our previous study (*14*, *15*). Gene expression was quantified using real-time PCR with the LightCycler. Target sequences were amplified using commercially available LightCycler Primer Sets (Search-LC, Heidelberg) with the LightCycler FastStart DNA Sybr Green I Kit (Roche Diagnostics) according to the manufacturer's protocol. The transcript concentration for the measured genes was calculated from a virtual standard curve, obtained by plotting a known input concentration of a plasmid to the PCR cycle number at which the detected fluorescence intensity reaches a fixed value. mRNA input was normalized by a constant expression value of two housekeeping genes (β-actin and cyclophilin B).

The residual gene expression after CsA intake was calculated as C2/C0*100, where C0 is the adjusted number of transcripts at CsA-predose level and C2 is the number of transcripts two hr after drug intake. For all three genes the residual expression was averaged and presented as "mean residual expression of NFAT-regulated genes."

In 82 patients without change of CsA dosage, repetitive measurements of NFAT regulated gene expression were performed in an interval between four weeks and 12 months.

Statistical Methods

Statistical analysis was performed using the SPSS 11.0 package (Chicago, IL). All statistical tests were two-tailed with a statistical significance level of 0.05. In the study population residual NFAT-regulated gene expression was not normally distributed, therefore non-parametric statistical tests were performed. The Mann-Whitney *U* test for comparison of sample medians was used, the chi-squared test for association between qualitative variables, Spearman's linear correlation coefficient for correlation between variables, and multivariate logistic regression analysis to determine the influence of several variables.

Results are expressed as percentages, means, and ranges. A target level for the residual NFAT regulated gene expression was defined by receiver operating curve (ROC) analysis of the combined infection and/or malignancy incidence.

RESULTS

Variability

In 82 cases, inhibition of NFAT-regulated gene expression was measured twice in an interval between four weeks to 12 months. Intraindividual variability in renal transplant patients on stable CsA immunosuppression was 9%. There was a strong correlation between the first and the second measurement ($r=0.77$, $P<0.001$). Interindividual variability in 31 patients with a daily CsA dose of 100 mg was 14%. The average variability of the reverse transcriptase polymerase chain reaction (RT-PCR) measurement was less than 11%.

Clinical Data

Mean serum creatinine of all patients was 1.4 (0.5–4.0) mg/dl. Mean CsA trough and two-hour blood levels in all 133 patients were 84 (25–181) and 521 (158–1228) μg/l, mean CsA peak/trough ratio was 6.7 (3.0–18.5). Mean residual NFAT-regulated gene expression in all patients was 16 (2–50)% with a mean residual gene expression of IL-2 of 14% (1–54), IFN-γ of 17% (2–55), and GM-CSF of 16% (2–58). Clinical data of renal transplant patients are given in Table 1.

Univariate Analysis

A total of 58 of 133 patients (44%) had recurrent infectious complications or opportunistic infections (34 patients had urinary tract infections, two patients developed urosepsis, 24 patients had respiratory tract infections—predominantly viral infections assumed, four patients had pneumonia, seven patients showed cytomegaly viremia, one patient had recurrent erysipelas, and two patients developed repetitive herpes zoster). Patients with infections showed a significantly stronger inhibition of NFAT-regulated gene expression as compared to patients with no infections (13% (2–47) vs. 18% (2–50), $P=0.004$, Table 2, Fig. 1). Importantly, CsA trough and two-hour blood levels as well as the CsA peak/trough ratio were not statistically differ-

TABLE 1. Clinical data of renal transplant patients

Characteristic	Value
Sex (male)	75 (56.4%)
Age (years)	56 (20–78)
Time after transplantation (months)	130 (60–306)
Body mass index (kg/m^2)	24 (18–41)
S-creatinine (mg/dl)	1.4 (0.5–4.0)
CsA trough level (μg/l)	84 (25–181)
CsA 2-hour level (μg/l)	521 (158–1228)
CsA peak/trough ratio	6.7 (3.0–18.5)
Residual NFAT-regulated gene expression (%)	16 (2–50)
Residual IL-2 gene expression	14 (1–54)
Residual IFN-γ gene expression	17 (2–55)
Residual GM-CSF gene expression	16 (2–58)

Data given as means (range) or n (%).

