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Pharmacodynamic monitoring of calcineurin inhibition therapy : investigation of the calcineurin activity marker

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Chapter 2

Spectrophotometric assay for calcineurin activity in leukocytes isolated from human blood

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Abstract

The calcineurin inhibitors, cyclosporine and tacrolimus, still constitute the cornerstone of immunosuppressive regimen after organ transplantation. An efficient and feasible way to measure calcineurin activity and inhibition by these drugs may improve therapeutic monitoring of these drugs in transplant recipients. Calcineurin activity was measured in leukocyte lysates isolated from human blood using spectrophotometric phosphate quantification. The dephosphorylation of a 19-amino acid peptide substrate of calcineurin was determined using the Malachite green phosphate reagent in the presence of okadaic acid and with and without the calcium chelator EGTA. Sample storage and lysis buffer components were among the variables optimized, and the inhibitory effect of calcineurin inhibitors was investigated. Observed loss of calcineurin activity during sample storage was eliminated by adding ascorbic acid to lysis buffer. The final inter- and intraassay variation coefficients were 10 and 4.5%, respectively, and the detection limit was $15 \text{ pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$, where WBC is white blood cells (leukocytes). In vitro IC_{50} values were 212 and 34 $\mu\text{g/L}$ for cyclosporine and tacrolimus, respectively. In vivo calcineurin inhibition was observed when calcineurin activity was measured in transplant recipients on maintenance therapy with cyclosporine and tacrolimus.

Introduction

A quarter-century ago, the launch of the fungal cyclic undecapeptide cyclosporin A (CsA) as a maintenance immunosuppressant revolutionized organ transplantation. This was followed by the introduction of the structurally unrelated but mechanistically similar drug tacrolimus (TRL) and a microemulsion formulation of CsA with a more predictable absorption profile. Both drugs are inhibitors of the calcium-dependent protein phosphatase calcineurin (CN) that ultimately regulates lymphocyte activation-associated cytokine production by dephosphorylation of the nuclear factor of activated T cells(1–3). Despite the marked improvement in acute rejection rates, unfavorable cardiovascular profile and nephrotoxic potential of CsA and TRL question the long-term applicability of these drugs(4). The balance between the prevention of acute rejection episodes and the chronic toxicity associated with high concentrations of these drugs is critical and is described as a narrow therapeutic index. Trough blood calcineurin inhibitor (CNI) concentration is routinely used for therapeutic drug monitoring of CNIs; however, correlation with clinical outcome is unsatisfactory, and cases of acute rejection and chronic nephrotoxicity remain(5). Many attempts are being made to improve this pharmacokinetic monitoring via the use of area under the curve (AUC) models(6) and samples taken 2h after drug administration(5,7). The use of a pharmacodynamic parameter to quantify the intracellular inhibitory effect of the CNIs has been proposed(8), and the first pharmacodynamic analyses of CNIs in transplantation patients have been published(9–13).

Most current techniques that attempt to measure CN phosphatase activity in clinical samples monitor the Ca^{2+} -dependent dephosphorylation of a phosphopeptide substrate derived from the RII subunit of protein kinase A(10–12,14–16). Many groups refer to the method published by Fruman and coworkers that involves an isotopic form of this peptide substrate and chromatographic separation and measurement of the ^{32}P -labeled phosphate product(17). Another technique, published by Enz and coworkers, uses HPLC to separate and quantify the phosphorylated substrate and dephosphorylated product(18).

We aimed to develop a more rapid and feasible CN assay that can be used to measure CN activity routinely in a clinical setting. As a result, we present a new protocol involving a nonradioactive spectrophotometric-based phosphate detection assay that has been optimized to enable monitoring of CN phosphatase activity and its inhibition by the immunosuppressants CsA and TRL in leukocytes isolated from human blood.

Materials and Methods

Materials

The following materials were used: dithiothreitol (DTT), ascorbic acid, Nonidet P40 (NP-40), 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); CsA microemulsion formulation from Novartis (Basel, Switzerland); and TRL from Fujisawa (Munich, Germany). The assay buffer, ethyleneglycoltetraacetic acid (EGTA) buffer, calmodulin, recombinant CN, okadaic acid, Malachite green reagent, and RII phosphopeptide substrate were obtained from the Biomol Green Cellular Calcineurin Assay Kit PLUS purchased from Biomol (Plymouth Meeting, PA, USA). All other chemicals and reagents were of the highest available grade.

