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RAPID COMMUNICATIONS

# MONITORING OF NFAT-REGULATED GENE EXPRESSION IN THE PERIPHERAL BLOOD OF ALLOGRAFT RECIPIENTS: A NOVEL PERSPECTIVE TOWARD INDIVIDUALLY OPTIMIZED DRUG DOSES OF CYCLOSPORINE A

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***Background.*** **With the introduction of cyclosporine A (CsA), long-term allograft function has significantly improved. Problems related to limited therapeutic margins and CsA toxicity remain unsolved. Until now there have been no reliable, practical markers to measure the biologic activity of CsA in vivo.**

***Methods.*** **Expression of NFAT (nuclear factor of activated T cells)-regulated genes (interleukin 2, interferon-γ, and granulocyte-macrophage colony-stimulating factor) in phorbol myristate acetate/ionomycin-stimulated peripheral blood from healthy volunteers (n=34) and from stable renal (n=25), cardiac (n=26), and liver (n=14) transplant recipients receiving CsA therapy was measured by quantitative real-time reverse transcriptase-polymerase chain reaction before and 2 hr after drug intake. Gene expression and CsA plasma levels were correlated.**

***Results.*** **Two hours after oral CsA ingestion, the mean suppression of induced interleukin 2, interferon-γ, and granulocyte-macrophage colony-stimulating factor gene expression was 85%. The individual decline of NFAT-regulated gene expression and the total drug exposure at this time point were closely related. Six hours after oral CsA uptake, gene expression levels reached predose values and subsequently increased further in some patients (rebound effect).**

***Conclusion.*** **Quantitative measurement of the inhibition of NFAT-regulated gene expression 2 hr after CsA intake represents a novel approach to assess the biologic effectiveness of CsA therapy and has the potential to enable individualized immunosuppressive regimens.**

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Cyclosporine A (CsA) has improved patient and organ graft survival, but the issue of benefit and toxicity remains unsolved (*1–3*). CsA treatment is still monitored according to CsA predose levels, which barely reflect its biologic effects. For instance, in renal allograft recipients, CsA levels poorly correlate with the frequency of acute rejection episodes (*4*). A more relevant parameter for CsA dosage is the measurement of total drug exposure (*5*). However, this method is virtually impractical for routine outpatient monitoring. Recent studies have shown that the CsA peak concentration (2 hr after oral administration; $C_2$) represents an appropriate, single-sample marker for total drug exposure (*6*,*7*). A reduced incidence and severity of rejection in de novo patients was demonstrated (*8*,*9*). Although the widely accepted measurements of CsA concentrations seem adequate for the majority of patients, it is not clear whether they reflect optimal functional immunosuppression.

Several approaches have been taken to measure the biologic consequences of CsA-based immunosuppression. Halloran et al. (*10*) showed that the immunosuppressive action of CsA (the inhibition of calcineurin in lymphocytes) is correlated with blood concentration and CsA peak approximately 2 hr after Neoral administration. In humans, peak CsA levels at 1 to 2 hr produced a 70% to 96% calcineurin inhibition, which correlated closely with the rise and fall of CsA levels. In a recent study, there was a significant decrease in the proportion of phorbol myristate acetate (PMA)/ionomycin-stimulated lymphocytes expressing interleukin 2 (CD4 IL-$2^+$) measured by flow cytometry (*11*).

The recently available technique of real-time polymerase chain reaction (PCR) provides a fast, highly reproducible, and sensitive tool for quantitative analysis of gene expression (*12*). In the present study we have used this method to measure directly the functional consequences of calcineurin inhibition, namely inhibition of transcriptional activities of NFAT (nuclear factor of activated T cells)-regulated genes in peripheral blood. This approach overcomes several limitations of the testing of CsA responsiveness in isolated peripheral blood mononuclear cells (PBMC) on the protein level.

## MATERIALS AND METHODS

### *CsA Determination*

All CsA predose and 2-hr levels were measured by EMID (Date-Behring) in the same laboratory. In seven renal transplant recipi-

ents, a CsA kinetic profile was measured at 0 (before oral uptake), 2, 6, and 10 hr after the intake of CsA. To assess the intraindividual variance, six additional kidney transplant patients (two men/four women) had repeated measurements of CsA (predose and 2 hr) levels.