TABLE 2. Residual expression of three key NFAT-regulated genes in patients with and without recurrent infections and with and without malignancy

	Mean (%)			IL-2 (%)			IFN-γ (%)			GM-CSF (%)		
	Yes	No	*P* value	Yes	No	*P* value	Yes	No	*P* value	Yes	No	*P* value
Recurrent infection (n=58)	13 (2–47)	18 (2–50)	0.004	11 (1–52)	16 (1–54)	0.003	14 (2–47)	19 (3–55)	0.008	13 (2–42)	18 (2–58)	0.008
Malignancy (n=26)	11 (2–37)	17 (2–50)	0.006	9 (1–34)	15 (1–54)	0.013	14 (2–47)	18 (3–55)	0.039	9 (2–30)	18 (2–58)	<0.001

Data are means (range). *P* values calculated by Mann-Whitney *U* test.

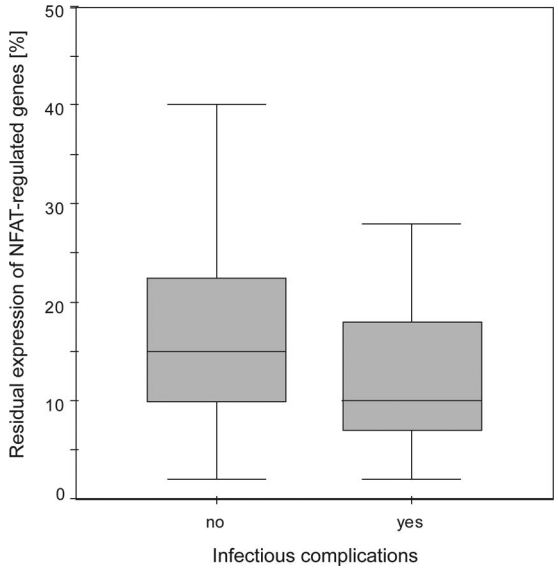

FIGURE 1. Boxplot of the residual NFAT-regulated gene expression in renal transplant patients without and with recurrent infectious complications shows the median, interquartile range and outliers (18% [2–50], SD 11%, 95% CI 15–21% vs. 13% [2–47], SD 9%, 95% CI 10–15%; $P=0.004$).

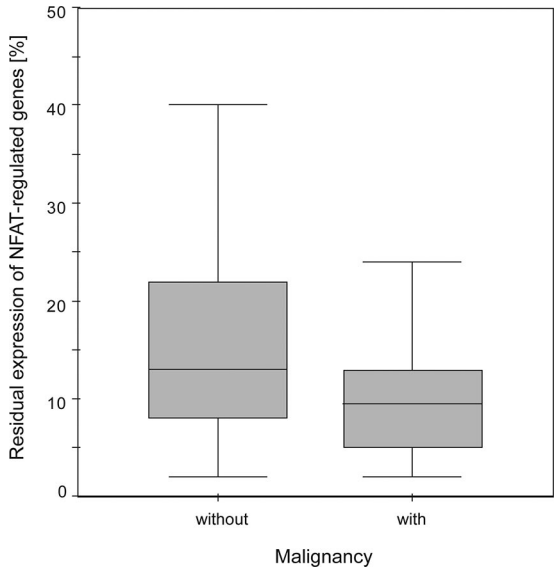

FIGURE 2. Boxplot of the residual NFAT-regulated gene expression in renal transplant patients without and with malignancies shows the median, interquartile range and outliers (11% [2–37], SD 8%, 95% CI 8–14% vs. 17% [2–50], SD 11%, 95% CI 15–19%; $P=0.006$).

ent between patients with/or without infections (CsA trough level: 83 μg/l [25–181] vs. 86 μg/l [25–163]; CsA 2-hour level: 504 μg/l [158–1122] vs. 543 μg/l [253–1228]; CsA peak/trough ratio: 6.4 [3.0–11.4] vs. 6.9 [3.0–18.5]).

Another 26 out of 133 patients (19.5%) developed malignancies (skin cancer: 13 patients with squamous cell carcinoma, partly recurrent and nine patients with basal cell carcinoma; other solid organ malignancies: one patient with an urothelial tumor, one female patient with breast cancer, and two patients with lung cancer). These 26 patients had a significantly stronger inhibition of NFAT-regulated gene expression (11% [2–37] vs. 17% [2–50], $P=0.006$; Table 2, Fig. 2). CsA trough levels were comparable (84 μg/l [25–181] vs. 86 μg/l [33–142]), CsA two-hour peak levels were higher in patients with cancer (579 μg/l [341–1103] vs. 507 μg/l [158–1228]; $P<0.05$), but the CsA peak/trough ratio was not statistically different between the groups (6.4 [3.0–18.5] vs. 7.3 [3.5–12.8], $P=0.07$).

