Chapter 3

Calcineurin activity and cytokine secretion at therapeutic cyclosporine whole blood concentrations

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Abstract

Background: Calcineurin (CN) activity has the potential to become a pharmacodynamic marker to monitor CN inhibitor (CNI) therapy after organ transplantation. We investigated the relation between CN activity and cytokine secretion at therapeutic cyclosporine (CsA) levels in order to visualize CsA based immunosuppression and the applicability of the CN activity marker.

Methods: Healthy volunteer whole blood incubated with CsA was used as a model for allograft recipient blood. CN activity and cytokine (IFNγ, TNFα) secretion after CD3/CD28 stimulation were determined.

Results: A concentration of 25000 μg/L CsA, used as CsA excess, showed only a minor increase in CN inhibition and no increase in cytokine (IFNγ, TNFα) inhibition when compared to 2500 μg/L CsA. Between therapeutic target concentrations (100, 250, 600, and 1700 μg/L CsA) significant differences in inhibition of cytokine secretion and CN activity were observed. Inhibition of cytokine secretion ranged from 0 to 90% while CN inhibition ranged from 25 to 48%. Additional Cyclophilin A could not increase CN inhibition by CsA in cell lysates, while addition of CsA and both CsA and Cyclophilin A could.

Conclusions: Whole blood concentrations of 2500 μg/L CsA approaches immunosuppressive saturation and is not yet limited by the CyP content. At the therapeutic CsA range, therapeutic target concentrations represented distinct immunosuppressive states. In addition, small increases in CN inhibition resulted in relative larger suppression of cytokine secretion. Altogether, CN activity as well as cytokine secretion after T cell specific stimulation is able to visualize CsA pharmacodynamics at relevant in vivo CsA concentrations.
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Introduction

The calcineurin inhibitors (CNI), cyclosporine (CsA) and tacrolimus (TRL), are still widely applied in immunosuppressive regimen to prevent allograft rejection after organ transplantation(1). Despite proven efficacy, their apparent lack in selectivity remains a major drawback, resulting in an unfavorable cardiovascular profile and lack of improvement on the long-term(2). To overcome these issues, CNI minimization and avoidance have been investigated with varying success(3-5). The wide variation in inter- and intra-individual pharmacokinetic parameters requires frequent therapeutic drug monitoring to guide dosing. Further optimization of CNI therapy by new monitoring strategies such as the use of pharmacogenetic and pharmacodynamic biomarkers are currently under investigation(6,7).

CNIs suppress allograft reactivity by blocking the intracellular T cell recognition signaling pathway(1). In T cells, CsA inhibits calcineurin (CN) phosphatase activity that is required for translocation of the transcription factor NFAT into the nucleus. Upon translocation, NFAT can transcribe cytokines, such as IL-2, IFNγ and TNFα that control T cell mediated immune responses(8). CN inhibition by CsA can only occur after dimerization with members of the cyclophilin (CyP) family(9). The CsA/CyP complex blocks substrate excess to the catalytic active binding site of CN and are therefore the true pharmacological inhibitors of CN(10). Since only partial inhibition of CN is observed in transplant recipients and, in vitro, excess of CsA only partially inhibited CN activity, it has been suggested that CyPs might limit CN inhibition at higher CsA concentrations(11,12). This view was supported by further in vitro experiments showing that addition of Cyclophilin A (CyPA), at high CsA concentrations, could further increase the degree of CN inhibition(12). The relevance of in vitro CyP limitation, for allograft recipients remains unknown.