### *Exclusion of Acute or Chronic Infections*

At the time when blood samples were drawn, none of the patients had acute or chronic infection. Infection was excluded by patient's history and physical examination and the determination of C-reactive protein (normal value <3.4 mg/dL). Cytomegalovirus viremia was excluded by the absence of viral antigen (pp65). None of the renal patients had chronic infection of the native kidneys or vasculitis. Patients with renal dysfunction were excluded from the study. None of the liver transplant patients had chronic hepatitis B or C.

### *Characteristics of the Kidney-Heart and Liver Transplant Patients*

Patients were studied at their regular follow-up in the transplant outpatient clinic. Blood samples were drawn predose and at 2-hr after oral uptake of CsA. All patients were advised to take their medication (immunosuppressives other than CsA, antihypertensives, and vitamin D analogs) after blood drawing for the 2-hr CsA blood level. The participating subjects gave informed consent, under a protocol approved by the institutional review board.

### *Kidney Allograft Recipients*

The study comprised 25 (14 men/11 women; 20 cadaveric, 5 living related) renal transplant recipients, with a median age of 49.5 (range, 27–72) years and a median body mass index of 25.5 (range, 17.7–32.6) kg/m$^2$; the median follow-up interval was 79 (range, 8–201) months after transplantation. Renal function measured by creatinine clearance was 70 mL/min/1.72 m$^2$ (range, 43–121). A history of acute rejection episodes (biopsy proven, <12 mo after transplantation) was noted in 3 (12%) of 25 patients. CsA nephrotoxicity (preglomerular vascular hyalinosis, striped fibrosis) was seen in 5 (20%) of 25 patients. Immunosuppression included methylprednisolone 3 (range, 1–4) mg and a median dosage of CsA of 1.85 (range, 0.9–3.8) mg/kg in all patients. Five patients additionally received azathioprine (1.2 mg/kg; range, 0.8–1.9), and another six patients received mycophenolate mofetil (1–1.5 g per day). Fifteen of 25 patients were receiving ACE inhibitors or angiotensin receptor blockers, and 11 of 24 patients were receiving inhibitors of cholesterol synthesis.

### *Cardiac Allograft Recipients*

The study included 30 blood samples from 26 (23 men/3 women) cardiac allograft recipients, with a median age of 59 (range, 46–71) years and a median follow-up after transplantation of 43 (3–155) months. Cardiac dysfunction was excluded by transthoracic echocardiography measured by the same examiner. Mild rejection episodes (biopsy proven, <12 months after transplantation) were reported in six patients (≤grade 1b). Immunosuppression consisted of CsA with a median dosage of 2.9 (range, 1.4–6.1) mg/kg in all patients. Additional immunosuppression included azathioprine in six patients (median dosage 1.89 mg/kg; range, 1.0–3.7); 13 patients received corticosteroids (median dosage 6.0 mg; range, 5–15), and 19 patients received mycophenolate mofetil (median dosage 2.9 g/day; range, 1–4.5).

### *Liver Allograft Recipients*

The study included 14 (9 men/5 women) liver allograft recipients with a median follow-up of 9 (range, 2–15) years. Median age and body mass index were 48 (range, 33–67) years and 26.5 (range, 18.4 - 30.9) kg/m$^2$, respectively. In nine patients (69%), acute biopsy-proven rejection episodes occurred during the first 12 months after transplantation. For immunosuppression, a median dose of 1.94 (range, 0.75–4.76) mg/kg CsA per day was given; no patients received corticosteroids.

### *Sample Preparation*

Heparinized peripheral blood (1 mL) was stimulated with 1 mL of complete RPMI 1640 containing 100 ng/mL PMA and 5 $\mu$g/mL ionomycin (Sigma) for 3 hr at 37°C. After red cell lysis with ACK buffer, leukocytes were lysed with 300 $\mu$L of MagNAPure lysis puffer (RAS, Mannheim), and the sample was frozen at −70°C. After thawing, mRNA was isolated with the MagNAPure-LC device using the mRNA standard protocol for cells. The elution volume was set to 50 $\mu$L. One aliquot of 8.2-$\mu$L RNA was reverse transcribed using AMV-RT and oligo-(dT) as 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 $\mu$L and stored at −20°C until PCR analysis.