CsA dose per kg body weight was not different in patients with and without malignancy (1.97 [0.71–3.25] vs. 1.87 [0.71–4.7] mg/kg body weight (BW) and in patients with or without infectious complications (1.86 [0.71–4.17] vs. 1.91 [0.71–4.7] mg/kg BW). Additional use of mycophenolic acid or azathioprine was not more frequent in patients with infectious complications or malignancy compared to patients without complications.

By ROC analysis of the combined infection and/or malignancy incidence, a critical cutoff for the increased risk of infectious and malignant complications could be found at 15% of residual NFAT-regulated gene expression.

In 60.2% of all patients, residual NFAT-regulated gene expression was below 15%. Patients with a residual NFAT gene expression below the cutoff level of 15% (which means with a stronger immunosuppression) had significantly more infectious complications compared to patients with residual NFAT activity above 15% (53 vs. 29%, $P=0.005$). In addition, patients with a residual NFAT-gene expression below 15% had significantly more malignancies compared to patients with residual NFAT activity above 15% (22 vs. 4%, $P=0.002$). Only in three patients with malignancy was a residual NFAT-regulated gene expression above 20% observed. It is of particular interest that patients with nonskin malignancy (lung, breast, and uroepithelial cancer) had significantly lower residual NFAT-regulated gene expression compared to patients with skin cancer (4% (*2–9*) vs. 10% (4–37), $P=0.02$).

Biopsy-proven acute rejection episodes (one borderline, one BANFF I) were documented in two patients; residual NFAT-regulated gene expression in both patients was 47%. There was no significant correlation between residual NFAT-regulated gene expression and kidney transplant function (serum creatinine; $r=-0.136$, $P=0.12$).

Multivariate Analysis

A multiple regression analysis was performed including the following variables: age, follow-up after transplantation, kind of immunosuppression, CsA trough level, CsA 2-hour level, and residual NFAT regulated gene expression. Mean residual NFAT regulated gene expression was independently associated with recurrent infection ($R=0.229$, beta $=-0.229$, $P=0.009$) and development of malignancies was significantly associated with age and NFAT regulated gene expression ($R=0.515$, beta [alter] $=0.458$, beta [NFAT regulated gene expression] $=-0.205$, $P<0.001$). Data for multivariate logistic regression analysis are given in Table 3.

DISCUSSION

Immunosuppression with CsA has improved patient and transplant survival in the past 20 years. Although the immunosuppressive efficacy of CsA is well proven, there is no rationale with respect to the optimal dose of CsA particularly in the long-term treatment of allograft recipients. The bene-

TABLE 3. Multivariate logistic regression analysis

	Coefficient β	*P* value	Odds ratio (95% CI)
Recurrent infection			
Age	0.308	0.481	1.36 (0.58–3.21)
Time after transplantation	0.118	0.662	1.13 (0.66–1.91)
Immunosuppression	0.497	0.181	1.64 (0.79–3.41)
CsA trough level	−0.006	0.984	0.99 (0.52–1.90)
CsA 2-hour level	−0.281	0.506	0.76 (0.33–1.73)
Residual NFAT-regulated gene expression	−0.521	<0.05	0.59 (0.39–0.90)
Malignancy			
Age	2.63	<0.001	13.86 (4.00–48.04)
Time after transplantation	0.10	0.783	1.10 (0.55–2.23)
Immunosuppression	−0.363	0.667	0.73 (0.17–3.11)
CsA trough level	−0.134	0.753	0.87 (0.38–2.02)
CsA 2-hour level	−0.363	0.504	0.70 (0.24–2.02)
Residual NFAT-regulated gene expression	−0.816	<0.05	0.44 (0.23–0.83)

Categories: age (cutoff 65 years), follow-up after transplantation (60–120 months, 121–180 months, >180 months), immunosuppression (CsA and steroids, CsA and MPA, CsA and azathioprine), CsA trough level (<50 μg/l, 50–99 μg/l, 100–149 μg/l, >149 μg/l), CsA two–hour level (<300 μg/l, 300–599 μg/l, 600–899 μg/l, >899 μg/l), and residual NFAT regulated gene expression (<10%, 10–14%, 15–19%, >19%).

