Whole-Blood Calcineurin Activity Is Not Predicted by Cyclosporine Blood Concentration in Renal Transplant Recipients

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Background: In transplant patients, current cyclosporine (CsA) dose monitoring with classic pharmacokinetics has demonstrated limitations. Evaluation of the activity of calcineurin (CN), the serine-threonine phosphatase enzyme target of CsA, has been proposed as a reliable way to optimize CsA dosing.

Methods: CN activity was measured in whole blood in an attempt to overcome the high variability of results obtained previously with peripheral blood mononuclear cells (PBMCs). We also explored, in vitro, a possible relationship between the CsA concentration and CN inhibition in whole blood. Finally, we assessed whether the CsA blood trough concentration correlates with whole-blood CN activity in kidney transplant recipients (n = 15) on maintenance immunosuppression with CsA.

Results: In 14 healthy individuals, less scattered CN activity values were documented in whole blood than in the PBMC fraction. Whole-blood CN activity was higher than the sum of the enzyme activity in each cell blood fraction. After ex vivo incubation of whole blood from healthy subjects (n = 5) with increasing concentrations of CsA (50 – 1000 μg/L for 1 h), a concentration-dependent inhibition of CN activity was found comparable to that in the PBMC fraction. Moreover, in 15 kidney transplant recipients, no relationship was found between CsA pharmacokinetic parameters and CN activity at time 0. However, a highly significant correlation was found between CN activity at time 0 and at 12 h postdosing (r = 0.96; P < 0.01).

Conclusions: Measuring CN activity in whole-blood samples is a reproducible method. In kidney transplant recipients, CsA trough concentrations do not predict baseline CN activity. Moreover, a single CN activity monitoring at baseline or at time 12 h post-CsA dosing may be a useful surrogate for the inhibition of this enzyme by CsA during 12 h.
exposure to CsA, it is seldom feasible to perform in routine outpatient clinic monitoring.

In contrast to pharmacokinetics, which measure the effects of the body on a drug, pharmacodynamics assess the effects of the drug on the body, thus providing a potential advantage for monitoring immunosuppressive drugs such as CsA. Pharmacodynamic monitoring may be performed using a surrogate laboratory measure of immunosuppressive activity. For CsA, this approach has been based on the measurement of inhibition of the enzyme calcineurin (CN), a serine-threonine phosphatase that represents a rate-limiting signal transduction pathway in the activation of T lymphocytes (13–15). Although the results from pharmacodynamic CsA monitoring have been encouraging, we are still in the early phase of development of a reliable and reproducible method for measuring CN activity. Indeed, evaluation of the activity of the enzyme as a way to optimize CsA dosing has been performed ex vivo in peripheral blood leukocytes (16, 17), but extremely variable results have been achieved in transplant recipients, probably because of the many factors affecting the reproducibility of the separation of mononuclear cells during sample preparation.

The present study was thus designed with the following aims: (a) to improve reproducibility of CN activity measurement by use of unseparated whole blood instead of peripheral blood mononuclear cells (PBMCs) as a matrix for the assay; (b) to establish in vitro a relationship between CsA concentrations and CN inhibition in whole blood; and (c) to assess whether CsA blood trough concentration, the commonly used index to monitor drug dosing, correlates with whole-blood CN activity in kidney transplant recipients on maintenance immunosuppression with CsA.

Materials and Methods

Optimization of CN Activity Measurement in Whole Blood

We assessed the reproducibility of measuring CN activity in whole blood using different assay conditions. Blood was withdrawn from the antecubital veins of healthy volunteers into heparin-containing (200 IU/mL of blood) Vacutainers and processed immediately. We first tested the effect of different dilutions of blood in the lysis buffer on CN activity. Dilution ratios of 1:3 to 1:30 (by volume) were used. Experiments were performed in triplicate at each dilution. Moreover, to evaluate the precision of the CN activity assay, blood samples collected from healthy individuals were divided into three aliquots; CsA was added at different final concentrations (0, 100, and 1000 µg/L, respectively) to the aliquots and incubated for 1 h at 37 °C. The precision of the method was assessed by measuring CN activity in 10 replicates of each aliquot of whole blood. Thereafter, we investigated the stability of CN activity in whole blood by performing the assay at different time points after blood collection (0–240 min) as well as after storage of lysed samples (0–15 days). In all cases, after dilution with lysis buffer samples were processed and finally stored at −80 °C until CN activity was assayed. The protein content of lysis buffer-treated samples was determined by the Coomassie blue dye method (18).

