Chapter 4

Variation in Leukocyte Subset Concentrations Affects Calcineurin Activity Measurement: Implications for Pharmacodynamic Monitoring Strategies

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Abstract

Background: In renal transplantation patients, therapeutic drug monitoring of the calcineurin (CN) inhibitor cyclosporin A (CsA) is mandatory because of the drug’s narrow therapeutic index. Pharmacodynamic monitoring of CN inhibition therapy could provide a tool to define and maintain the therapeutic efficacy of CsA therapy. We investigated the effect of variation in cell counts of leukocyte subsets on leukocyte CN activity measurement in renal transplant recipients.

Methods: We measured leukocyte CN activity, whole blood CsA concentrations, and leukocyte subset cell counts in 25 renal transplant recipients. Blood was collected before graft implantation and CsA therapy, 1 day before transplantation when CsA therapy was already started, and 5 days after transplantation. Monocyte, granulocyte, CD4+ T cell, CD8+ T cell, B-cell, and natural killer cell CN activities and CsA inhibition sensitivities were determined in vitro by a spectrophotometric CN assay.

Results: Leukocyte CN activity was inhibited after drug intake. Inter- and intrapatient variation in leukocyte subset cell counts resulted in variation of sample composition. The mean (SD) CN activity varied among leukocyte cell subsets, ranging from 650 (230) to 166 (26) pmol/min./10^6 cells for monocytes and CD4+ T cells, respectively. CsA half maximal inhibitory concentration (IC_{50}) values ranged from 15 to 78 μg/L for monocytes and B cells, respectively.

Conclusion: Inter- and intraindividual leukocyte subset cell count variation can affect measured CN activity independent of CsA concentration. Cell-specific activity and drug sensitivity should be considered for sample validation to optimize method specificity when pharmacodynamic monitoring strategies are applied in a clinical setting.
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Introduction

Immunosuppression by calcineurin (CN) inhibition therapy is essential for prevention of graft rejection early after renal transplantation. The 2 CN inhibitors (CNI) used in transplantation medicine, cyclosporin A (CsA) and tacrolimus, inhibit CN after formation of a complex with their corresponding binding proteins, and result in suppression of lymphocyte activation(1,2). Unfortunately, side effects, such as nephropathy, neuropathy, diabetogenesis, malignancies, and cardiovascular disease occur in CNI-treated patients(3–7). Because of the unpredictable kinetic profiles of CNIs and potential drug-drug interactions, patients receiving CNI therapy require intensive drug monitoring(8). Pharmacokinetic monitoring is performed by measuring blood drug concentrations at different time points after drug intake(9–11). However, more accurate drug monitoring strategies are desired to maximize efficacy of these drugs(12,13).

Pharmacodynamic monitoring of CNI therapy is a new approach for the optimization of CNI monitoring(8), and several methods are available for measurement of CN enzyme activity as a pharmacodynamic marker. All methods use the same substrate, a 19-amino acid peptide (RII-peptide), which corresponds to a part of the regulatory subunit of bovine cAMP dependent protein kinase(14), but methods differ in the detection techniques used, such as HPLC-ultraviolet measurement of dephosphorylated peptide(15,16), radioactive measurement of $^{32}$P-labeled phosphate(1,17,18), and absorbance measurement of malachite green phosphate reagent(19). In addition, various blood fractions, such as whole blood, peripheral blood mononuclear cells (PBMCs), and leukocytes are used as the specimen. Although clear relationships between drug concentration and CN activity have been observed, no clear relationship between clinical outcome and CN activity has been reported(17,20,21). We investigated whether variations in leukocyte subset counts would directly affect CN activity measurement independent of CNI concentration and potentially confound the use of CN activity as a predictive marker. To investigate the effect of sample composition variation on CN activity measured in renal transplantation patients, we determined cell-specific CN activities and CsA inhibition sensitivities for granulocytes, monocytes, CD4+ T cells, CD8+ T cells, B cells, and natural killer (NK) cells.
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Materials and Methods

