

Delayed Cytokine mRNA Expression Kinetics after T-Lymphocyte Costimulation: A Quantitative Measure of the Efficacy of Cyclosporin A-based Immunosuppression

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Background: Because cyclosporin A (CsA) and glucocorticoids inhibit the production of interleukin-2 (IL-2) and other cytokines, quantitative analysis of cytokine mRNA might constitute a pharmacodynamic measure for immunosuppressive drug effects. We investigated whether immunosuppressive drugs influence cytokine mRNA expression kinetics during T-cell costimulation.

Methods: We used a human whole blood assay to determine basal (unstimulated) IL-2, IL-4, and tumor necrosis factor- α (TNF- α) mRNA concentrations and expression kinetics after anti-CD3/anti-CD28 monoclonal antibody costimulation in kidney transplant recipients undergoing CsA-based immunosuppressive triple therapy and in healthy controls (ex vivo study I). The effect of CsA on IL-2 mRNA expression kinetics was also determined ex vivo in patients undergoing CsA monotherapy (ex vivo study II) and after in vitro addition of CsA.

Results: In ex vivo study I, basal TNF- α mRNA but not IL-2 and IL-4 mRNA was decreased in kidney transplant patients. We observed shifts in peak IL-2 and IL-4 (from 8 to 24 h) and TNF- α (from 4 to 8 h of costimulation) mRNA expression in kidney transplant patients after T-cell costimulation. In patients undergoing CsA monotherapy (ex vivo study II), the inhibitory effect of CsA was detectable as an individually delayed increase in IL-2 mRNA during costimulation. In vitro addition of

CsA also induced a dose-independent displacement of IL-2 mRNA expression kinetics (i.e., a delay).

Conclusions: A delayed increase in cytokine mRNA expression during T-cell costimulation may represent a sensitive effect of immunosuppression. The single analysis of one absolute or peak mRNA value could be misleading. For prospective studies involving measurement of cytokine mRNA, we therefore suggest the parameter "area of cytokine mRNA expression over time", which should include absolute cytokine mRNA values at two different time points of mRNA kinetics.

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Standard protocols for maintenance therapy after organ transplantation involving cyclosporin A (CsA)³ have led to a marked improvement in graft survival (1, 2). However, despite adjustment of dosing to achieve blood concentrations within therapeutic ranges, some individuals will receive inappropriately low amounts of immunosuppression and be subject to an increased risk of transplant rejection. Other individuals may be overtreated and are more likely to develop side effects, such as opportunistic infections, nephrotoxicity, and cancer (3). Previous studies have demonstrated that the clinical response to immunosuppressive drugs, in terms of both efficacy and drug-related toxicity, may vary substantially even in patients with similar blood concentrations of immunosuppressive drugs (4, 5). Laboratory tests to monitor the functional impact of CsA and other drugs on immune reactivity might represent valuable tools to optimize immunosuppressive treatment. The molecular mecha-

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Received May 25, 2002; accepted September 12, 2002.

³ Nonstandard abbreviations: CsA, cyclosporin A; IL, interleukin; NF, nuclear factor; RT-PCR, reverse transcription-PCR; mAb, monoclonal antibody; and TNF- α , tumor necrosis factor- α .

nisms of action of most immunosuppressive agents are well documented: CsA specifically targets the signal transduction pathway of interleukin-2 (IL-2) by inhibiting the calcium-dependent phosphatase calcineurin and, thereby, nuclear translocation of the transcription factors nuclear factor-AT (NF-AT) and nuclear factor- κ B (NF- κ B) (6). Corticosteroids have multiple effects, including inhibition of IL-2 and other cytokine gene transcription by interference with the binding of transcription factors activator protein-1 and NF-AT to their nuclear targets and down-regulation of the IL-2 receptor (7). Azothioprine suppresses T-lymphocyte proliferation through inhibition of the biosynthesis of purines, essential substrates for RNA and DNA synthesis (8). However, the lack of sensitive pharmacodynamic measures limits the quantitative determination of immunosuppressive drug effects on early events of T-cell activation (1, 5).

In this report we describe a human whole blood assay based on quantitative real-time cytokine reverse transcription-PCR (RT-PCR) for the pharmacodynamic assessment of immunosuppressive drug effects. To investigate the extent to which immunosuppressive drugs influence early increases of cytokine mRNA expression, we studied cytokine mRNA concentrations after anti-CD3/anti-CD28 monoclonal antibody (mAb) costimulation in kidney transplant recipients receiving CsA-based triple therapy (ex vivo study I), in patients undergoing CsA monotherapy before kidney transplantation (ex vivo study II), and after in vitro addition of CsA.