Blood collection

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood from the antecubital veins of healthy volunteers was used for assay optimization and validation experiments. In addition, EDTA anticoagulated blood from the antecubital veins of patients on CsA and TRL maintenance therapy following kidney transplantation was used to study in vivo CN inhibition. Four patients (two males and two females) received CsA (100–125 mg twice a day [BID]) as

part of their immunosuppressive therapy, and four other patients (three males and one female) received TRL (2–4 mg BID) as part of their immunosuppressive therapy. The CsA-treated patients (ages 25–61 years) had undergone transplantation 3 months before, and the TRL-treated patients (ages 27–68 years) had undergone transplantation 6 months to 3 years before. Blood was taken immediately prior to drug intake (C0) and 2 h after drug intake (C2). From one patient on CsA (63-year-old male, 7 days after transplantation, 250 mg CsA BID) and one patient on TRL (47-year-old female, 7 days after transplantation, 6 mg TRL BID), blood was collected at multiple time points after drug intake.

Formal approval from the institutional ethics committee was obtained at the participating sites, and written informed consent was given before enrollment in the study.

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Preparation of total leukocyte lysates

Leukocyte lysates were prepared at 4°C from fresh blood and always within 24 h after sampling. To remove erythrocytes and platelets, 2.5 ml blood was added to 37.5 ml NH₄Cl lysis buffer (8.4 g/L NH₄Cl and 1.0 g/L KHCO₃, pH 7.3) and was incubated on ice for 10 min with inversion every 2 min. Intact leukocytes were spun down (350g, 10 min, 4°C), and supernatants containing erythrocyte and platelet lysates were discarded. Cell pellets were washed twice in 10 ml Hepes-buffered saline (9.0 g/L NaCl and 10 mM Hepes, pH 7.5), spun down (350g, 10 min, 4°C), and then resuspended in 1.5 ml Hepes-buffered saline. A 250-μl aliquot was taken for a leukocyte count performed on a Sysmax XE2100 (Mundelein, IL, USA), and 2 million leukocytes were aliquoted per microcentrifuge tube and were spun down (500g, 10 min, 4°C). Supernatants were removed, and leukocytes were resuspended in 200 μl lysis buffer (50 mM Tris-HCl [pH 7.7], 1.0 mM DTT, 5.0 mM ascorbic acid, 0.02% [v/v] NP-40, 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulfonyl fluoride, 5.0 mg/L leupeptin, and 5.0 mg/L aprotinin) and were lysed by three freeze-thaw cycles (liquid N₂/30°C). Cell debris was spun down (10000g, 10 min., 4°C), and supernatant was collected. Lysates were snap-frozen in liquid N₂ and stored at -80°C until they were assayed for CN activity.

CN activity assay

Two phosphate quantifications were performed per sample using a 96-well plate (half area, Costar, Corning): one in the presence of EGTA and the second in the presence of Ca²⁺ and calmodulin.

EGTA wells contained 40 μl of 12.5 mM EGTA, 0.375 mM RII phosphopeptide substrate, 75 mM Tris (pH 7.5), 150 mM NaCl, 9 mM MgCl₂, 0.75 mM DTT, 0.0375% NP-40, 0.625 μM okadaic acid, 0.125 mM CaCl₂, and 5.0 mM ascorbic acid. In the calmodulin/Ca²⁺ wells, EGTA was excluded and replaced by 0.313 μM calmodulin and 0.75 mM CaCl₂. To correct for background absorbance, blanks were performed for both the EGTA and calmodulin/Ca²⁺ wells for every sample. In these wells, substrate was absent. A phosphate series of 0 to 5 nmol was used for calibration, and one aliquot of 50 U (specific activity was determined using Biomol assay kit protocol) pure recombinant human CN was used as an interassay control. Reactions were initiated via the addition of 15 μl thawed leukocyte lysate sample to all wells, resulting in a final assay volume of 55 μl. After 30 min, reactions were stopped by adding 100 μl Malachite green reagent, the plate was mixed, and color was allowed to develop for 50 min at room temperature before absorbance was read at 620 nm using a 96-well plate spectrophotometer (SpectraMax 250,

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Molecular Devices, Downingtown, PA, USA). The Softmax Pro (version 2.4.1) program was used for curve fitting (four-parameter fit).