### *Quantitative Analysis of Gene Expression*

Gene expression was quantified using real-time PCR with the LightCycler. This technology has several advantages. Data are obtained during the exponential phase of the amplification and are therefore quantitative. The method is very fast, and the closed tube approach limits contamination by PCR products (*12*). The system can be automated with quick turnarounds. 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. To use gene expression analysis in a dependable fashion, it is important to investigate several genes of one group simultaneously, to adjust for random variations in the expression of individual genes. 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 (CP) at which the detected fluorescence intensity reaches a fixed value.

The amount of isolated mRNA from 1 mL of peripheral blood is too low to be reliably determined using standard techniques of quantification. To correct for variations in the yield and to control the integrity of mRNA, two control genes were analyzed in parallel. These control genes—$\beta$-actin and cyclophilin B—are differentially expressed in different tissues, but importantly, minimally regulated during the short stimulation period of 3 hr. The mean expression of the two genes was indistinguishable between patients with CsA therapy and healthy controls (6496 vs. 6695 and 405 vs. 432 transcripts/$\mu$L cDNA for $\beta$-actin and cyclophilin B, respectively). Therefore, mRNA input was normalized by a constant expression value of the two housekeeping genes. Because this factor remained constant, all samples are comparable to each other. The adjusted transcript concentration was calculated as follows: adjusted transcript=measured transcript/mean ($C_{actin}$/7000; $C_{CBP}$/450). The data of the two independent analyses for each sample and parameter were averaged and presented as adjusted transcripts/$\mu$L cDNA. This concentration multiplied by a factor of 3,000 gives an estimate of the total transcript level in 1 mL of whole blood. The inhibition of gene expression was calculated as $100-C_2/C_0*100$, where $C_0$ is the transcript concentration at CsA predose level and $C_2$ is the concentration 2 hr after drug intake.

## RESULTS

### *Inhibition of Gene Expression by CsA in Healthy Controls*

Experimentally, NFAT activation can be achieved by exposure of leukocytes to phorbolester and ionomycin (PMA/ionomycin) in vitro. Preliminary experiments showed a significant discrepancy between therapeutically effective doses of CsA and the concentrations that inhibit gene expression in isolated PBMC. In addition, during the isolation step, the

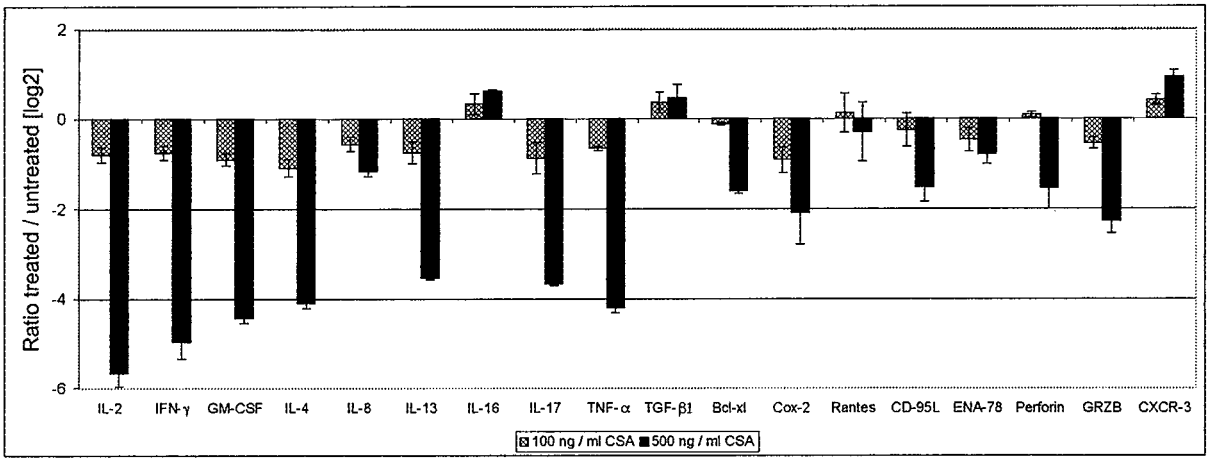


**FIGURE 1. Changes in gene expression after exposure of peripheral blood to CsA in vitro. Blood from three healthy individuals was incubated with 100 or 500 ng/mL CsA for 30 min followed by stimulation with PMA/ionomycin for 3 hr. After red cell lysis, the gene expression was measured as described in *Material and Methods*.**

"wash out effect" of CsA varied significantly between experiments. To avoid in vitro artifacts related to the PBMC purification, we established a stimulation protocol for whole blood samples.