Materials and Methods

WHOLE BLOOD CULTURE

Human whole blood cultures were performed in 6-well cluster tissue culture dishes (35 mm diameter; Costar). For each sample, duplicate 1-mL aliquots were diluted in 9 mL of IMDM supplemented with penicillin (100 kilounits/L), streptomycin (100 mg/L), and 10 mmol/L L-glutamine and stimulated with 1 mg/L anti-CD3 mAb (CLBT3/4E) and 1 mg/L anti-CD28 mAb (CLB-CD28/1; Hiss Diagnostics) for 4, 8, and 24 h. Unstimulated whole blood cultures were used for analysis of basal cytokine mRNA concentrations.

RNA ISOLATION

Total RNA from whole blood leukocytes or peripheral blood mononuclear cells was isolated by use of the Purescript RNA isolation reagent set (Gentra Systems) according to the manufacturer's protocol. The resulting RNA was resuspended in 300 μ L of diethylpyrocarbonate water and stored at -80°C until use.

TaqMan RT-PCR

The RT-PCR protocol for the quantitative detection of cytokine mRNA has been described recently (9). In brief, the PCR reaction mixture contained 5 μ L of 10 \times TaqMan A-buffer (500 mM KCl, 100 mM Tris-HCl, 100 mM EDTA, 600 nM passive reference dye ROX, pH 8.3; Perkin-Elmer);

3.5 mM MgCl₂; 300 μ M dATP, dCTP, and dGTP; 600 μ M dUTP; 100 nM forward and reverse primer; 100 nM fluorogenic probe; 20 units of RNase inhibitor (Invitrogen); 25 U of murine leukemia virus reverse transcriptase (Perkin-Elmer); 1.25 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer); and 20 μ L of water control, diluted calibrators, or unknown RNA template in a total volume of 50 μ L. Sequence-specific primer pairs and fluorogenic probes were obtained from TIB Molbiol [IL-2, IL-4, and tumor necrosis factor- α (TNF- α) (9)] or Perkin-Elmer Cetus (β -actin reagent set for cDNA samples). All PCR reactions for patient RNA samples and calibrators were performed in triplicate in optical reaction tubes (Applied Biosystems), and conditions were 2 min at 50 $^{\circ}\text{C}$ and 30 min at 48 $^{\circ}\text{C}$ for reverse transcription, 10 min at 95 $^{\circ}\text{C}$ for DNA polymerase activation, 40 cycles of 15 s at 95 $^{\circ}\text{C}$ and 1 min 30 s at 60 $^{\circ}\text{C}$, and a final 25 $^{\circ}\text{C}$ hold.

QUANTIFICATION OF CYTOKINE mRNA EXPRESSION

All PCR reactions for cytokine mRNA quantification were performed in an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). For calibrators, cDNA fragments encoding β -actin, IL-2, IL-4, interferon- γ , and TNF α were cloned using an Original TA Cloning Kit (Invitrogen). Serial logarithmic dilutions of β -actin or the respective cytokine calibrators containing known amounts of molecules (1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , and 1×10^2 molecules diluted in water from a stock solution of known concentration) were prepared in triplicate to generate calibration curves. The same master mixture for samples and calibrators was used to minimize tube-to-tube variation. The fluorescence signals of each well were collected every 7 s, and threshold cycles (C_t) were calculated by determining the point at which the fluorescence intensity was 10 times larger than the SD of the baseline fluorescence. The ratio between the β -actin content in calibrator samples (10^6 copies) and test samples was defined as the normalization factor. Standardized cytokine mRNA quantities (cytokine copies/ 10^6 β -actin copies) were determined by dividing the interpolation-derived values from the cytokine calibration curve by the normalization factor.

EX VIVO STUDY I

Heparinized whole blood was obtained from healthy volunteers ($n = 10$) and clinically stable kidney transplant recipients with excellent graft function who received an immunosuppressive therapy including CsA (CsA dosed to reach trough concentrations of 150–200 $\mu\text{g/L}$), methylprednisolone alone (12–20 mg/day), and azothioprine (2 mg/kg of body weight per day) 14 days after transplantation ($n = 8$; local transplantation center at the University of Lübeck Medical School) at the same time of day before additional immediate processing. None of the transplant recipients had viral or bacterial infections or experienced transplant rejection during clinical observation.