Patients and Samples
Twenty-five patients who underwent living-donor renal transplantation at the Leiden University Medical Center were included in this study. Formal approval from the institutional ethics committee was obtained, and informed consent for blood sampling was obtained from all participants, both patients and healthy volunteers. The patient group consisted of 17 male and 8 female recipients with a mean (SD) age of 49 (13) years at the time of transplantation. All patients received quadruple immunosuppressive therapy with basiliximab, prednisolone, mycophenolate mofetil, and CsA (4 mg/kg twice a day), with the latter therapy started 3 days before transplantation. EDTA-anticoagulated blood samples from the antecubital veins were collected at 3 different time points; 5 days before transplantation (day -5), when no immunosuppressive therapy had begun; 1 day before transplantation (day -1), after initiation of oral CsA therapy but without administration of the other immunosuppressive drugs; and 5 days after transplantation (day 5), when full immunosuppressive therapy was given. On the day before transplantation (day -1), samples were obtained immediately before drug intake (12 hours after previous dose), and 2 and 3 hours after drug intake. On day 5 after transplantation samples were obtained immediately before and 2, 3, 4, and 6 hours after drug intake.

Blood leukocyte and lymphocyte subset measurement
Hematologic profiling was performed on a Sysmex XE2100. Lymphocyte subsets were analyzed by flow cytometry. The lymphocytes gated in the side-scatter/CD45 dot-plot were analyzed for CD3+/CD4+, CD3+/CD8+, CD3+/CD19+ and CD3+/CD16/56+ populations using fluorescein isothiocyanate (FITC)-labeled CD4 (clone SK3), phycoerythrin (PE)-labeled CD8 (clone SK1), APC-labeled CD3 (clone SK7) or APC-labeled CD19 (clone SJ25Cl), FITC-labeled CD3, and PE-labeled CD16 and PE-labeled CD56 panels on a FACSCalibur (all from BD Biosciences) equipped with CellQuest.

Sample preparation for calcineurin activity measurement
EDTA-anticoagulated blood (2 mL) was treated with erythrocyte lysis buffer (8.4 g/L NH₄Cl, 1.0 g/L KHCO₃, pH 7.3). The leukocytes were then washed twice with 10 mL Hepes buffered saline (HBS, 9.0 g/L NaCl, 10 mmol/L Hepes pH 7.5) before resuspension in 1.5 mL HBS for cell count on the Sysmex XE2100. Leukocyte cell lysis was performed for 2·10⁶ cells that 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% (vol/vol) 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 lysed by 3 freeze-thaw cycles (liquid N₂, 30°C). Cell debris was separated by centrifugation (10 min, 10000g, 4°C), and supernatants were snap-frozen in liquid N₂ and stored at -80°C before calcineurin activity measurement.

Isolation of leukocyte and lymphocyte subsets for absolute CN activity measurement

Granulocyte and PBMC fractions from healthy volunteers were separated by Ficoll density gradient centrifugation. The PBMC fraction was washed with HBS and resuspended in 0.1% BSA in HBS at a final concentration of 15·10⁶ to 20·10⁶ cells/mL. In the granulocyte fraction erythrocytes and thrombocytes were removed by erythrocyte lysis with NH₄Cl lysis buffer, washed once with HBS, and resuspended in HBS. Purity of granulocyte and PBMC fractions was analyzed and found to be at least 95% for the PBMC fraction and 90% for the granulocyte fraction. Cell fractions were divided into aliquots of 2·10⁶ cells and resuspended in 165 μL lysis buffer for cell disruption.

CD3+/CD4+ (CD4+ T cells), CD3+/CD8+ (CD8+ T cells), CD3+/CD19+ (B cells), CD3+/CD16/56+ (NK cells), and monocyte fractions were sorted from the PBMC fraction using an ARIA sorter (BD Biosciences). The PBMC fraction was incubated for 30 min with FITC-labeled CD4, PE-labeled CD8, and APC-labeled CD3 or APC-labeled CD19, FITC-labeled CD3, and PE-labeled CD16/56 before cell sorting. Isolated fractions were collected in 5-mL BD FACS tubes (BD Biosciences) containing 500 μL 50% heat inactivated fetal calf serum in Roswell Park Memorial Institute medium. The purity of sorted fractions was analyzed and found to be at least 95% for every fraction. Truecount tubes (BD Biosciences) were used for controlling cell counts. Sorted cells were spun down (10 min, 500g, 4°C), washed once with 250 μL HBS, and resuspended in 165 μL lysis buffer for cell disruption.