To investigate CsA effects on gene expression, in a first set of experiments we individually stimulated peripheral blood from three healthy controls with PMA/ionomycin in the presence of 100 and 500 ng/mL CsA and measured gene expression levels by real-time PCR. The doses correspond to predose and $C_2$ serum concentrations of CsA. As shown in Figure 1 the strongest inhibition was observed for the genes of IL-2, interferon (IFN)-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The observed slightly increased expression of transforming growth factor-β1, IL-16, and CXCR3 in peripheral blood upon CSA exposure indicates that CSA does not reduce the transcriptional activity in general (*13*,*14*).

### *Expression of NFAT-Regulated Genes in Transplant Patients Receiving CsA Therapy*

Figure 2 shows the expression of the selected NFAT-regulated genes at the CsA predose level. In kidney and heart transplant patients, a median threefold reduction with a significant overlap to the control group was observed. In contrast, despite comparable therapeutic drug levels (100 mg/dL), we found no difference in the expression of these same genes in the group of CsA-treated liver transplant patients in comparison to untreated control patients. Importantly, CsA was the single immunosuppressive therapy in this latter group, suggesting that the observed reduction in gene expression in the other groups is a result of the concomitant use of other immunosuppressive drugs. However, no correlation between the individual gene expression level and the use of steroids could be observed. In addition, the PMA/ionomycin-induced gene expression was, at least in vitro, insensitive to the effects of corticosteroids or azathioprine (data not shown).

### *Kinetics of CsA-Induced Inhibition of Gene Expression*

We analyzed the PMA/ionomycin-induced expression of NFAT-regulated genes at 2, 6, and 10 hr after drug intake in seven kidney transplant patients. Two hours after Neoral ingestion, a significant decrease in gene expression was observed. Interestingly, the recovery occurred rapidly and was complete by 6 to 10 hr. Some patients showed a rebound effect at the latter time point. The kinetics of gene expression was strongly correlated with the individual CsA blood levels, confirming the biologic relevance of this assay: minimal gene expression occurred at the maximal drug level (Fig. 3). We therefore continued to investigate individual responsiveness to CsA at predose concentrations and at 2 hr after drug intake. Figure 4 shows the strong inhibition of IL-2, IFN-γ, and GM-CSF genes 2 hr after drug intake in kidney, heart, and liver transplant patients, respectively.

### *CsA Pharmacodynamics*

Although intraindividual measurements show a clear dose-response relation, interindividual expression levels of NFAT-regulated genes did not always correlate with corresponding blood concentrations of CsA. In addition, it must be mentioned that the interindividual levels of gene expression are highly variable. Therefore, we normalized the drug responsiveness with the level of gene expression at predose CsA concentrations. Repeated measurements were performed for six kidney transplant patients and for two heart transplant patients to show the intraindividual variability of the assay. The results remained constant in patients with therapeutic CsA blood concentrations (data not shown). In one patient we analyzed the inhibition of gene expression up to 10 months later and observed only a minor variation (data not shown).

The currently most predictive marker for the individual state of immunosuppression is the total drug exposure measured as area under the curve (AUC). We calculated a mini-AUC from the $C_0$ and $C_2$ concentrations. For most patients, we found a close correlation with the individual degree of immunosuppression based on the mean suppression of IL-2, IFN-γ, and GM-CSF transcripts after intake of CsA (Fig. 5).

## DISCUSSION

The introduction of calcineurin inhibitors that target the transcription factor NFAT resulted in improved long-term allograft function (*1*). At present, measurement of the inten-