Whole blood cultures were then processed for RNA isolation before basal mRNA concentrations and the kinetics of IL-2, IL-4, and TNF- α mRNA expression after costimulation were determined by quantitative real-time PCR (TaqMan technology) as described above.

EX VIVO STUDY II

To investigate the effects of CsA alone on IL-2 mRNA expression kinetics after anti-CD3/anti-CD28 mAb costimulation *ex vivo*, we selected living donor transplant patients who had been pretreated with peroral application of Neoral (administered twice daily; total dose, 5 mg \cdot kg⁻¹ \cdot day⁻¹) before transplantation (n = 3). Heparinized whole blood was collected before participants received CsA for the first time, after 96 h of CsA monotherapy, and 14 days after transplantation with immunosuppressive triple therapy (CsA, methylprednisolone, and azothioprine). None of the transplant recipients had viral or bacterial infections or experienced transplant rejection during the clinical observation (Table 1).

CsA dosage was determined from trough concentrations (c_0) and concentrations in samples collected 2 h after dosing (c_2). To exclude a wash-out phenomenon of immunosuppression in culture conditions, CsA concentrations were verified before and after whole blood incubation by fluorescence polarization (TDx; Abbott Diagnostics; kindly performed by Professor Heiko Iven, Institute of Pharmacology and Toxicology, Medical University of Lübeck). Whole blood cultures were processed as described above and assayed for IL-2 mRNA expression kinetics.

IN VITRO STUDY

To determine how CsA influences IL-2 mRNA expression kinetics *in vitro*, we prepared heparinized whole blood samples from healthy volunteers (n = 5) for culture as described above and preincubated them for 2 h with 0, 250, 500, or 1000 μ g/L CsA (Sandimmun, Batch 8003; Novartis Pharma) at 37 °C in a humidified 95% air–5% CO₂ atmosphere before costimulation with anti-CD3 and anti-CD28 mAbs for 4, 8, and 24 h. IL-2 mRNA expression was analyzed by quantitative real-time PCR.

All performed *ex vivo* and *in vitro* studies were approved by the Ethics Commission of the Lübeck University School of Medicine.

STATISTICAL ANALYSIS

Statistical analysis was performed with a Mann–Whitney *U*-test (SPSS for Windows, Release 6; SPSS). A two-tailed *P* value <0.05 was the criterion for statistical significance.

Results

EX VIVO STUDY I: KINETICS OF CYTOKINE mRNA EXPRESSION IN PATIENTS UNDERGOING IMMUNOSUPPRESSIVE TRIPLE THERAPY

In *ex vivo* study I, we compared basal cytokine mRNA concentrations and expression kinetics after T-cell costimulation in kidney transplant patients receiving immunosuppressive triple therapy and healthy controls. Basal IL-2 and IL-4 mRNA expression did not reveal a significant difference between transplant patients and healthy controls, whereas TNF- α mRNA was significantly decreased in transplant patients ($P = 0.02$; Fig. 1). As shown in panels A and B of Fig. 2, peak IL-2 and IL-4 mRNA expression was observed after 8 h in healthy controls, whereas patients receiving immunosuppressive therapy had significantly lower IL-2 and IL-4 mRNA concentrations after 4 and 8 h of anti-CD3/anti-CD28 mAb costimulation: IL-2 values at 4 h were 26.8% of those of controls ($P = 0.02$), and at 8 h, they were 33.9% of controls ($P = 0.02$); IL-4 values at 4 h were 6.9% of those of controls ($P = 0.002$), and at 8 h, they were 24.4% of controls ($P = 0.001$). IL-2 and IL-4 mRNA expression reached peak values in patients after 24 h of costimulation. In contrast to healthy controls, who had peak TNF- α mRNA expression after 4 h of costimulation, patients showed a kinetic shift of peak expression to 8 h of costimulation: TNF- α values at 4 h