To determine the potential of CN activity as a pharmacodynamic biomarker, it is relevant to gain insights in the relation between the achieved CNI inhibition at therapeutic relevant target levels and the resulting immunosuppressive state, measured by cytokine secretion. We therefore investigated the effect of representative CsA concentrations in whole blood on CN activity and T cell specific cytokine secretion, determined by IFNγ and TNFα measurement after CD3/CD28 stimulation.
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Materials and Methods

Healthy volunteers and kidney transplant recipients blood
EDTA-or heparin-anticoagulated blood of healthy volunteers was obtained via Sanquin Bloodbank (Amsterdam, NL). For simulation of recipient blood, 2 mL of EDTA-anticoagulated blood or 1 mL of heparin anticoagulated blood of healthy volunteers was incubated (1h, 37°C) with various CsA stock solutions resulting in final ethanol concentrations of 0.25% (v/v). For investigation of CsA pharmacodynamics, CsA concentrations were used that represented trough and peak concentrations regularly found in renal allograft recipients. These conditions are referred to as T0 and T2 respectively. As representative CsA concentrations: 100 μg/L (late T0), 250 μg/L (early T0), 600 μg/L (late T2) and 1700 μg/L (early T2), were chosen.

Six patients on CsA immunosuppressive therapy after living donor renal transplantation were included in this study. The patients underwent renal allograft implantation within 2 weeks prior to sample date and received a quadruple regimen comprising basiliximab (20 mg, days 0 and 4), prednisolone (10 mg/bid), mycophenolate mofetil (1 g/bid) and CsA starting 4 mg/kg bid. EDTA-anticoagulated blood was taken just prior to dose (T0, 12 hours after the previous dose) and 2 hours after drug intake (T2). Mean CsA whole blood concentrations at T0 was 204 ± 71 μg/L and 983 ± 278 μg/L at T2. Formal approval from the institutional ethics committee was obtained and informed consent for blood sampling was obtained from all participants. CsA concentrations of renal allograft recipient blood were measured by fluorescence polarization immunoassay using an Abbott AxSYM system (Abbott Park, IL, USA). The assays were performed according to the manufacturer’s instructions.

Preparation of leukocyte lysate for CN measurement
Leukocyte lysates of CN inhibitor incubated EDTA-anticoagulated blood were prepared as previously described(13). In brief; 2 mL of EDTA-anticoagulated blood was lysed with NH₄Cl lysis buffer (8.4 g/L NH₄Cl, 1.0 g/L KHCO₃, pH 7.3) and washed twice with 10 mL HBS before resuspension in 1.5 mL Hepes buffered saline (HBS, 9.0 g/L NaCl, 10 mmol/L Hepes pH 7.5) for cell count on a Sysmex XE2100.

Cell lyses was performed at a cell concentration of 10·10⁶ cells/mL, therefore 2·10⁶ cells were resuspended in 200 μL lysis buffer (50 mmol/L Tris HCl pH 7.7, 1.0 mmol/L dithiothreitol, 5.0 mmol/L ascorbic acid, 0.02% (v/v) NP-40, 50 mg/L
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soybean trypsin inhibitor, 50 mg/L phenylmethylsulphonyl fluoride, 5.0 mg/L leupeptin and 5.0 mg/L aprotinin) and lysed by 3 freeze thaw cycles (liquid N₂ / 30°C). Cell debris was spun down (10 min, 10000g, 4°C) and supernatants were snap frozen in liquid N₂ before storage at -80°C till cyclophilin A lysate experiments and/or CN activity measurement.

For cyclophilin A (CyPA) / CsA experiment in cell lysate, lysate either or not prepared from CsA pre-incubated whole blood or patient blood was incubated with CsA and/or CyPA. Final ethanol concentrations were 1%. Samples were incubated for 1 hour at 37°C and CN activity was determined directly after incubation.

CN activity measurement

CN enzyme activity was determined by spectrophotometric quantification of phosphate released after incubation with the phosphorylated RII substrate as has been described before(13). In brief, 15 μL of lysate was added to 40 μL of assay buffer (0.313 μmol/L calmodulin, 0.375 mmol/L RII phosphopeptide substrate, 75 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 9 mmol/L MgCl₂, 0.75 mmol/L dithiothreitol, 0.0375% NP-40, 0.625 μmol/L okadaic acid, 0.75 mmol/L CaCl₂ and 5 mmol/L ascorbic acid) in presence and absence of substrate. Phosphatase reaction was run for 30 min at 30°C, the reaction was stopped by adding 100 μL malachite green reagent and the absorbance (620 nm, SpectraMax 250, Molecular Devices, Downingtown, PA, USA)) was read after 50 min incubation at RT. Background absorbance from blank measurement was subtracted and phosphate was quantified out of a 0-1.5 nmol phosphate calibration curve.