CN Activity in Whole Blood and Different Blood Fractions

Venous blood samples from five healthy individuals were collected, and CN activity was measured in the whole blood and in each cellular fraction [erythrocytes, PBMCs, polymorphonuclear cells (PMNs), and platelets (PLTs)] as well as in plasma. Blood samples were centrifuged, and the collected plasma was diluted with two volumes of lysis buffer (50 mmol/L Tris; pH 7.5; 0.1 mmol/L EGTA; 1 mmol/L EDTA; 0.5 mmol/L dithiothreitol; 50 mg/L phenylmethylsulfonyl fluoride; 5 mg/L leupeptin; 5 mg/L aprotinin; and 50 mg/L soybean trypsin inhibitor). The remaining buffy coat was discarded, and precipitated red blood cells (RBCs) were resuspended in lysis buffer (1:4 dilution). PBMCs were prepared from whole blood by Ficoll gradients as described previously (16), and 1.5–2 × 10^6 cells were pelleted and suspended in 100 µL of lysis buffer. The remaining pellet, which contained RBCs and PMNs, was mixed with Emagel (Hoechst, Marion Roussel); the RBCs were allowed to sediment and were removed by ammonium chloride lysis. PMN-enriched supernatant was centrifuged, and the pellet (1.5–2 × 10^6 PMNs) was suspended in 100 µL of lysis buffer. PLT-rich plasma was also obtained by blood centrifugation at 150g for 20 min. PLT-rich supernatant was sedimented, and the pellet was lysed immediately (final concentration, 500 × 10^6 PLTs). All samples were then processed as above and stored at −80 °C until CN activity was assayed.

Expression of CN Subunits in Cell Blood Fractions

To investigate the distribution of CN activity in whole blood, expression of the α subunits of the enzyme (19) was determined in the different blood cell fractions by Western blot analysis (20). Briefly, 50–200 µg of lysates of RBCs, PBMCs, PMNs, or PLTs was loaded on 12.5% sodium dodecyl sulfate-polyacrylamide gels and electrophoresed. Gels were electroblotted onto the nitrocellulose membrane with a Mini blotting apparatus (Bio-Rad). The membrane was incubated for 2 h with mouse monoclonal anti-CN (α subunit) IgG1 (Sigma) diluted 1:10 000 in Tris-buffered saline-Tween (TBST; 10 mmol/L Tris, pH 8.0; 150 mmol/L NaCl; 0.5 mL/L Tween 20). After washing, the membrane was incubated for 1 h with horse biotinylated anti-mouse IgG (Vector Laboratories) at a 1:200 dilution in TBST. The membrane was then transferred into a plate containing ABC solution (avidin and biotinylated horseradish peroxidase in 1:1 ratio in TBST, prepared according to the manufacturer’s instructions; Vector Laboratories) and incubated for 30 min. After
additional washing, the membrane was incubated in 20 mL of fresh reagent solution [one tablet of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Merck) and 6.5 μL of 300 mM/L H2O2] until color development.

IN VITRO EFFECT OF CSA ON WHOLE-BLOOD CN ACTIVITY
The effect of CsA on CN activity was first evaluated in vitro using whole-blood samples collected from healthy volunteers (n = 5) and compared with that of the enzyme activity in PBMCs. Increasing concentrations of CsA (50–1000 μg/L; Novartis) or vehicle (ethanol; Merck) were added to aliquots of whole blood, which were then incubated at 37 °C for 1 h. From each aliquot of blood, PBMCs were isolated. Whole-blood and PBMC lysates were immediately frozen and stored at −80 °C until the CN activity was assayed.