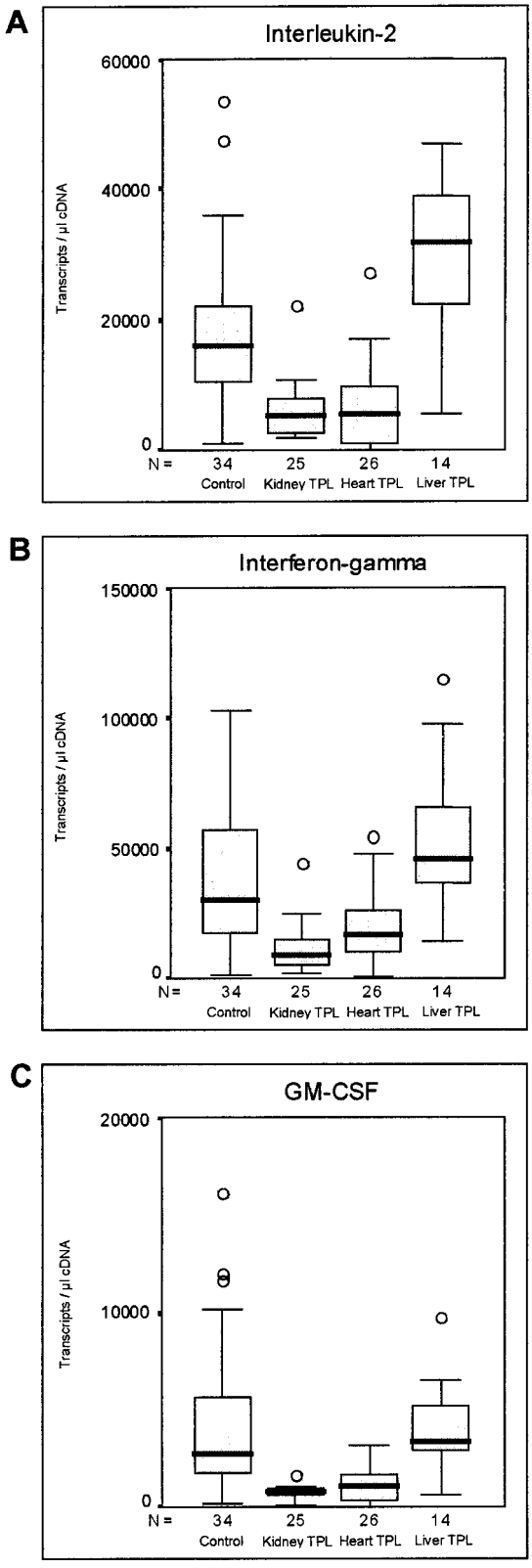


**FIGURE 2. Gene expression in PMA/ionomycin-stimulated whole blood samples from healthy volunteers and stable transplant recipients. One milliliter of peripheral blood taken at predose CsA levels was stimulated with PMA/ionomycin for 3 hr. After red cell lysis, the gene expression was measured as described in *Material and Methods*. Data are presented as box-plot analysis.**

sity of CsA-based immunosuppression relies almost exclusively on predose CsA levels (*10*). Recently, efforts have been made to improve the accuracy of the CsA dosage by measuring the CsA blood level at 2 hr after oral uptake. The 2-hr postoral uptake blood level is more closely correlated to the total CsA load necessary to achieve adequate immunosuppression (*6*). An appropriate 2-hr CsA level assessment reduces the risk of acute rejection (*6*,*8*,*9*). Despite this more rational dosage of CsA, minimal information exists on its biologic effects in the individual allograft recipient.

Recent work by Sindhi et al. (*11*) and Halloran et al. (*10*) confirmed the need to consider the biologic effect of CsA in vivo. Of particular interest is the correlation of whole blood CsA concentration, CsA tissue concentration, and the inhibition of calcineurin in spleen and kidney. It is assumed that CsA concentrations and calcineurin inhibition in the kidney may be relevant for nephrotoxicity. These results clearly argue for an individualized CsA dosing for optimal immunosuppression. Because calcineurin inhibition by CsA prevents the activation of the transcription factor NFAT, we measured the expression of NFAT-regulated genes (IL-2, IFN-γ, and GM-CSF) by quantitative real-time PCR technique in activated lymphocytes from patients with stable allograft function (kidney, heart, liver) who were treated with CsA. Gene expression of these cytokines is highly correlated with protein expression (data not shown). However, it seems that gene expression is more dynamic than protein expression and, therefore, more suitable for drug monitoring. The genes were selected for their high sensitivity toward calcineurin inhibition, rather than as markers for alloreactivity. However, genes that are reported to be up-regulated during rejection episodes, such as perforin or granzyme B, can also be monitored using this assay (Fig. 1). It is important to note that this assay is performed in whole blood, avoiding the possible variations caused by PBMC isolation and differences in drug responsiveness in vitro versus in vivo (*15*,*16*).