Leukocyte and lymphocyte subset isolation for CsA inhibition curves

Granulocyte and PBMC fractions were isolated from buffycoats (Sanquin) using Ficoll density gradient separation. PBMC subsets were isolated using CD4, CD8, B cell, NK cell, and monocyte-negative isolation kits (Invitrogen). We incubated 1.5·10⁶ to 2·10⁶ cells/mL in Roswell Park Memorial Institute medium containing 20% inactivated fetal calf serum and 1% ethanol for 1 hour at 37°C, with final CsA concentrations of 0, 0.1, 0.32, 1, 3.2, 10, 32, 100, 320, 1000, 3200, and 10000 μg/L. Cells were washed once with HBS before cell lysis.
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Calcineurin activity assay
Calcineurin activity was determined as previously described(19). 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) and to 40 μL of assay buffer without substrate to correct for background absorbance. The enzyme reaction was performed at 30°C and terminated after 30 min by adding 100 μL of malachite green reagent, and then the color was allowed to develop for 50 min at ambient temperature. Absorbance was read at 620 nm using a 96-well plate spectrophotometer (SpectraMax 250, Molecular Devices), and phosphate was quantified using a 0–5 nmol phosphate calibration curve using SoftmaxPro (Version 2.4.1) software.

CsA blood concentration
CsA blood concentrations were quantified using a fluorescence polarization immunoassay on the Abbott AxSYM system according to the manufacturer’s instructions.

Data analysis
Means and SD are used in the text of this manuscript. Data presented in the Figs. Are shown as either mean, mean (SE), or box plot. Paired 2-tailed Student t-tests were performed to test for statistical significance, which was defined as P < 0.05. Inhibition curves and IC₅₀ (half maximal inhibitory concentration) values were fitted and analyzed using sigmoidal dose–response curves with GraphPad software.

Results
Leukocyte CN activities and blood CsA concentrations
The observed leukocyte CN activities and blood CsA concentrations for 25 renal transplant recipients, measured at the 3 different time points, are shown in Figure 4.1. CN activities measured in the patients before CsA therapy ranged from 168 to 385 pmol/min./10⁶ leukocytes. The range of CN activities after oral treatment with CsA was 48 to 464 pmol/min./10⁶ leukocytes, and blood CsA concentrations ranged from 90 to 2016 μg/L.
Figure 4.1. Leukocyte CN activity before and shortly after renal transplantation in patients on CsA therapy. Box plots of CsA blood concentration (A) and leukocyte CN activities (B) at 3 different time points: day -5, before CsA treatment and graft implantation; day -1, before graft implantation at initiation of CsA treatment; and day 5 after transplantation. T0, T2, T3, T4, T5 and T6 represent the time (hours) after drug intake.
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An inverse relationship between CsA drug concentration and CN activity was observed on day -1 and day 5. On day -1 and day 5, maximal CN inhibition values of 42% and 31%, respectively, were observed 2 hours after drug intake compared to T0 CN activities, with mean (SD) T0 CsA concentrations of 195 (86) and 245 (100) µg/L and T2 concentrations of 1070 (399) and 1037 (312) µg/L, respectively. Compared to pretherapy CN activity, a maximum inhibition of 52% and 38% on day -1 and 5, respectively, was observed 2 hours after drug intake. CN activity 6 hours after drug intake was not significantly different from T0 CN activity on day 5 (P = 0.12), whereas at 2 hours (P < 0.001), 3 hours (P < 0.001), and 4 hours (P = 0.001) after drug intake significantly lower CN activities were found compared to T0. When average T0 CN activities were compared to predrug (day -5) CN activities, 15% and 13% lower CN activities were observed on day -1 and day 5 respectively, though only day -1T0 (P = 0.03) was found to be significantly lower (P = 0.08 for day 5 T0).

Peripheral blood leukocyte and lymphocyte subset concentrations
Leukocyte and lymphocyte subset cell counts were monitored in parallel with CN activity. The mean peripheral blood concentrations for leukocyte subsets at all sampling points are shown in Figure 4.2A and 4.2B.