CSA PHARMACOKINETICS AND EX VIVO INHIBITION OF CN ACTIVITY IN RENAL TRANSPLANT PATIENTS
A total of 15 adult patients (3 females and 12 males) who had received a kidney transplant at least 1 year prior were studied. All patients had stable renal function and were on chronic immunosuppression with CsA, prednisone, and azathioprine. The mean CsA dosage was 3.1 ± 1.2 mg·kg⁻¹·day⁻¹ in two divided doses. The study protocol was described in detail to patients before admission, and informed consent to perform the study was obtained.

On the morning of the study, a blood sample was collected from each patient, via an antecubital vein, into heparin-containing tubes for determination of baseline (trough) CsA concentration and whole-blood CN activity. Each patient was then given the morning dose of CsA and underwent evaluation of a CsA pharmacokinetic profile in parallel with sequential determinations of CN activity in whole blood. For these measurements, blood was drawn at 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 h after CsA dosing. Both the area under the CsA blood concentration–time curve (CsA-AUC0–12 h) and the area under the CN activity–time curve (CN-AUA0–12 h) from time 0 to the last sampling point were calculated by trapezoidal rule. In eight of these patients, the CN activity profile in whole blood was compared with that in PBMCs.

As a control for the possible daily variation of CN activity, the kinetic profile of the enzyme activity was assessed in three healthy individuals.

CN ACTIVITY ASSAY
The assay was performed in duplicate as described previously (16). Briefly, 20 μL of lysate sample, 35 μL of analysis buffer [final concentration, 20 mmol/L Tris-HCl (pH 8), 100 mmol/L NaCl, 6 mmol/L MgCl2, 0.1 mmol/L CaCl2, 0.5 mmol/L dithiothreitol, 0.1 g/L bovine serum albumin, 500 nmol/L okadaic acid], and 5 μL of 32P-labeled phosphopeptide (final concentration, 5 μmol/L) as substrate were used. The synthetic peptide DLD-VPIPGFRDRRVSVAE (Sigma), corresponding to the phosphorylation site on the RII subunit of cAMP-dependent protein kinase (21), was phosphorylated on the unique serine residue by the catalytic subunit of cAMP-dependent protein kinase, using [γ-32P]ATP.

Samples were evaluated for their ability to dephosphorylate a 32P-labeled 19-amino acid peptide substrate in the presence of okadaic acid to inhibit phosphatase type 1 and type 2A (22). Background phosphatase 2C activity (CsA- and okadaic acid-resistant activity) was determined and subtracted from each sample, with the assay performed in the presence and absence of excess CsA (10 μmol/L) or ethanol. The remaining phosphatase activity was taken as CN activity. CN activity was expressed as picomol of 32P released per minute per milligram of protein in the lysate (or milliliter of sample or 10⁶ cells). The protein content of the cell lysates was determined by the Coomassie blue dye method (18).

CSA DETERMINATION
Blood CsA concentrations were measured using a previously described modified method (23, 24). Briefly, collected blood was frozen immediately and stored at −20 °C before liquid extraction and reversed-phase HPLC analysis (mobile phase, 370 mL/L acetonitrile–340 mL/L methanol–290 mL/L water–0.1 g/L ammonium sulfate). Results were expressed as μg/L.

STATISTICAL ANALYSIS
The 50% inhibitory concentrations (IC₅₀s) were determined by variable slope, nonlinear regression curve fitting and analyzed using EasyFit software. Data were analyzed using the t-test or the Wilcoxon test with Stat View 4.0 on an iMac computer (Apple). Values are reported as mean ± SD or as median and interquartile (IQ) range, as appropriate. Statistical significance was defined as P < 0.05.

RESULTS
EFFECTS OF DIFFERENTLY DILUTED BUFFER AND ASSAY TIME ON IN VITRO CN ACTIVITY IN WHOLE BLOOD
Increasing the dilution ratio of blood samples in lysis buffer from 1:3 to 1:12 was not associated with any significant change in CN activity [CN activity (as pmol 32P·min⁻¹·mL⁻¹), 104% ± 8% for the 1:6 dilution and 104% ± 5% for the 1:12 dilution compared with the 1:3 baseline dilution]. However, increasing the dilution ratio to 1:30 produced a significant reduction of the enzyme activity (73% ± 5%; P < 0.05). On the basis of these findings, all subsequent assays of CN activity in whole blood were performed at the 1:3 dilution with lysis buffer.