Figure 2 shows the adjusted transcript numbers of IL-2, IFN-γ, and GM-CSF genes in healthy controls, as well as kidney, heart, and liver allograft recipients at CsA predose levels. In kidney and heart transplant patients, the levels of transcripts for all three cytokines are reduced in comparison

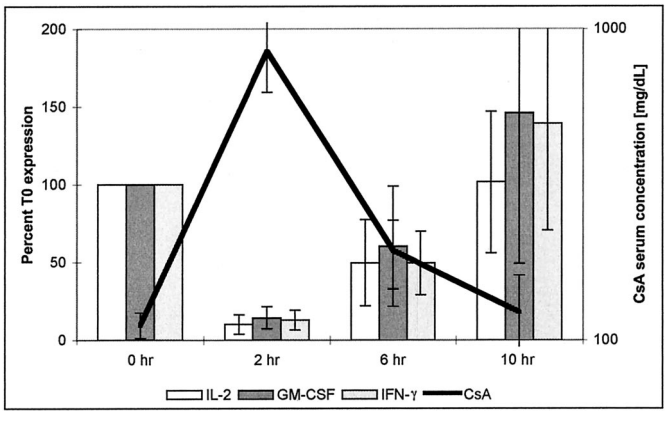


**FIGURE 3. Rapid recovery of induced gene expression after CsA treatment. Gene expression of NFAT-regulated genes was analyzed at 2, 6, and 10 hr after drug intake in seven patients who received CsA after renal transplantation. For comparison, the measured CsA plasma level is also shown.**

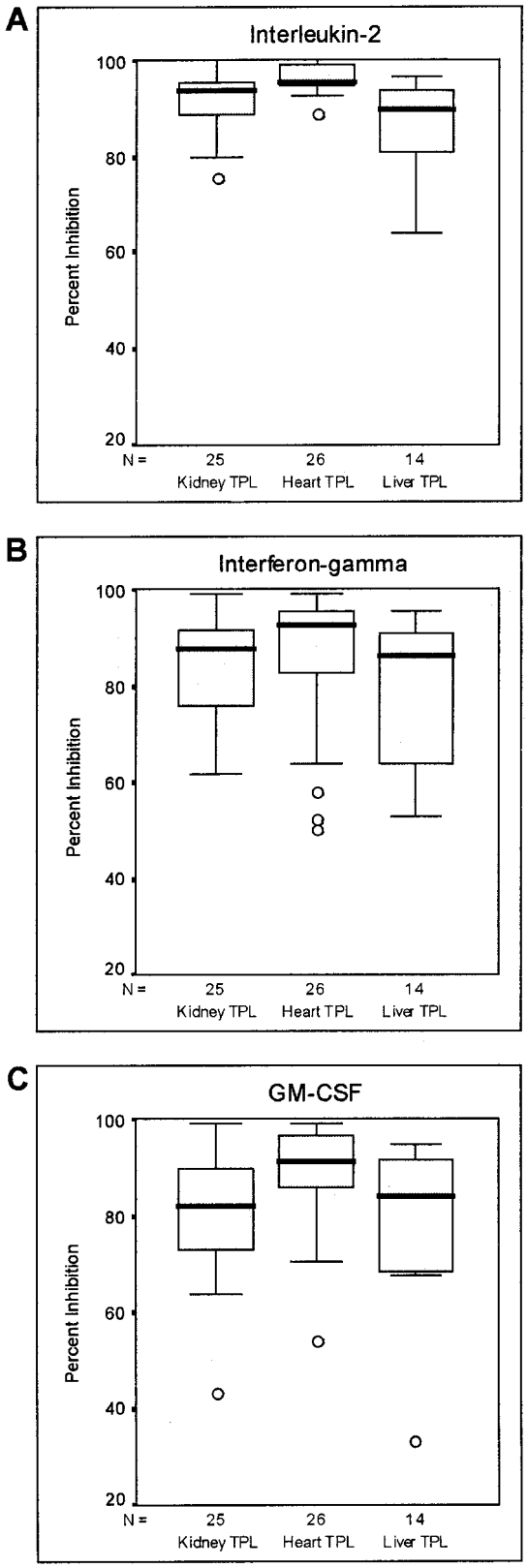


**FIGURE 4. Inhibition of IL-2 (A), IFN-γ (B), and GM-CSF (C) gene expression 2 hr after CsA intake. Peripheral blood of transplant patients was stimulated before and 2 hr after CsA ingestion. After red cell lysis, the gene expression was measured as described in *Material and Methods*. Data are presented as box-plot analysis.**