fits of CsA treatment decline in a considerable portion of patients due to severe side effects, such as malignancy, infection, and nephrotoxicity. It is therefore of particular importance to identify measurable parameters to assess the effect of CsA on the patient and the allograft survival. In general, measuring the trough level drug concentration is used to monitor CsA treatment. However, to achieve a more accurate CsA exposure, measurement of AUC for various durations (2 to 12 hr) was introduced but its clinical implementation is impracticable. Despite these efforts, side effects are not avoided and it is presumed that long-term allograft loss is mainly due to CsA toxicity. Several approaches have been undertaken to measure the biological impact of CsA-based immunosuppression. Calcineurin is a key component of T-cell activation and serves as the target of the CsA cyclophilin complex (*16*). Halloran et al. (*11*) demonstrated that the immunosuppressive action for CsA, when measured as inhibition of calcineurin activity in lymphocytes, is inversely correlated with CsA blood levels. In humans, peak CsA blood levels at one to two hours after oral CsA uptake resulted in a 70 to 96% inhibition of calcineurin, which was rapidly reversed when CsA blood levels came down to the trough levels (*10*, *11*). The results were further supported by the observation of Pai et al. (*17*) that a lower calcineurin activity resulted in a higher rate of graft versus host disease in bone marrow recipients.

Despite the increasing knowledge of the relationship between calcineurin activity and CsA levels, unanswered questions remain: What should be the standard level of calcineurin inhibition and at what level the toxicity exceeds the efficacy of immunosuppression? Most recent studies focused on the measurement of cytokines, such as IL-2 or IFN-γ. Stein et al. proposed a simple assay by measuring IL-2 protein in mitogen-activated whole blood (*12*). Sindhi et al. (*18*) demonstrated a significant decrease of IL-2 synthesis by flow cytometry in PMA/ionomycin stimulated lymphocytes under CsA treatment. In a recent study, Härtel et al. described an assay based on differential gene expression kinetics in CD3/CD28 stimulated lymphocytes (*13*).

We recently introduced a precise, highly reproducible, and dynamic assay to measure the individual responsiveness of T-lymphocytes to CsA exposure in vivo. The assay is based of the quantitative analysis of IL-2, IFN-γ, and GM-CSF gene expression in PMA/ionomycin stimulated lymphocytes in whole blood samples of renal transplant recipients at CsA trough levels and two hr after oral uptake of CsA (*14*, *15*). The assay is not sensitive toward other commonly used immunosuppressive drugs such as corticosteroids, azathioprine, mycophenolic acid, or rapamycin. Therefore, the reduction of gene expression after two hr postoral uptake of CsA compared to the gene expression prior to CsA uptake reflects only the strength of CsA based immunosuppression. In order to make the test more powerful towards the interindividual variations in gene expression, a ratio between the values at peak and trough drug concentration was used. In addition, this opens the possibility to establish target values independent of the technical platform for gene expression analyses. The test can be performed semiautomated and highly standardized at relative low cost in a specialized laboratory. Overnight shipment of samples is possible. Results are available within one working day.

As we showed previously, the reduction of NFAT-dependent gene expression varies considerably between patients but is consistent within a given patient with a variability of 9% in the present study. It is of note that about 60% off all patients had a residual NFAT-regulated gene expression of less than 15%, which documents that the immune system in the majority of patients is more than necessary suppressed. In addition, the variability among patients reflects the individual immunosuppressive responsiveness to CsA. We studied patients beyond the fifth year after transplantation when the immunological risk is relative low compared to drug specific side effects such as malignancy and infections. In the present

study we detected a higher rate of malignancy, mainly nonmelanoma skin tumors (squamous and basal cell carcinomas), in patients with a significantly stronger reduction of NFAT gene expression compared to nontumor bearing long-term allograft recipients. There was no correlation between treatment for primary disease in patients with or without posttransplant malignancy. Furthermore, none of the patients with cancer was under cytotoxic induction therapy. The number of retransplants, both second and third, was not higher in tumor-bearing vs. nontumor-bearing patients. With respect to tumor genesis, we could demonstrate that a stronger inhibition (<90%) of NFAT gene expression two hr after CsA uptake is associated with a higher rate of malignancy, particularly skin cancer. It has been shown previously that patients on a high CsA-dose regimen are at increased risk for the development of cancer compared to patients on a low-dose regimen (*19*). However, the patients in this study would match only the low dose group (median CsA trough concentration, 80 μg/l; only three out of 133 patients had a CsA trough concentration above 150 μg/l and none of those developed tumors). This might explain why we could not see in our study a correlation between malignancies and CsA trough levels or CsA peak/trough ratios.

Not only the rate of malignancy is augmented but also the frequency of infections is approximately doubled in patients with low residual NFAT expression two hr after CsA uptake. Patients with malignancy and/or infection received similar CsA doses per body weight. Patients with or without infections did not differ at CsA trough blood levels and CsA two hr blood levels, suggesting that this test better reflects the degree of immunosuppression in individual patients compared to drug concentration measurements.