For CN activity calculation, background absorbance for EGTA and Ca^{2+} /calmodulin wells was subtracted and phosphate was quantified with the calibration curve. The difference in phosphate between EGTA and Ca^{2+} /calmodulin wells (expressed as $\text{pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$, where WBC is white blood cells [leukocytes]) was defined as CN activity.

In vitro CN inhibition

CsA and TRL were diluted to appropriate concentrations using phosphate-buffered saline (154 mM NaCl and 1.4 mM phosphate, pH 7.5). Then 2.5 μl diluted inhibitor stock solution was added to 2.5 ml fresh blood to yield final concentrations of 0 to 5000 $\mu\text{g/L}$ CsA and 0 to 150 $\mu\text{g/L}$ TRL. These samples were incubated for 1 h at 37°C before leukocyte lysate preparation.

Concentrations of CsA and TRL in peripheral blood

CsA concentration was measured in whole blood by fluorescence polarization immunoassay using an Abbott AxSYM system (Abbott Park, IL, USA). TRL concentration was measured in whole blood by microparticle enzyme immunoassay using an Abbott IMx system. The assays were performed according to the manufacturer's instructions.

Statistical analysis

Inhibition curves and IC_{50} values were fitted and analyzed using sigmoidal dose–response curves with GraphPad Prism (GraphPad Software, San Diego, CA, USA) and are described within the 95% confidence interval (CI) range. Other results are reported as mean values, with errors representing 1 standard deviation of the mean. In addition, t tests were used to test significance, and statistical significance was defined as $P < 0.05$.

Results

Optimization of assay conditions

Progress curves were performed to ensure that the initial rate of CN phosphatase activity was measured and to enable optimization of assay duration and cell lysate concentration. During a 30 min reaction using a lysate of $1.5 \cdot 10^5$ WBC, activity rate

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was observed to remain linear and within the range of the phosphate standard curve. These conditions were used in all subsequent experiments (not shown).

Maintaining CN activity during storage

A loss of CN activity was observed repeatedly when leukocyte lysate samples were stored at -80°C prior to assay performance. Ascorbic acid subsequently was added to our lysis buffer in an attempt to maintain CN in its reduced and active state. Samples were lysed and stored in lysis buffer with or without 5.0 mM ascorbic acid and then were assayed for CN activity after 0, 1, 2, 7, and 21 days storage at -80°C (Figure 2.1).

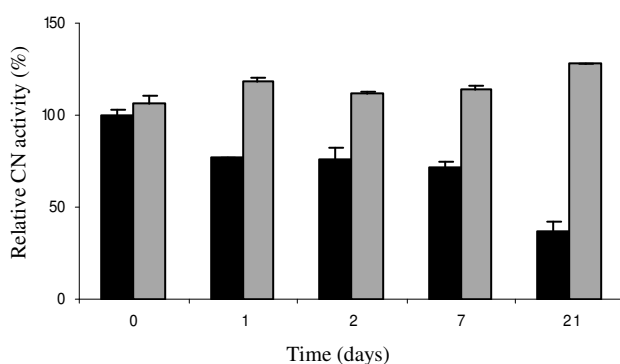


Figure 2.1. CN activity after storage of cell lysate at -80°C . Black bars: without ascorbic acid in the lysis buffer; gray bars: with ascorbic acid (5.0 mM) in the lysis buffer. Columns represent the means of duplicates, and error bars show +1 SD.

The loss of CN activity observed in time was eliminated with the addition of ascorbic acid to the lysis buffer. In this way, the CN activity measured in our samples could be maintained during storage at -80°C for at least 21 days. Subsequent assays always were performed within three weeks of storage.