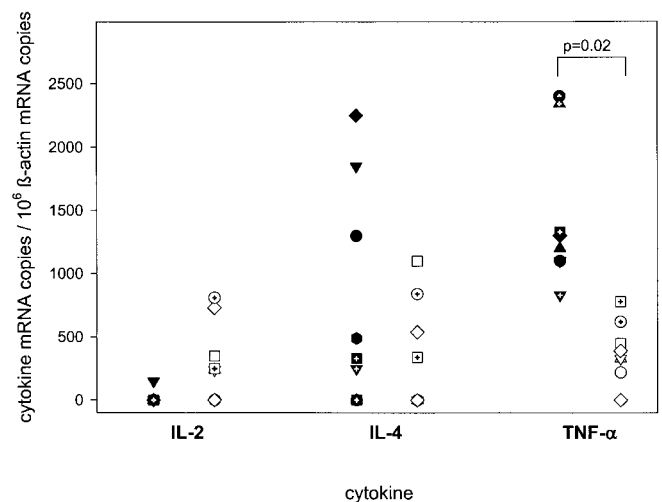


Fig. 1. Basal cytokine mRNA expression of patients undergoing immunosuppressive therapy compared with healthy controls.

Whole blood samples were obtained from posttransplantation patients (n = 8; open symbols) and healthy controls (n = 10; filled symbols). Each symbol corresponds to one individual donor. IL-2, IL-4, and TNF- α mRNA expression was determined by real-time RT-PCR and normalized with respect to β -actin mRNA (cytokine mRNA expression/ 10^6 β -actin mRNA copies). Statistical calculations were performed using the Mann–Whitney *U*-test; *P* values <0.05 were regarded as significant.

Table 1. Clinical data of patients in *ex vivo* study II.

Patient	CsA, μ g/L				Serum creatinine, μ mol/L
	CsA monotherapy		CsA triple therapy		
	C_0	C_2	C_0	C_2	
1	125	800	425	955	78
2	140	1160	155	1055	150
3	220	1245	150	1295	95