Patients with a stronger inhibition of NFAT expression had a tendency to higher serum creatinine levels. To exclude an influence of transplant function on NFAT gene expression, we calculated the relative reduction of NFAT gene expression.

With the present study, we document the individual functional response of renal transplant recipients to CsA. A stronger inhibition of NFAT gene expression in the long-term follow-up is associated with a higher rate of cancer and infection. Patients at risk for these side effects can be identified and the CsA dose can be reduced accordingly while maintaining organ protective immunosuppression.

ACKNOWLEDGMENT

We thank Ms. Daniela Merklinger for excellent technical assistance.

REFERENCES

1. Hariharan S, Johnson CP, Bresnahan BA, et al. Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 2000; 342(9): 605.
2. Nankivell BJ, Borrows RJ, Fung CL, et al. Calcineurin inhibitor nephrotoxicity: longitudinal assessment by protocol histology. *Transplantation* 2004; 78(4): 557.
3. Sommerer C, Hergesell O, Nahm AM, et al. Cyclosporin A toxicity of the renal allograft–a late complication and potentially reversible. *Nephron* 2002; 92(2): 339.
4. Kovarik JM, Mueller EA, van Bree JB, et al. Cyclosporine pharmacokinetics and variability from a microemulsion formulation–a multicenter investigation in kidney transplant patients. *Transplantation* 1994; 58(6): 658.
5. Pescovitz MD, Barbeito R. Two-hour post-dose cyclosporine level is a better predictor than trough level of acute rejection of renal allografts. *Clin Transplant* 2002; 16(5): 378.
6. Morris RG, Russ GR, Cervelli MJ, et al. Comparison of trough, 2-hour, and limited AUC blood sampling for monitoring cyclosporin (Neoral) at day 7 post-renal transplantation and incidence of rejection in the first month. *Ther Drug Monit* 2002; 24(4): 479.
7. Di Paolo S, Teutonico A, Schena A, et al. Conversion to C2 monitoring of cyclosporine A exposure in maintenance kidney transplant recipients: results at 3 years. *Am J Kidney Dis* 2004; 44(5): 886.
8. Einecke G, Schutz M, Mai I, et al. Limitations of C2 monitoring in renal transplant recipients. *Nephrol Dial Transplant* 2005; 20(7): 1463.
9. Mahalati K, Belitsky P, West K, et al. Approaching the therapeutic window for cyclosporine in kidney transplantation: a prospective study. *J Am Soc Nephrol* 2001; 12(4): 828.
10. Kung L, Batiuk TD, Palomo-Pinon S, et al. Tissue distribution of calcineurin and its sensitivity to inhibition by cyclosporine. *Am J Transplant* 2001; 1(4): 325.
11. Halloran PF, Helms LM, Kung L, Noujaim J. The temporal profile of calcineurin inhibition by cyclosporine in vivo. *Transplantation* 1999; 68(9): 1356.
12. Stein CM, Murray JJ, Wood AJ. Inhibition of stimulated interleukin-2 production in whole blood: a practical measure of cyclosporine effect. *Clin Chem* 1999; 45(9): 1477.
13. Hartel C, Fricke L, Schumacher N, et al. Delayed cytokine mRNA expression kinetics after T-lymphocyte costimulation: a quantitative measure of the efficacy of cyclosporin A-based immunosuppression. *Clin Chem* 2002; 48(12): 2225.
14. Giese T, Zeier M, Meuer S. Analysis of NFAT-regulated gene expression in vivo: a novel perspective for optimal individualized doses of calcineurin inhibitors. *Nephrol Dial Transplant* 2004; 19 Suppl 4: IV55.
15. Giese T, Zeier M, Schemmer P, et al. Monitoring of NFAT-regulated gene expression in the peripheral blood of allograft recipients: a novel perspective toward individually optimized drug doses of cyclosporine A. *Transplantation* 2004; 77(3): 339.
16. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 1992; 357(6380): 695.
17. Pai SY, Fruman DA, Leong T, et al. Inhibition of calcineurin phosphatase activity in adult bone marrow transplant patients treated with cyclosporine A. *Blood* 1994; 84(11): 3974.
18. Sindhi R, LaVia MF, Paulling E, et al. Stimulated response of peripheral lymphocytes may distinguish cyclosporine effect in renal transplant recipients receiving a cyclosporine+rapamycin regimen. *Transplantation* 2000; 69(3): 432.
19. Dantal J, Hourmant M, Cantarovich D, et al. Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens. *Lancet* 1998; 351(9103): 623.