to healthy controls, whereas in liver transplant patients, the number of transcripts is comparable to healthy donors. Note that the latter group of patients had undergone steroid withdrawal and CsA was the only immunosuppressive drug. However, in individual patients, no correlation between the use of other immunosuppressive drugs and the level of gene expression could be established. Importantly, the PMA/ionomycin-induced expression of genes investigated here seems to be insensitive toward steroids, azathioprine, and rapamycin. Therefore, this test reflects the immunosuppressive potential of calcineurin inhibitors. To exclude day-to-day variability, repeated measurements of cytokine transcripts in renal transplant recipients were performed and showed reproducible results.

In allograft recipients 2 hr after CsA intake, the expression of NFAT-regulated gene transcripts was strongly inhibited (Fig. 3). This finding is comparable to the results of Halloran et al. (*10*) who showed an inverse relationship between CsA blood levels and calcineurin activity during a 12-hr monitoring. Maximum inhibition was seen during the first and second hour after oral CsA uptake. Figure 3 depicts the percentage of gene expression in correlation to CsA blood levels after oral uptake of CsA. CsA blood levels are inversely correlated to the magnitude of gene expression, and the profile is similar to that described for the inhibition of calcineurin. In contrast, however, gene expression of IL-2, IFN-γ, and GM-CSF is higher in some patients at 10 hr after drug intake, suggesting a rebound phenomenon, which has not been reported for calcineurin activity as yet (*10*). However whether this observation has any clinical relevance or describes just an ex vivo phenomenon needs to be further investigated.

Currently, the most closely correlated parameter to functional immunosuppression is the total drug exposure (AUC). Unfortunately, AUC monitoring is not feasible in routine clinical practice. Several limiting sampling strategies have been developed to estimate the AUC (*17*). We also used one of those estimates to correlate individual suppression of gene expression with total drug exposure. In the three different allograft groups (kidney, heart, liver), we observed a close

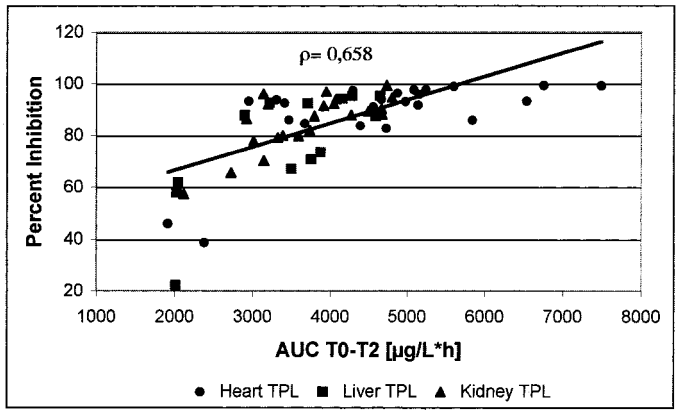


**FIGURE 5. Correlation between inhibition of gene expression 2 hr after CsA intake and exposure to CsA. The mean inhibition of IL-2, IFN-γ, and GM-CSF gene expression 2 hr after CsA ingestion was plotted against an estimate for the drug exposure calculated as follows: AUC==990+10,74*$C_0$+2,28*$C_2$. Nonparametric correlation was estimated by Spearman's analysis. The Spearman's ρ of 0.658 is significant at the 0.01 level.**

relationship between these parameters for most patients. One patient with recurrent acute rejection episodes, despite high CsA blood concentrations, showed no suppression of the GM-CSF gene and less than average suppression of IL-2 (20%) and IFN-$\gamma$ (40%), whereas all patients with previous rejection episodes who remained stable did not differ from other patients with stable renal graft function. Therefore, the method presented here might be a better estimate for functional immunosuppression in individual patients. Quantification of gene expression will not replace current drug monitoring but might assist in defining appropriate dosages of CsA to achieve optimal therapeutic effects. This method, in contrast to other functional studies, allow same-day results and is highly reproducible. The potential benefit from individually optimized drug doses will compensate for the additional costs.

In a prospective study, we are presently investigating how individual levels of immunosuppression, as assessed here, correlate with clinical outcome. In a separate study, we are examining how dosage adjustments will influence the level of NFAT-regulated gene expression in vivo. These studies should also provide clues to the level of functional immunosuppression for which we should aim.

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