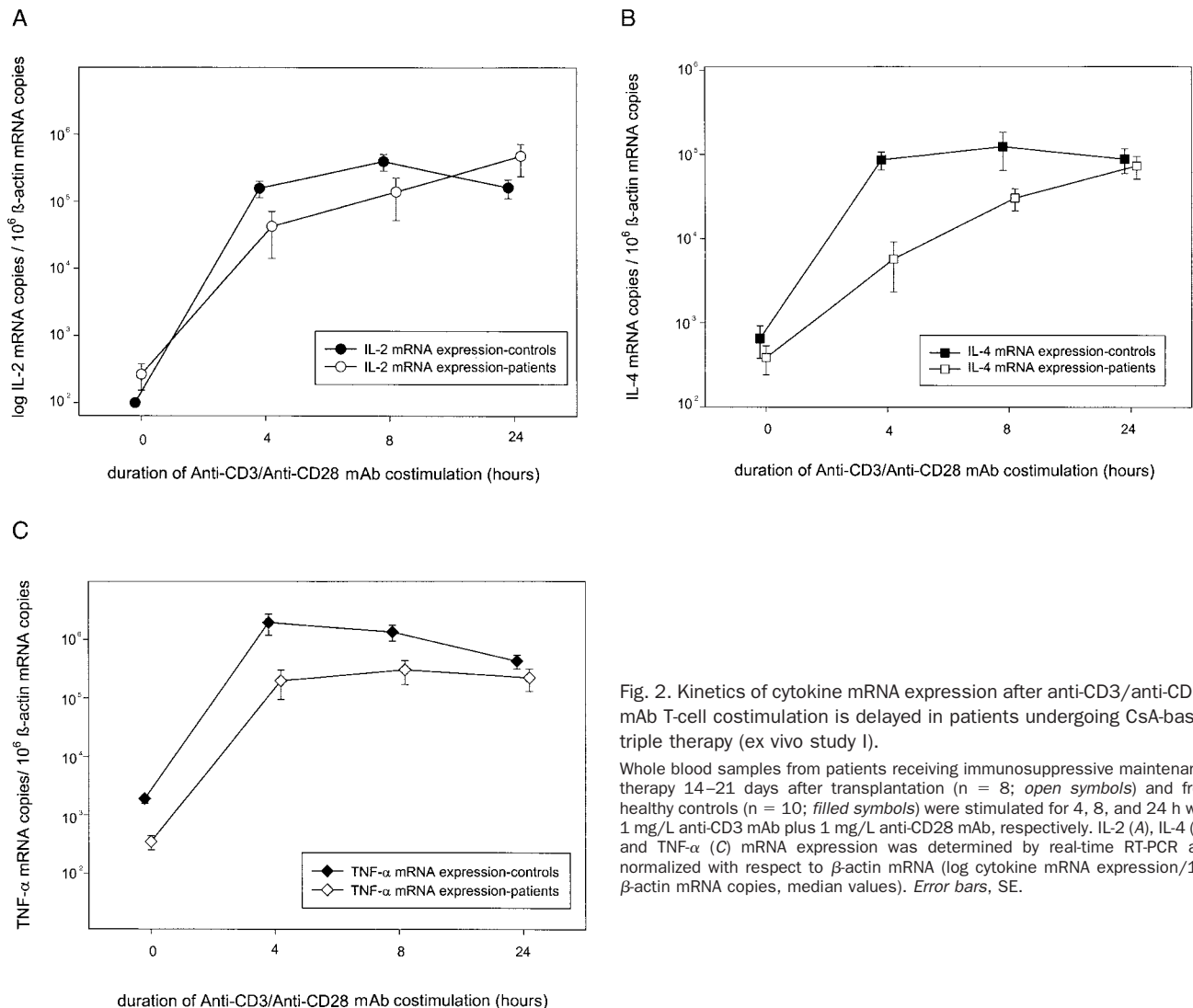


Fig. 2. Kinetics of cytokine mRNA expression after anti-CD3/anti-CD28 mAb T-cell costimulation is delayed in patients undergoing CsA-based triple therapy (ex vivo study I).

Whole blood samples from patients receiving immunosuppressive maintenance therapy 14–21 days after transplantation ($n = 8$; open symbols) and from healthy controls ($n = 10$; filled symbols) were stimulated for 4, 8, and 24 h with 1 mg/L anti-CD3 mAb plus 1 mg/L anti-CD28 mAb, respectively. IL-2 (A), IL-4 (B), and TNF- α (C) mRNA expression was determined by real-time RT-PCR and normalized with respect to β -actin mRNA (log cytokine mRNA expression/ 10^6 β -actin mRNA copies, median values). Error bars, SE.

were 10.1% of those of controls ($P = 0.009$), and at 8 h, they were 22.5% of controls ($P = 0.03$; Fig. 2). Notably, peak IL-2, IL-4, and TNF- α mRNA expression was not significantly diminished in patients compared with controls after 24 h of T-lymphocyte costimulation. However, we found no correlation between CsA concentrations in patients (c_0 range, 88–375 $\mu\text{g/L}$; c_2 range, 800–1590 $\mu\text{g/L}$) and mRNA expression.

EX VIVO STUDY II: THE EFFECT OF CSA MONOTHERAPY ON IL-2 mRNA EXPRESSION KINETICS

In ex vivo study II, we investigated the extent to which the effect of CsA alone influences IL-2 mRNA expression kinetics during T-cell costimulation. In the blood samples drawn after 96 h of continuous CsA monotherapy, IL-2 mRNA expression after anti-CD3/anti-CD28 costimulation showed distinct differences in the kinetics of IL-2 mRNA expression in all three individuals compared with whole blood drawn before they had received CsA for the first time (control). After 4 h of costimulation, patients 1

and 2 had unaffected IL-2 mRNA concentrations after CsA monotherapy, whereas even higher IL-2 mRNA concentrations were observed in patient 3. In contrast, after 8 h of costimulation, IL-2 mRNA was inhibited in all three individuals (49.5–71.3% inhibition) when CsA monotherapy was administered. After 24 h of costimulation, however, only patient 1 showed inhibited IL-2 mRNA expression, whereas patients 2 and 3 had similar IL-2 mRNA concentrations after CsA monotherapy compared with control concentrations before treatment. After receiving CsA-based triple therapy for 14 days after transplantation, all three living donor kidney transplant recipients demonstrated inhibited IL-2 mRNA expression after 8 h of anti-CD3/anti-CD28 mAb costimulation, but after 24 h of costimulation, patients 1 and 3 showed inhibited IL-2 mRNA expression, whereas patient 2 reached control IL-2 concentrations (Fig. 3).

Confirming the data of ex vivo study I, the inhibitory effect of CsA was detectable as an individually delayed increase in IL-2 mRNA during whole blood costimulation.