When lysate samples were added to the reaction mixture during the CN activity assay, the additional ascorbic acid in the lysis buffer was diluted from 5.0 mM to 1.4 mM. To compensate for this, extra ascorbic acid was added directly to the reaction mixture, leading to a 60% increase in the CN activity level. Further observations included nearly total inactivation of CN at ascorbic acid concentrations above 10.0 mM. Ascorbic acid subsequently was added to a final concentration of 5.0 mM in the assay.

A time-dependent loss of CN activity was also observed when whole donor blood was stored at 4°C for a period of 72 h. Aliquots of the stored blood were taken, prepared, and assayed for CN activity after 0 (fresh), 24, 48, and 72 h of storage.

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During the 72-h period, a loss of 47% CN activity was observed despite the presence of ascorbic acid in the lysis buffer. Subsequently, all blood was prepared as soon as possible after sampling and always within 24 h, when activity loss was less than 15%.

Components of the lysis buffer

In addition to ascorbic acid, various other components of the lysis buffer were observed to have a significant effect on CN activity levels. Although many groups add the cation chelators EDTA and EGTA to their lysis buffers to inhibit cation-dependent protease activity, the Ca^{2+} -dependent activity of CN is also affected. The effect of 0.1 mM EDTA or 0.1 mM EGTA in the lysis buffer on CN activity was tested, and it was shown that CN activity was decreased to 21 ± 9 and $52 \pm 12\%$, respectively ($n = 4$), indicating that these cation chelators cause a decrease in activity and must be removed.

The nonionic detergent NP-40 was present in assay buffer and was also observed to increase CN activity when added to our lysis buffer. An optimal increase in CN activity was observed between concentrations of 0.01 and 0.025% (v/v) NP-40 in the lysis buffer. In comparison with the sample lysis without NP-40, increases of 237 ± 30 and $257 \pm 23\%$ in CN activity were achieved by adding 0.01 and 0.025% (v/v) NP-40, respectively ($n = 2$). For subsequent experiments, we chose to add 0.02% NP-40 to our lysis buffer, resulting in an assay NP-40 concentration of 0.033%.

Standard curve

A typical phosphate standard curve is shown in Figure 2.2A. Softmax Pro software was used for curve fitting. To check linearity of our assay, leukocyte lysate was serially diluted and resulted in a linear relation between dilution and CN activity (Figure 2.2B).

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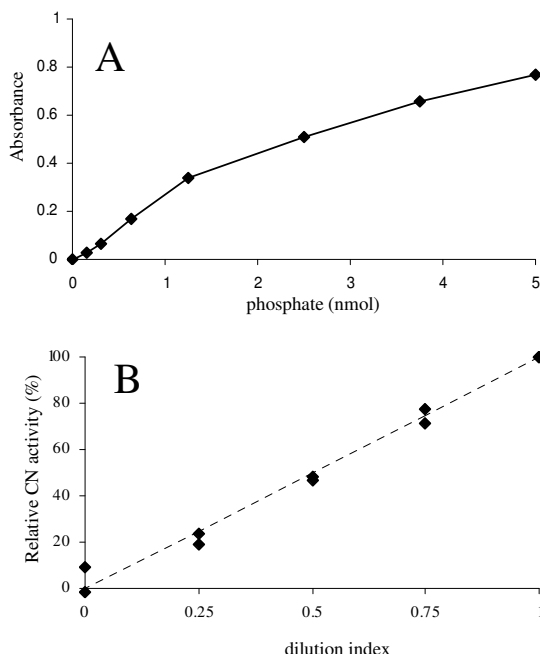


Figure 2.2. (A) Typical phosphate standard curve. (B) Dilution series of sample. The CN activity is calculated with the phosphate standard curve. Diamonds represent sample dilutions, and the dotted line indicates the expected dilution line. This experiment was performed in duplicate.

CN assay validation

To identify the interassay variation, five blood samples of one volunteer taken within a 2-month period were prepared separately and analyzed on different days (including intraindividual variation). This resulted in a coefficient of variation (CV) of 10%. One leukocyte lysate sample was also assayed repeatedly within one experiment at both high and low concentrations to provide intraassay CVs of 4.5 and 2.2%, respectively ($n = 9$).