The assay showed good precision for whole-blood samples with high CN activity [CN activity (207.0 ± 8.5 pmol 32P·min⁻¹·mL⁻¹; CV = 4%). The imprecision was greater but still acceptable at a lower CN activity (62.9 ± 7.5 pmol 32P·min⁻¹·mL⁻¹; CV = 12%).

We also found that in whole-blood samples from four healthy individuals processed >15 min after collection
and stored at room temperature, CN activity was markedly reduced (78.1% at 15 min; 74.6% at 240 min) with a large CV for the measured values (47% at 15 min; 14% at 240 min). Thus, blood samples were processed immediately after being collected.

When CN activity was assayed in lysates stored at $-80^\circ$C for $>5$ days ($n = 7$), a progressive decrease in the enzymatic activity was documented compared with day 2 (100% at baseline; 98.3% on day 3; 95.5% on day 5; 61.9% on day 15). Therefore, all samples were subsequently assayed within 5 days of storage, a time at which the CV of the CN measurement was acceptable (CV = 5.7%).

To assess the best way to express CN activity, the enzyme activity in whole-blood fractions from healthy volunteers ($n = 14$) was normalized for milliliter of blood or for milligram of protein. When we evaluated the intraindividual variability of basal CN activity in healthy individuals, we documented greater scatter of values when the enzyme activity was expressed as pmol $^{32}\text{P} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (CV = 38%) than when it was expressed as pmol $^{32}\text{P} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$ (CV = 25%).

**CN Activity and Expression in Whole Blood and Different Blood Fractions in Healthy Individuals**

The total recovered CN activity, obtained by adding each individual cell and plasma fraction, accounted for only 61.9% of the enzyme activity measured directly in unfractionated whole blood (Fig. 1). RBC CN activity per $10^6$ cells was negligible (0.02 ± 0.01 pmol $^{32}\text{P} / \text{min per} 10^6$ cells) and was lower than in PBMCs (1.4 ± 1.5 pmol $^{32}\text{P} / \text{min per} 10^6$ cells). Among the fractions, RBCs contained 56% of the whole-blood CN activity because the RBC count is at least three orders of magnitude higher than that of white blood cells per milliliter of blood. The concentration of CN protein in each cell blood fraction paralleled the CN activity. As shown in Fig. 2, the CN $\alpha$ subunit in RBCs was documented only after the lysate dose was increased up to 200 $\mu$g of protein. On the other hand, at a low loading dose (50 $\mu$g) of lysate, Western blot analysis clearly detected the CN $\alpha$ subunit in PBMCs, whereas it was minimally detected in PMN fractions.

**In Vitro Effect of CSA on Whole-Blood CN Activity**

The concentration–response curves for the in vitro effect of CSA on CN activity in whole blood and PBMCs from healthy volunteers are shown in Fig. 3. Exposure of whole blood to increasing concentrations of CSA was associated with a concentration-dependent inhibition of CN activity in both whole-blood and PBMC extracts. In whole-blood samples, CN activity decreased from 144.4 ± 32.9 pmol $^{32}\text{P} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$ without CSA to 35.5 ± 25.3 pmol $^{32}\text{P} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$ in the presence of 1000 $\mu$g/L CSA ($P < 0.05$). A similar inhibition profile for CN activity was found in the PBMC fraction, where a progressive inhibition of the enzyme activity was documented with increas-
ing concentration of CsA and residual CN activity of 0.5 ± 0.6 pmol $^{32}$P·min$^{-1}$·mL$^{-1}$ at 1000 μg/L CsA was found. These profiles demonstrated that in whole blood, the concentration of CsA in the incubation medium producing the IC$_{50}$ for CN activity was, on average, 394 μg/L (95% confidence interval, 360–428 μg/L). A lower IC$_{50}$ was found in PBMCs (239 μg/L; 95% confidence interval, 212–266 μg/L).