IFNγ and TNFα excretion

For cytokine determination, 30 μL of anti-CD3/anti-CD28 stimulation solution was added to the CN inhibitor / Heparin-anticoagulated blood resulting in final concentrations of 0.5 mg/L anti-CD28 and 0.12 mg/L anti-CD3. Samples were incubated for another 4 hours at 37°C under gentle rotation. After centrifugation (1550g, 20 min, 20°C), plasma was collected and stored at -80°C until cytokine concentration determination. IFNγ and TNFα plasma concentrations were determined using ELISA technique and performed according to manufacturers’ protocol, based on(14).

Data analysis

Data was normalized and expressed as % of blank measurement in order to correct for inter-individual variation. Data in the text are presented as mean ± SD.
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Student t-tests were used to test significance and significance was defined as p<0.05.

Materials
The following materials were used; cyclosporine A, dithiothreitol (DTT), ascorbic acid, NP-40, Cyclophilin A (CyPA), and soybean trypsin inhibitor from Sigma Aldrich (Steinheim, Germany); phenylmethylsulfonyl fluoride from Merck (Darmstadt, Germany), Leupeptin from Roche (Basel, Switzerland); aprotinin from Bayer (Leverkusen, Germany); Phosphate standard concentrate from Fluka (Buchs, Switzerland); mouse anti-CD28 and mouse anti-CD3 from BD Biosciences (San Jose, CA, USA); CN assay components from the Biomol Green Cellular Calcineurin Assay Kit Plus from Biomol (Plymouth Meeting, PA, USA). Buffycoats, ELISAs (IFNγ, and TNFα) were from Sanquin (Amsterdam, NL).

Results

Extent of CsA mediated immunosuppression
First, the extent of CsA mediated immunosuppression for in vivo conditions was investigated. Therefore, the pharmacodynamic effects of high range relevant CsA in vivo whole blood concentrations were determined relative to baseline level and a condition of CsA excess. Whole blood from healthy volunteers was incubated with 2500 µg/L and 25000 µg/L CsA. Whole blood incubated with final CsA concentrations of 2500 µg/L represented a relevant high range in vivo concentration and 25000 µg/L CsA was used as a condition of CsA excess. CN enzyme activity and plasma cytokine concentrations after CD3/CD28 stimulation were determined, see figure 3.1.

Compared to 2500 µg/L CsA, a minor but significant decrease in CN inhibition was observed for 25000 µg/L CsA (p=0.004). For IFNγ and TNFα no differences in plasma cytokine concentrations were observed between 2500 and 25000 µg/L CsA with p=0.18 for IFNγ and p=0.25 for TNFα. These results indicated that, in the system used, a whole blood CsA concentration of 2500 µg/L approaches the maximum immnosuppressive effect of CsA.
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Figure 3.1. The extent of CsA pharmacodynamics. Healthy volunteer whole blood was incubated with final CsA concentrations of 0 (BL), 2500 and 25000 μg/L. A; CN activity (n=14). B; IFNγ (n=6) and C; TNFα (n=6). Data are expressed relative to Baseline (BL) within each volunteer and presented as mean + SD. ** for p<0.01 and NS (not significant) for p≥0.05.

Pharmacodynamics under representative in vivo CsA concentrations

Next, the immunosuppressive dynamics at simulated representative CsA T0 and T2 conditions for allograft recipients at early and late stages after graft implantation were investigated. The results are plotted in figure 3.2.