IN VITRO STUDY: CSA CONCENTRATION EFFECT ON IL-2 mRNA EXPRESSION KINETICS

The in vitro addition of CsA (250–1000 $\mu\text{g/L}$) to whole blood samples of healthy individuals ($n = 5$) inhibited IL-2 mRNA expression after 4 h of anti-CD3/anti-CD28 mAb costimulation (range of inhibition, 63.3–92.1%) and to a lesser extent after 8 h (range of inhibition, 19.5–51.3%). However, after 24 h, the inhibitory effects of CsA were no longer detectable, confirming the delay of IL-2

mRNA expression kinetics seen in our ex vivo investigations (Fig. 4).

Discussion

Predicting immunosuppressive drug efficacy and toxicity in individual patients is difficult and requires a biologically relevant, pharmacodynamic measure. This report describes a human whole blood assay that quantitatively detects cytokine mRNA expression for determination of sensitive immunosuppressive drug responses. We observed (a) decreased basal mRNA expression of TNF- α but not IL-2 and IL-4 in patients undergoing CsA-based immunosuppressive triple therapy, and (b) delayed cytokine mRNA expression kinetics during T-cell costimulation not only in patients receiving immunosuppressive triple therapy, but also in patients receiving CsA monotherapy and after CsA addition in vitro. These data suggest that distinct shifts in peak cytokine mRNA expression may represent a sensitive pharmacodynamic marker of individual CsA response.

Several efforts have been made to quantify the pharmacodynamic effects of immunosuppressive drugs after organ transplantation based on the molecular mechanism of immunosuppressive action. Most studies have focused on measuring the production of T-cell cytokines such as IL-2, which is central to the inhibitory effect of CsA (1, 10–12). However, T lymphocytes in a resting or non-activated stage usually show minimal or undetectable cytokine expression, which makes systematic investigation of basal cytokine profiles a difficult task. In this study, we applied the technology of quantitative real-time RT-PCR, a straightforward approach that was highly sensitive for the quantification of cytokine mRNA expression (13–15). Our data revealed no significant difference

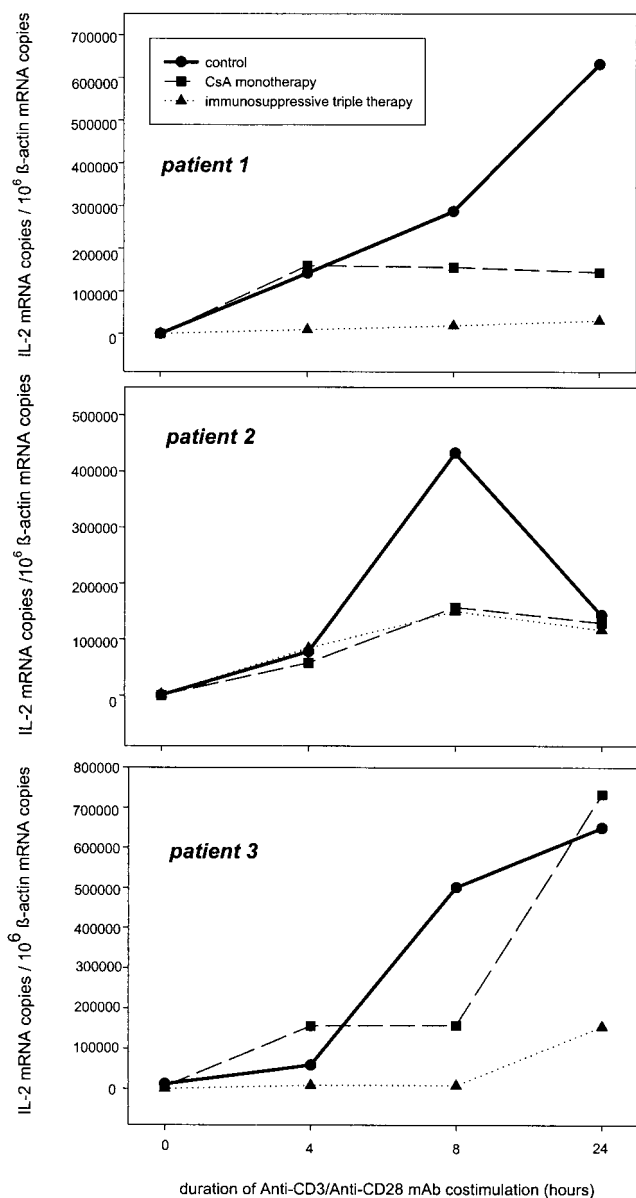


Fig. 3. Effects of CsA on anti-CD3/anti-CD28-induced IL-2 mRNA expression kinetics in patients undergoing CsA monotherapy (ex vivo study II).