**EFFECT OF CSA ON CN ACTIVITY IN KIDNEY TRANSPLANT RECIPIENTS**

A comparison of the time profile of the CsA concentration in blood and the profile of CN activity in whole blood from kidney transplant recipients with stable renal function is shown in Fig. 4. After drug administration, CsA concentrations in blood increased progressively from trough (median, 135 μg/L; IQ range, 105–156 μg/L) to peak values reached, on average, 1 h after dosing (median, 722 μg/L; IQ range, 499–896 μg/L). Thereafter, CsA concentrations in blood progressively decreased toward preadministration values in the following 8–10 h [median, 129 μg/L (IQ range, 79–194 μg/L) at 8 h; median, 95 μg/L (IQ range, 74–152 μg/L) at 10 h].

In these patients, the kinetics of the mean CN activity showed an inverse correlation with mean CsA pharmacokinetics ($r = -0.96; P < 0.01$). CN activity reached a nadir, on average, 1 h after CsA dosing [basal value, median, 81.3 (IQ range, 58.4–142.4) pmol $^{32}$P·min$^{-1}$·mL$^{-1}$; nadir, median, 32.4 (IQ range, 14.5–43.4) pmol $^{32}$P·min$^{-1}$·mL$^{-1}$]. At this time point, the mean enzyme activity in whole-blood extracts was reduced by 67% compared with the pre-CsA dosing value (time 0). Thereafter, a slow but progressive recovery of CN activity was observed, approaching baseline values between 6 and 8 h after CsA dosing [at 6 h, median, 83.6 (IQ range, 55.5–125.8) pmol $^{32}$P·min$^{-1}$·mL$^{-1}$; at 8 h, median, 83.5 (IQ range, 62.2–114.4) pmol $^{32}$P·min$^{-1}$·mL$^{-1}$].

As shown in Fig. 5, a comparison of CN activity profiles in whole blood and PBMC fractions after CsA administration was performed in a subgroup of these patients. Each point is the median and IQ range (error bars) of eight patients. $+$, $P < 0.05$ vs basal values. The median CN activity in whole blood from 16 healthy individuals was 167.3 pmol $^{32}$P·min$^{-1}$·mL$^{-1}$ (IQ range, 140.2–184.7 pmol $^{32}$P·min$^{-1}$·mL$^{-1}$).
patients. The time profile of the mean enzyme activity measured in PBMC extracts did not parallel that in the whole-blood fraction and was less negatively correlated with the CsA pharmacokinetic profile \((r = -0.53; P, n o t \) significant). In PBMC extracts, the nadir of CN activity inhibition occurred 3 h after CsA dosing (median, 0.58 pmol \(32P \cdot \text{min}^{-1} \cdot \text{mL}^{-1}\); IQ range, 0.24–1.03 pmol \(32P \cdot \text{min}^{-1} \cdot \text{mL}^{-1}\)) compared with the basal values (median, 2.99 pmol \(32P \cdot \text{min}^{-1} \cdot \text{mL}^{-1}\); IQ range, 1.61–4.73 pmol \(32P \cdot \text{min}^{-1} \cdot \text{mL}^{-1}\)). With the decline in CsA blood concentration, PBMC CN activity progressively increased and reached basal values between 7 and 8 h post-CsA administration (at 8 h, median, 1.74 pmol \(32P \cdot \text{min}^{-1} \cdot \text{mL}^{-1}\); IQ range, 0.69–3.93 pmol \(32P \cdot \text{min}^{-1} \cdot \text{mL}^{-1}\)).

Considering the mean profile of CN activity, the estimated IC50 of CsA inhibition on CN activity was 80 \(\mu g/L\) in PBMCs and 137 \(\mu g/L\) in whole-blood fractions. These results, however, cannot be extended to individual patients because of the high dispersion of IC50 values (IC50 range, 32–347 \(\mu g/L\) in PBMCs, 40–419 \(\mu g/L\) in whole blood).

In three control individuals, CN activity did not change significantly during the 12-h observation period in both whole blood and PBMCs (data not shown).