Figure 3.2. Pharmacodynamics of CsA for whole blood target conditions. A; relative CN inhibition (n=8) and B; relative TNFα and IFNγ plasma concentrations (n=7). Data are all expressed relative to baseline (BL) condition per volunteer and are presented as mean + SD. Statistics presented in the figure on the bars represent t-testing with respect to the lower CsA concentration. *** for p<0.001, ** for p<0.01, * for p<0.05 and NS (not significant) for p≥0.05

A CN inhibition of 25 ± 8% was observed at 100 μg/L CsA and a CsA concentration dependant increase in CN inhibition was found with 48 ± 8% of CN inhibition at
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1700 µg/L. For IFNγ and TNFα the CsA concentration range was responsible for much larger relative dynamic effects between conditions chosen and covered almost the full dynamic range from no inhibition to total inhibition of cytokine excretion. At 100 µg/L CsA a small (IFNγ, 11 ± 6%, p= 0.004) or no reduction (TNFα, p=0.27) was observed, while at 1700 µg/L, cytokine inhibition was 88 ± 11% for IFNγ and 79 ± 12% for TNFα. Each increase in CsA concentration led to a further significant decrease in IFNγ and TNFα.

CyP limitation

Since the dynamic range of CN inhibition was more narrow than observed for inhibition of cytokine secretion, we investigated whether cyclophilin content could be responsible for limiting CN inhibition at high CsA concentrations. Limitation of CyP on CsA based CN inhibition at a whole blood concentration of 2500 µg/L CsA was investigated by (cyclophilin A) CyPA and CsA addition experiments in leukocyte lysate. The leukocyte lysate used was obtained from whole blood previously incubated with 2500 µg/L CsA (L2500). The lysates were incubated with and without additional 5000 µg/L CsA and/or 40000 µg/L CyPA. Addition of CsA to L2500 lysate could further increase CN inhibition (p=0.027) and an additional increase in CN inhibition was observed by the addition of CyPA and CsA (p=0.003). Addition of CyPA itself did not significantly affect CN activity (p=0.066), see figure 3.3.

Figure 3.3. Cyclophilin limitation. Sample lysate of full blood incubation with 2500 µg/L CsA. was incubated with 40000 µg/L CyPA and 5000 µg/L CsA. BL is leukocyte lysate of whole blood without CsA and L2500 is leukocyte lysate prepared from whole blood incubated with 2500 µg/L CsA. Data are presented as mean ± SD. * is used for 0.05>p>0.01 and ** is used for p<0.01.
Peripheral blood obtained from 6 renal allograft patients treated with CsA was used to investigate the relevance of CN inhibition limitation by cyclophilins for allograft recipients. Patient samples were taken within the first two weeks after transplantation, when whole blood target concentrations or CsA are highest. Sample lysate was prepared and lysate prepared from T2 sample was incubated with or without 20000 µg/L CsA, 40000 µg/L CyPA or both. After addition of either CyPA or CsA to sample lysate, a decrease in CN activity of 8 ± 6% for CyPA (p=0.04) and 30% ± 8% for CsA (p=0.004) were observed. Addition of both CsA and CyPA led to additional inhibition of CN activity when compared to previous conditions.

**Relation between CN inhibition and cytokine inhibition**

To investigate the relation between CN inhibition and inhibition of T cell cytokine excretion, we plotted the calcineurin inhibition and cytokine excretion inhibition data from the previous experiments in one graph, see figure 3.4. Here it is shown that at over a certain CN inhibition threshold, which is located around 100 µg/L CsA, a small increase in CN inhibition resulted in relative large increase in cytokine secretion inhibition. At 2500 µg/L, CsA approaches its maximal immunosuppressive state.

![Figure 3.4. Relation between CN and cytokine inhibition.](image-url)

Relative TNFα (A) and IFNγ (B) inhibition is plotted against the CN inhibition observed at different CsA concentrations. The numbers in the graph represent CsA concentration in µg/L. Error bars represent SEM values.
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Discussion

To further optimize CNI therapy, pharmacodynamic monitoring strategies are currently investigated as potential tool to guide dosing. Previously we developed a CN assay to monitor CN activity in allograft recipients treated with CNIs(13). In the present study we assessed the relation between CN activity and the decrease in T cell related cytokine secretion at therapeutically relevant CsA whole blood concentrations. The results show that at relevant CsA concentrations for renal allograft recipients, small changes in CN inhibition resulted in a significant change in cytokine secretion and, at high CsA concentrations, approached immunosuppressive saturation.