The detection limit was $15 \text{ pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$, calculated as the mean of the activity measured for sample blanks plus 3 standard deviations ($n = 9$). The lower limit of quantification, calculated as the mean of blanks plus 6 standard deviations, was $31 \text{ pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$.

Analytical recovery was determined by adding recombinant CN to leukocyte solution after erythrocyte lysis, resulting in a recovery of $101 \pm 13\%$ ($n = 3$).

In vitro inhibition of CN activity

The inhibition of CN by CsA and TRL was studied in vitro by adding different concentrations of these immunosuppressive drugs to blood obtained from healthy

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volunteers. The inhibition curves are shown in Figure 2.3. IC_{50} values were calculated to be 212 $\mu\text{g/L}$ CsA (95% CI, 67–668 $\mu\text{g/L}$) and 34 $\mu\text{g/L}$ TRL (95% CI, 13–88 $\mu\text{g/L}$).

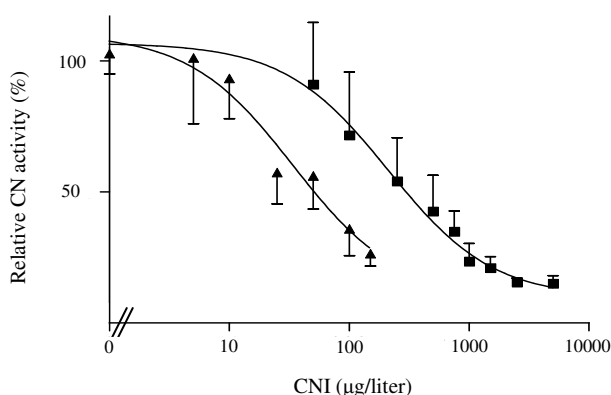


Figure 2.3. In vitro inhibition of CN by CsA (squares) and TRL (triangles). The means $\pm$ 1 SD of four healthy volunteers are displayed. Curves were fitted and analyzed using GraphPad Prism.

In vivo inhibition of CN activity

The in vivo activity and inhibition of CN was also studied using blood samples obtained from kidney transplant patients treated with CN inhibitors CsA and TRL. The CN activities obtained from patients, both prior to (C0) and 2 h after (C2) oral administration of CsA and TRL, are displayed in Figure 2.4.

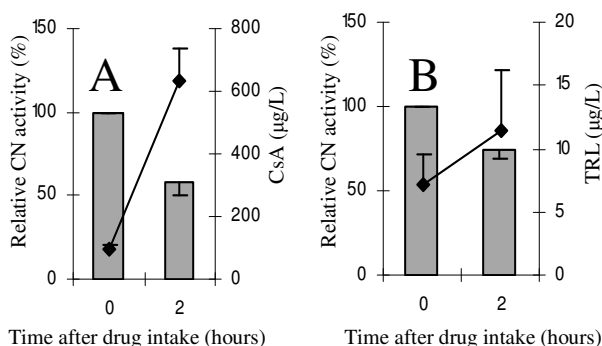


Figure 2.4. In vivo CN inhibition in patients who received CsA and TRL therapy. Bars represent relative CN activity, and diamonds represent full blood drug concentration. (A) C0 and C2 CsA concentrations and relative CN activities of four different patients on CsA therapy. (B) C0 and C2 TRL concentrations and relative CN activities of four different patients on TRL therapy.

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Blood CsA concentration increased from a mean of $94 \pm 13 \mu\text{g/L}$ at C0 to $636 \pm 105 \mu\text{g/L}$ at C2 and resulted in a $42 \pm 8\%$ inhibition of baseline CN activity at C2. Blood TRL concentration increased from a mean of $7.1 \pm 2.4 \mu\text{g/L}$ at C0 to $11.5 \pm 4.8 \mu\text{g/L}$ at C2 and resulted in a $26 \pm 5\%$ inhibition of baseline CN activity at C2.