**Relationship between CN inhibition and CsA pharmacokinetics**

Regression analysis of CN activity in whole blood from kidney transplant recipients plotted against blood CsA concentrations showed no relationship between baseline enzyme activity and blood CsA trough concentration (Fig. 6A). Similarly, no significant correlation was found between CN activity at baseline \((CN_{0}, r = 0.103; P = 0.71; \) not shown) or at 2 h post-CsA dosing \((CN_{2}, r = -0.27; P = 0.34; \) not shown) and CsA blood concentration at 2 h \((C_{2})\). Moreover, baseline CN activity did not correlate with the CsA-AUC0–12 h \((r = 0.17; P = 0.54; \) not shown), nor was any correlation documented between the CN-AUC0–12 h and CsA-AUC0–12 h \((r = 0.07; P = 0.81; \) not shown) and CsA blood concentration 2 h postdose \((C_{2}; r = 0.02; P = 0.93; \) not shown).

Furthermore, by regression analysis, no significant correlation was found between percentage of CN inhibition and percentage increase in CsA concentration at 2 h after drug dosing compared with trough values in both whole-blood \((r = -0.09; P = 0.83)\) and PBMC \((r = -0.23; P = 0.59)\) samples.

Similarly, no correlation was reported between abso-

![Fig. 6. Correlation between inhibition of whole-blood CN activity and CsA pharmacokinetic parameters (A and B) and between CN activity parameters (C and D) in 15 kidney transplant recipients.](image-url)
lute blood CsA concentrations at 2 h and the percentage of CN inhibition over trough enzyme activity (100%; whole blood, \( r = 0.30, P = 0.13; \) PBMCs, \( r = -0.20, P = 0.65 \)).

However, a highly significant relationship was found between CN-AUA \(_{0-12} h\) and CN activity at baseline (\( r = 0.79; P < 0.01; \) Fig. 6C) or at 12 h post-CsA dosing (\( r = 0.96; P < 0.01; \) Fig. 6D).

**Discussion**

The first finding of the present study was the demonstration that, in healthy individuals, the CVs for measurement of CN activity in whole-blood extracts were lower than those for PBMC extracts from the same individuals. This indicates that the whole-blood matrix is more reliable for monitoring this enzyme activity than the originally proposed peripheral blood lymphocytes (16, 17). That this may be attributable to the avoidance of fractionating blood compartments is supported by the fact that only 61% of the total activity in fresh whole blood was recovered when the CN activities of all of the cell and plasma fractions were added together. This observation confirms a recent finding that CN inhibition can be measured in fresh whole blood with no cell separation procedures (25). Disruption of the in vivo equilibrium among the different cellular and plasma components of whole blood, loss of CsA during separation of PBMCs and other cellular components, and the low precision of the CN activity assay at extremely low activities in PBMCs, as we have documented in the present study, are possible explanations for the discrepancy between CN activity measured in unfractionated vs fractionated whole blood. Moreover, recent data pointed out that during the extraction of CN enzyme from crude tissue, generation of oxygen free radicals occurred, which reduced the stability of the enzyme activity (26). This is also supported by our data in PBMCs (F. Gaspari and colleagues, unpublished observations) and by previous observations that in crude tissue extract, the stability of CN activity is increased when the antioxidant \( \alpha \)-ascorbic acid is added to lysis buffer (26). Thus, the complex procedure of whole-blood cell fractionation might also favor production of oxygen radicals in excess of that in unfractionated samples, ultimately increasing the uncertainty of CN activity measurements in PBMCs.

By use of in vitro studies, we have documented that CN inhibition by CsA in whole blood closely reflected the concomitant effect of the drug. The profile of the inhibition of the enzyme activity by CsA in whole blood closely paralleled that in PBMCs. The lag observed with CN inhibition in both matrices may be attributable to the fact that CsA initially binds extracellular components in the plasma (27). However, the CsA concentration that allowed 50% inhibition (IC\(_{50}\)) was different, being higher in whole blood than in the PBMC fraction. This reflects the competition for CsA-binding sites outside of the leukocytes, such as in erythrocytes (28, 29), that occurs in whole blood and makes the use of whole blood for in vitro CN activity assays closer to in vivo conditions.