IL-2 mRNA expression data at baseline (before participants had received CsA; ●), after oral CsA monotherapy for 96 h (■), and after undergoing immunosuppressive triple therapy for 14 days after transplantation (▲) are shown for patient 1 (top), patient 2 (middle), and patient 3 (bottom).

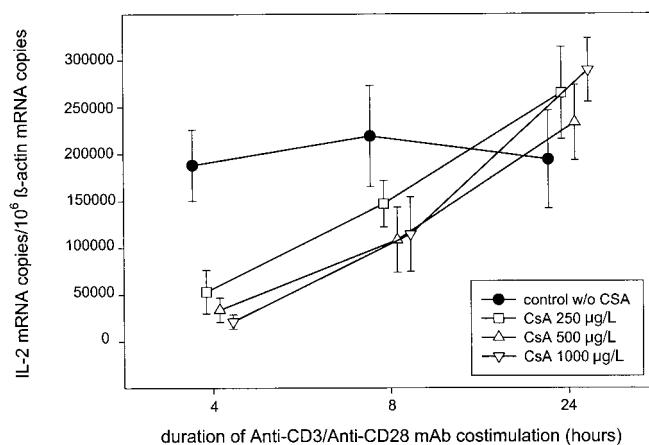


Fig. 4. In vitro CsA concentration effect on IL-2 mRNA expression kinetics.

Whole blood samples from healthy controls ($n = 5$) were preincubated for 30 min without (●) or with 250 $\mu\text{g/L}$ (□), 500 $\mu\text{g/L}$ (△), or 1000 $\mu\text{g/L}$ (▽) CsA and stimulated for 4, 8, and 24 h with 1 mg/L anti-CD3 plus 1 mg/L anti-CD28 mAb. IL-2 mRNA expression copies were determined by real-time RT-PCR and normalized with respect to β -actin mRNA (log cytokine mRNA expression/ 10^6 β -actin mRNA copies, median values). Error bars, SE.

in basal *ex vivo* IL-2 and IL-4 mRNA concentrations between kidney transplant patients and healthy controls. In contrast, basal TNF- α mRNA expression was decreased in patients. Because the effect of CsA-based triple therapy may already be detectable at basal concentrations of whole blood TNF- α mRNA, the effect on monocyte function and, potentially, TNF- α could also be relevant for immunosuppressive drug monitoring.

In the search for a pharmacodynamic measure of immunosuppressive drug effects, most laboratory studies have relied on demonstrating the potential for cellular cytokine synthesis by use of a strong supraphysiologic activation stimulus such as phytohemagglutinin, phorbol-myristate acetate, or ionomycin (10–12), all of which may exceed or conceal the original drug effect. Such studies using endpoint measurements have generally shown that IL-2 production is inhibited by 35–40% in patients receiving CsA. Other groups have introduced the assessment of IL-2 mRNA for pharmacodynamic monitoring of immunosuppressive drugs (16) and found significant inhibition in kidney transplant patients. Rather than endpoint measurements, this study investigated the influence of immunosuppressive drugs on the time course of cytokine mRNA expression after anti-CD3/anti-CD28 T-cell costimulation. We observed significantly delayed IL-2, IL-4, and TNF- α peak mRNA expression in kidney transplant patients. In contrast, the peak expression was not diminished in patients undergoing CsA-based triple therapy compared with healthy controls (*ex vivo* study I). Similarly, in patients undergoing CsA monotherapy (*ex vivo* study II), the inhibitory effect of CsA was detectable as a delayed increase in IL-2 mRNA during whole blood costimulation; however, all three investigated patients had individually different IL-2 mRNA kinetics. After 8 h of T-cell costimulation, IL-2 mRNA expression was inhibited in all patients. In contrast, after 24 h of T-cell costimulation, two of three patients reached control concentrations after CsA monotherapy. Notably, after CsA-based triple therapy, two of three patients also demonstrated inhibited IL-2 mRNA expression after 24 h of T-cell costimulation, thus contradicting results from study I.