In addition, *t* tests were performed to identify differences in drug concentrations and CN activities between C0 and C2. For patients treated with CsA as well as patients treated with TRL, significantly higher CN activities were observed at C0 compared than at C2 (*Ps* = 0.004 and 0.01, respectively), whereas significantly lower drug concentrations were observed at C0 than at C2 (*Ps* = 0.0015 and 0.04, respectively). For all patients, higher drug concentrations were observed at C2 than at C0, and inhibition of CN was observed at C2.

To illustrate the potential of this assay for CN monitoring in CNI-treated patients, two CN inhibition curves of patients who had undergone kidney transplantation are shown in Figure 2.5. In these patients, inverse relations between CN activity and drug concentrations are observed.

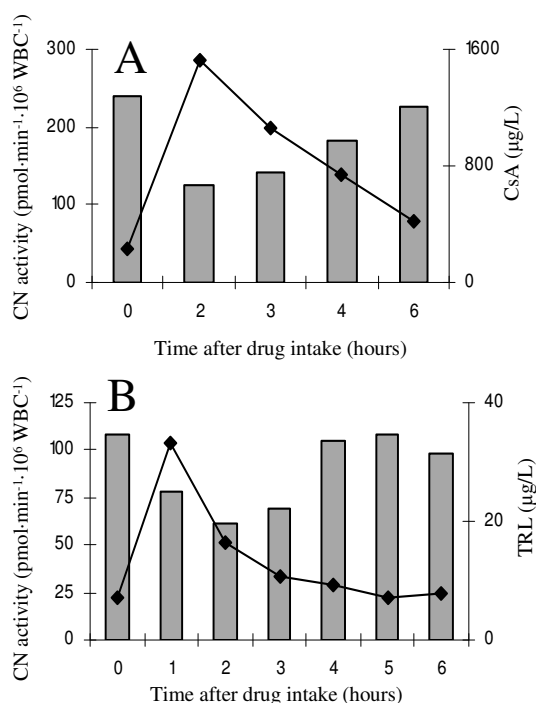


Figure 2.5. In vivo CN monitoring. Bars represent CN activity, and diamonds represent full blood drug concentration. (A) Example of CN inhibition in time by CsA (63-year-old male, 7 days after transplantation, 250 mg BID CsA). (B) Example of CN inhibition in time by TRL (47-year-old female, 7 days after transplantation, 6 mg BID TRL).

Discussion

The main reason for developing the spectrophotometric CN activity assay was to enable assessment of pharmacodynamic CN monitoring in patients treated with the CNIs CsA and TRL. The spectrophotometric detection principle allows us to avoid the use of radioactive materials and organic solvents. A linear increase of phosphate in time was observed for at least 40 min, indicating that substrate is in excess, and we decided to use a 30 min running time.

The concentration of okadaic acid in the final reaction mixture was 0.45 μM , inhibiting protein phosphatase 1 (PP1) and PP2A, which have okadaic acid IC_{50} values lower than 10 nM. Meanwhile, the IC_{50} value of CN exceeds 10 mM(19), resulting in selective inhibition of these other protein phosphatase enzymes. The CN activity was distinguished from okadaic acid-resistant, calcium-independent PP2C activity by subtracting the phosphatase activity measured in the presence of the calcium chelator EGTA from that measured in excess calcium, resulting in calcium-dependent phosphatase activity. PP2C activity is the calcium- independent phosphatase activity measured in the presence of okadaic acid and was found to be less than 5% of the CN activity in healthy volunteers.

Optimization studies involved modifications to the lysis buffer. Because these resulted in significantly higher activity levels and subsequently in lower detection limits, we reduced the requirement for blood and increased lysate storage times. To preserve in vivo conditions, and in particular the state of CN inhibition within the patient, as much as possible, we chose to maintain samples at 4 °C throughout sample preparation. However, the separation of peripheral blood mononuclear cells (PBMCs) using a Ficoll–Hypaque gradient, a technique used by many groups, should be performed at room temperature to maintain the density of the Ficoll solution. Due to this temperature limitation and to the variable amounts of erythrocytes, platelets, and granulocytes observed to remain after Ficoll separation(10), we decided to simply remove erythrocytes and platelets at 4 °C using ammonium chloride lysis buffer and to measure CN activity in total leukocyte lysates that contain PBMCs and granulocyte fraction. This technique requires a lower volume of blood, and separation is less cumbersome and more reproducible than it is with the Ficoll gradient procedure. Support for using material additional to PBMCs comes from work by other groups, such as Halloran and coworkers(20), who have shown that CN inhibition patterns in whole blood and PBMCs are in accord.