Although this is the first formal report of an in vitro comparison between the IC\(_{50}\) for CN activity in whole blood and the PBMC fraction, other investigators have shown IC\(_{50}\) values in PBMC samples quite lower than the value we estimated here (30). It should be noted, however, that in the other study (30), the 95% confidence interval for the IC\(_{50}\) was quite wide (42–245 \( \mu g/L \)) compared with our finding (212–266 \( \mu g/L \)). This may reflect the interindividual variability of basal CN activity, which translates into different CsA concentrations required to achieve the IC\(_{50}\). Together these findings indicate that, in vitro, a narrow window of CsA concentrations that would provide 50% inhibition of CN activity cannot be defined, thus probably hampering the in vivo use of target IC\(_{50}\) values for a given population of transplant patients as a guide for drug dosing.

Previous studies have documented that inhibition of cellular CN by CsA closely parallels the inhibition of T-cell activation, measured as interleukin-2 generation (15), suggesting that measurement of the enzyme activity might help in monitoring of the immune effects of CsA in vivo. This is indirectly supported by the present in vivo findings that baseline CN activity in whole-blood samples from renal transplant patients on chronic CsA who did not experience recent graft rejection was numerically lower than in healthy individuals. We also found in these patients a progressive reduction of CN activity in whole blood associated with a parallel increase in CsA blood concentration after drug administration; this decrease in CN activity rapidly reversed as CsA concentrations fell. The temporal relationship between CN inhibition and changes in CsA concentration was, however, closer when the enzyme activity was measured in whole blood rather than in the PBMC fraction. This again may reflect the competitive effect of CsA-binding sites outside the leukocytes, which leads to a delay of CN inhibition in white blood cells. This is at variance with a previous observation in children with renal transplants in whom no lag between peak CN inhibition in peripheral blood leukocytes and CsA blood concentration was documented (31). Minimal data are also available in adult patients, but inconsistent results were achieved concerning the relative time of peaking of these two variables (17, 25). These discrepancies may reflect a different CsA metabolism in children vs adult patients (32), or in adults, interindividual variability in blood microenvironment that may affect the rapidity at which CsA reaches the leukocytes and thus inhibits CN enzyme activity.

Finally, we evaluated the possible relationship between CN activity in whole blood and CsA blood trough concentration, the currently used variable to monitor drug dosing in solid organ transplant recipients. The lack of a significant correlation between baseline CN activity and trough CsA concentration indicates that basal enzyme activity in whole blood in a given patient is not a function...
of CsA blood concentration. Recently it was shown that, in patients given Neoral, measurement of the CsA blood concentration 2 h post-drug dosing (C2) represented the best correlation of an individual time point with CsA-
AUC0–12 h (33–35) and had the potential to provide a more effective monitoring of CsA immunosuppression than the trough concentration (34, 36–38). Although we have confirmed here a correlation between the CsA blood concentration 2 h post-drug dosing, we failed to show any significant relationship between CN activity at baseline or at 2 h post-drug dosing and CsA C2 blood concentration. A poor correlation was also found between CN-AUA0–12 h and CsA-AUC0–12 h which represent the extent of the CN daily inhibition and the overall daily exposure to the drug, respectively. Thus, although the more recent pharmacokinetic results substantially improve the value of CsA blood measurements, our present findings tend to reduce the significance of current monitoring of CsA blood concentrations as a means to judge the immunosuppressive state of graft recipients with adequate confidence. Nevertheless, prospective studies are needed to formally document that measuring CN activity in transplant patients is a more relevant surrogate marker than pharmacokinetic drug monitoring to optimize immunosuppression and minimize the risk of rejection.

In conclusion, we have shown that (a) measuring CN activity in whole-blood samples is a reliable method that largely overcomes the variability of enzyme activity results in PBMC preparations; (b) in vitro incubation of whole blood or PBMCs from healthy individuals with increasing concentrations of CsA produced a comparable profile of inhibition of CN activity; and (c) in kidney transplant recipients, CsA trough concentrations did not predict baseline CN activity in whole blood, nor was a relationship found between CsA blood concentrations and enzyme activity at 2 h post-drug dosing; moreover, a single CN activity determination at baseline or 12 h post-CsA dosing is an useful surrogate for the daily inhibition of the enzyme by CsA. The CN inhibition test in its current form is, however, too complex for clinical use. Refinements made with the whole-blood approach represent an important step in this direction and will serve as a stepping stone in reaching the ultimate goal for the simplest, but effective, immunosuppressive monitoring.

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References