These interindividual variations may be explained by (a) differences in the underlying disease, secondary diseases, medications, or immune status before transplantation, including the influence of dialysis; (b) variable pharmacodynamic responses to steroids within a CsA-based multidrug immunosuppressive regimen; (c) the influence of surgery; (d) the differential sensitivity of primed vs naive T cells; and (e) the functional impact of cytokine and cytokine receptor polymorphisms, which should be thoroughly investigated in future prospective studies. Notably, we found no correlation between CsA concentrations and cytokine mRNA expression in patients. This confirms previous investigations that indicated that CsA concentrations may not predict the pharmacodynamic response (i.e., calcineurin inhibition) for individual patients (1, 17). Furthermore, the correlation between CsA trough concen-

trations and true systemic exposure may also not be very precise. Therefore, the determination of CsA concentrations is needed to avoid dosage outside of the “therapeutic range”; however, its use for the assessment of pharmacodynamic responses is limited.

Confirming the data of *ex vivo* studies I and II, the *in vitro* addition of CsA to whole blood cultures also induced a dose-independent delay of IL-2 mRNA expression kinetics. Notably, after 24 h of costimulation, an *in vitro* CsA effect on T-cell reactivity was no longer evident. This may be attributable to accumulation effects of IL-2 mRNA transcripts and secondary changes in the culture that no longer reflected minor cytokine mRNA changes after CsA treatment. Rather, the demonstrated sensitive effect of CsA early in the time course of IL-2 mRNA expression points to the importance of kinetic measurements because little is known about its influence on the sequence of crucial events subsequently leading to T-cell proliferation.

This human whole blood assay is potentially useful for studying early and subtle effects of immunosuppressive therapy. It offers several advantages. The assay tends to resemble the human whole blood situation most closely, because other essential factors for T-lymphocyte activation are present in physiologic concentrations (18). Moreover, CsA is thought to equilibrate among a complex set of extralymphocytic binding sites (e.g., red blood cells), which are present in whole blood just as they are *in vivo* (17). Indeed, separation procedures for the application of peripheral blood lymphocytes or purified T lymphocytes, as used in other studies (10, 19, 20), may in fact deplete intracellular CsA concentrations. Furthermore, such methods might also cause preactivation, as we found recently (15), so that they might interfere with the detectable pharmacodynamic effects of immunosuppressive treatment. Despite these positive aspects, the assay does have some limitations: One limitation is that, in contrast to the advantageous measurement of CsA effects in undiluted whole blood, as shown by Stein et al. (1) for inhibition of phytohemagglutinin-induced IL-2 production, adequate cytokine RNA yield, RNA processing, and RT-PCR tend to require dilution of whole blood in our experience. Another limitation is that the determination of cytokine mRNA kinetics in patients must be compared with the kinetics in healthy control groups and that more data need to be generated concerning whether exposure of both control and experimental groups to infections influences the results. The third limitation is that a relationship between different pharmacodynamic parameters (i.e., calcineurin inhibition and “area of cytokine mRNA expression over time”) has not been established. Finally, cytokine mRNA concentrations in the transplanted organ may serve as a confounding factor and should therefore be included in prospective studies. Although this assay is not yet able to facilitate the distinction of single drug effects pertaining to a multidrug immunosuppressive therapy after transplantation, our investigations clearly

demonstrate that data on cytokine mRNA expression kinetics are necessary for adequate interpretation of the monitoring of both CsA monotherapy and CsA-based immunosuppressive triple therapy. For conclusive interpretation, future prospective studies with an appropriate number of patients are necessary to investigate the differences in cytokine mRNA kinetics between patients with functioning grafts and patients with acute/chronic rejection.

In summary, we believe that analysis of a single absolute cytokine mRNA concentration could be misleading for the interpretation of a drug's efficacy. Furthermore, peak cytokine mRNA values after T-cell costimulation are not necessarily physiologically relevant because the response of a T cell *in vivo* may be determined much earlier in time. This test system may become helpful to assess the response of patients receiving immunosuppressive therapy as minor changes in cytokine mRNA (i.e., a delay). For prospective studies on cytokine mRNA concentrations, we therefore suggest the parameter "area of cytokine mRNA expression over time", which should include absolute cytokine mRNA concentrations measured at two different time points.

We thank Brigitte Ebel and Kerstin Mussehl for excellent technical assistance and Una Doherty and Dr. Julian Keogh for carefully editing the manuscript.

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