Complete inhibition of cytokine excretion with only partial inhibition of CN enzyme activity has been reported previously(12). These studies have been very valuable in understanding the pharmacologic effects of CNIs, but since they have been performed in culture systems their clinical relevance and applicability has remained unclear. We chose whole blood incubated with CsA as a model to simulate allograft recipient blood treated with CsA. Due to the absence of relevant CsA binding sites, CsA-related pharmacodynamic parameters under culture conditions may not correspond to these markers in whole blood(15). In addition, by using healthy volunteer blood, the pharmacodynamic responses could be investigated without interference of other concomitantly drugs present and can be expressed relative to their baseline level.

Between the high end of the regular therapeutic range and high excess of CsA, only a small increase in CN inhibition was noted, but no differences in cytokine secretion, indicating that 2500 μg/L CsA represented the optimal immunosuppressive effect in the whole blood system. Using CsA concentrations from 100 to 1700 μg/L, covering the range of relevant T0/T2 conditions for renal allograft recipients, the decrease in secretion of T cell related cytokine reached 90% relative to baseline values. In contract, for CN inhibition the relative differences were much smaller. In addition, 100 μg/L CsA had only a marginal effect on the suppression of cytokine secretion, while already a relative large degree of CN inhibition around 25% was found. This could imply that CN inhibition has to cross a certain threshold, located CsA concentrations around 100 μg/L or a CN inhibition of 25%, before CN inhibition actually results in inhibition of T cell cytokines. Thereafter, however, a small additional increase in CN inhibition resulted in a relative large increase in suppression of cytokine secretion. The cytokine response range was covered by the different representative T0 and T2 conditions.
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chosen and responses to these different concentrations were significantly separated. These results indicate that different late and early CsA C0 and C2 target concentrations represent distinct immunosuppressive conditions.

It has been demonstrated that cyclophilins can limit CN inhibition by CsA in cell culture test systems(12). In addition, partial CN inhibition has been observed in all studies that monitored CN activity in allograft recipients(16). These two observations led us to investigate the role of cyclophilin as rate limiting step at high CsA concentrations. Leukocyte lysate prepared from whole blood incubated with 2500 μg/L CsA was incubated with and without additional CsA and CyPA. Addition of CsA resulted in a further increase in CN inhibition, that was not observed when CyPA was added. This confirmed that CyPA did not limit CsA inhibition at clinically relevant concentrations. In agreement with previous observations, however, adding both CyPA and CsA resulted in significantly less CN activity, confirming that, at higher CsA concentrations CyPs, may indeed be rate-limiting(12). Therefore our results indicate that up to 2500 μg/L CN inhibition by CsA is not limited by lack of CyPs.

To investigate whether cyclophilin limitation is relevant for allograft recipients, we repeated these experiments in leukocyte lysate isolated from renal transplant recipients treated with CsA-based immunosuppression. Leukocyte lysates were obtained just before drug intake and two hours thereafter, representing trough and peak levels respectively(17). Addition of CsA to T2 lysates resulted in a further decrease in CN activity illustrating that maximum CN inhibition by CsA was not achieved under the corresponding whole blood conditions. The number of patients used here, are however too small to exclude cyclophilin limitation for CsA therapy in allograft recipients.

Even though these data illustrate the immunosuppressive dynamics of CsA in recipients, one important assumption is made, namely that whole blood is a relevant and representative matrix to assess the immunosuppressive state. In peripheral whole blood there is a large fluctuation in CsA concentrations that may not necessarily reflect concentrations at the tissue level.

In summary, despite redundancy in the immune system, and calcineurin inhibition in particular, there was a significant and dose-dependent suppression of cytokine synthesis as assessed by secretory capacity. Thus far, pharmacodynamic biomarkers have mainly contributed to study drug interactions and visualizing the degree of clinical immunosuppression in allograft recipients using culture systems(11,18,19). The results of the present study add to our understanding of biological variances in CN inhibition and revealed that the degree of clinical
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Immunosuppression can be assessed either by CN activity or T cell cytokine secretion.

References

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