When the leukocyte subset cell counts before graft implantation (day -5 and day -1 T0) were compared to subset counts after implantation (day 5 T0), an increase of total leukocytes (P <0.001), granulocytes (P <0.001), monocytes (P <0.001), and B cells (P = 0.009) was observed. On day -1 no significant changes of leukocyte subsets were observed, whereas on day 5 a significant decrease in PBMC cell counts (starting 4 hours after drug intake, P = 0.012), B cells (starting 3 hours after drug intake, P = 0.040), CD4+ T cells (starting 3 hours after drug intake, P = 0.011), and CD8+ T cells (starting 4 hours after drug intake, P = 0.026) were found. All of these changes in peripheral leukocyte subset cell counts affect leukocyte sample composition. Figure 4.2C and 4.2D show the relative fractions of leukocyte subsets that made up a leukocyte and PBMC sample, which is used as sample in other CN activity methods.
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Figure 4.2. Peripheral blood cell counts of leukocyte subsets in renal transplantation patients and the effect on sample composition. Granulocyte and PBMC cell counts in peripheral blood (A) and PBMC subset cell counts in peripheral blood (B). Data points represent mean cell counts of all patients, and error bars represent SE. (C,D), box plots of subset fractions within leukocyte and PBMC sample, respectively. All sampling time points for all patients are included. Sample compositions were calculated using full blood differentiates.

CN activities of different leukocyte and lymphocyte subsets
To investigate whether the changing sample composition within a patient, and the variation of sample composition between patients, affects CN activity measurement, we determined the cell-specific CN activity for the leukocyte subsets. Cell-specific CN activities for 4 healthy volunteers are shown in Figure 4.3.

The PBMC fraction seemed to have higher CN activity than the granulocyte fraction. The monocytes subset had the highest CN activity in at least 3 healthy volunteers. Within the lymphocyte subsets variation in CN was also observed in these volunteers. The NK-cell fraction seemed to have the highest CN activity, and CD8+ T cells had slightly higher CN activity than CD4+ T cells, which had the lowest cell specific CN activity in these individuals.
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Figure 4.3. Cell-specific CN activity. Cell-specific CN activity determined for 4 healthy volunteers and expressed per cell. Every bar style represents one individual and columns represent the means of duplicates.

CsA inhibition curves for leukocyte subsets

We determined cell-specific CsA inhibition sensitivities to investigate whether the changing sample composition affects the CN inhibition profile of leukocytes. CsA inhibition curves obtained for the different leukocyte subsets are shown in Figure 4.4. Table 4.1 lists CsA IC₅₀ concentrations, which were obtained from the inhibition curves in Figure 4.4 for the leukocyte subsets.

Discussion

Investigation of the usefulness of CN activity measurement as a pharmacodynamic marker for CNI therapy requires a clear relationship with therapy outcome. Most studies have focused on the relationship between pharmacokinetics and pharmacodynamics(22–24), or evaluation of CN inhibition profiles by CsA and tacrolimus at different time points after transplantation(25–29). To our knowledge only one study, by Fukudo et al., has provided evidence that CN activity is related to clinical responses in organ transplantation patients(17), and one other study by Sanquer et al. showed that CN activity might be a predictive marker for graft-vs-host-disease in allogeneic stem-cell transplantation(30). Both groups used PBMC samples that were isolated from peripheral blood by Ficoll density gradient.
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Figure 4.4. Cell specific inhibition of CN by CsA. CsA inhibition curves for different leukocyte subsets fitted with sigmoidal dose–response curves using GraphPad software. CN activity is expressed as percentage of CN activity without CsA. Means of duplicates are displayed.