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The observed loss of CN activity on sample storage(10,14) was hypothesized to be due to inactivation of CN via oxidation of the binuclear $\text{Fe}^{2+}\text{-Zn}^{2+}$ center found at the CN active site(21,22). This same mechanism was proposed to cause a 10- to 20-fold loss of activity during the purification of CN from human tissue(21,23). Various antioxidants, including ascorbic acid, were found to have an activating effect on CN(23). When ascorbic acid was added to our lysis buffer, the level of CN activity could be maintained during the storage of lysate samples at -80°C for a minimum of 21 days (Figure 2.1). Activity levels were further increased when ascorbic acid was added directly to the reaction mixture and an optimal activity was achieved in the presence of 5.0 mM ascorbic acid, in agreement with data for purified CN published by Sommer and coworkers(23). The effect of the reducing agent DTT on enzyme activity and storage was also investigated, but no significant effect was observed.

The cation chelators EDTA and EGTA originally were included in the lysis buffer of Fruman and coworkers to inhibit cation-dependent proteases(17). That study stated that these subsequently were omitted (in some experiments) because they were found to have no protective effect on cellular phosphatase activity. Considering that the removal of Ca^{2+} is known to inactivate CN and that EGTA is used in this way to define Ca^{2+} -dependent phosphatase activity in the assay, it does not seem sensible to add cation chelators to the reaction mixture.

The nonionic detergent NP-40 was added to the lysis buffer at the concentration at which we observed maximum CN activity. In one study, NP-40 was used to extract CN from the T lymphocyte membrane with which it is reported to be associated(24). The release of membrane-associated CN during lysis would explain the rise in CN activity observed. However, at NP-40 concentrations higher than 0.025% (v/v), the level of CN activity gradually decreases, probably due to partial denaturation.

The phosphate standard curve used for this assay is not linear and becomes insensitive at higher phosphate concentrations. Therefore, we recommend that samples with an absorbance higher than 0.75 be diluted to ensure assay performance. Assay linearity was observed in sample dilution and standard addition experiments. In vitro CN inhibition experiments resulted in CsA and TRL IC_{50} values of 212 and 34 $\mu\text{g/L}$, respectively. These values are similar to those described previously(10,11) and agree with reports that TRL is more potent than CsA(2).

CN activity and inhibition was also measured in samples obtained from four kidney transplantation patients on CsA therapy and four kidney transplantation patients on TRL therapy. In both CsA and TRL patients, CN inhibition is observed given that

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significantly higher drug concentrations at C2 resulted in significantly lower CN activities when compared with C0. Interestingly, no total inhibition of CN activity was observed for any patient, a phenomenon that was observed previously by others(25). The data presented show that in vivo binding of CNIs to their immunophilins and calcineurine is reflected in the CN assay. In vivo measurement of CN activity in patients who undergo immunosuppressive therapy by CNIs has the potential to guide drug dosing and improve clinical outcome, although the benefits of pharmacodynamic monitoring have not yet been proven. The ability of this assay for pharmacodynamic monitoring of CN inhibition therapy is illustrated in Figure 2.5, where CN activity is measured at multiple time points after drug intake. In conclusion, the protocol presented requires 2.5 ml fresh blood and standard laboratory equipment. Multiple samples can be prepared and assayed for CN phosphatase activity within 1 working day with high intra- and interassay reproducibility. The spectrophotometric assay is flexible and can be used to measure CN activity in lysates of leukocytes, PBMCs, and combinations of other blood cell subsets as well as in other cell types. The assay can be used to monitor CN activity in patients that undergo CN inhibition therapy and to assess the role of pharmacodynamic CN monitoring in clinical practice.

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