<table>
<thead>
<tr>
<th>Leukocyte subset</th>
<th>IC\textsubscript{50} CsA (μg/L)</th>
<th>95% CI (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>21</td>
<td>15 - 31</td>
</tr>
<tr>
<td>Monocytes</td>
<td>15</td>
<td>11 - 21</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>36</td>
<td>18 - 71</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>25</td>
<td>18 - 36</td>
</tr>
<tr>
<td>NK cells</td>
<td>43</td>
<td>29 - 62</td>
</tr>
<tr>
<td>B cells</td>
<td>78</td>
<td>43 - 142</td>
</tr>
</tbody>
</table>

Table 4.1: CsA IC\textsubscript{50} values for different leukocyte subsets. CsA IC\textsubscript{50} values and CI were obtained from the inhibition curves in Figure 4.4 analyzed by GraphPad software.

We monitored CN activity in the leukocyte fraction because its preparation requires less blood and is less cumbersome than PBMC isolation. In a previous study we validated our analytical method(19), and as a follow-up we investigated and validated our sample choice by monitoring inter- and intrapatient compositional variation of the leukocyte fraction and determination of cell-specific activity and CsA-inhibition sensitivity.

Parallel to leukocyte CN activity, peripheral leukocyte subset cell counts were monitored in 25 renal transplantation patients (Figure 4.2), and large interpatient variation was observed. Significant intrapatient changes were also observed before
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and after graft implantation, and in time after drug intake when monitored after implantation. The granulocytosis, monocytosis, and lymphopenia observed after transplantation are caused by surgical trauma and steroid therapy. These changes in peripheral blood leukocyte subset concentrations caused time dependent shifts in sample composition. To investigate the effect of these changes and interindividual variations in leukocyte sample composition on the leukocyte CN activity measured, cell-specific CN activities and CsA inhibition sensitivities were determined. On average, monocytes have the highest CN activity per cell, as has been reported previously(31). Within the lymphocyte fraction, differences in CN activity were observed, with the highest CN activity observed in NK cells and the lowest CN activity in the T cell subsets for our 4 volunteers. CsA inhibition sensitivity profiles for the different leukocyte populations were similar and comparable with cell IC$_{50}$ values reported by Kung et al.(32), a finding that indicates that measurement of leukocyte CN inhibition by CsA is not affected by variation in sample composition. Taken together, these results show that variation in sample composition affects CN activity measured in patient samples, whereas CsA inhibition is not affected. Variation in monocyte cell count could have a particularly influential effect on CN measurement because monocytes have the highest cell-specific CN activity. When linear regression was performed between day -5 (pre CsA) CN activities measured in the renal transplantation patients and the percentage of cell subsets present in the sample, no clear relationship (max R$_2$ = 0.15, for monocyte fraction) was found, and therefore no effect of sample composition on leukocyte CN activity was observed. Variation in monocytes could have a greater effect on PBMC CN activities because monocytes account for a larger proportion of the PBMC sample, and the inter- and intrapatient variation in PBMC fraction composition is much larger than that of the leukocyte fraction (see Figure 4.2D). In PBMC samples, monocytes were found to constitute 20% to 80% of the sample, and this large variation, based on the larger cell-specific CN activity, can significantly influence CN measurement. Halloran et al. compared the PBMC fraction with the leukocyte fraction in healthy volunteers and found similar CsA inhibition affinities, but larger and more variable inhibition in the PBMC fraction(26). When leukocytes are used as sample, the majority of the cells [mean (SD) 75% (10%)] are granulocytes, and sample composition changes are relatively small. A disadvantage is that a large proportion of the CN activity used for monitoring CNI pharmacodynamics is from the granulocyte fraction. Ideally, one would like to selectively monitor in the compartment where the drug exerts its effect(21). The effector compartment of CsA therapy is thought to be the lymphocyte or, more specifically, the T cell populations, and monitoring of these fractions could
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theoretically enable avoidance of the influence of changing sample composition on CN activity. Absolute CN activities would then be more characteristic of immunosuppressive status, and observed CN inhibition after drug intake would reflect drug pharmacodynamics within the effector compartment and could therefore be more relevant. In practice the procedure for routinely sorting and preparing these cell fractions for CN activity analyses is very complicated and time consuming. In addition, when T cell depletion by anti-thymocyte globulin is used in, for instance, treatment of acute rejection episodes, collection of these cells from the peripheral blood is very difficult because they are therapeutically removed. The CN activities observed in 25 renal transplantation patients were inversely related to whole blood CsA concentrations, and for every CN activity time curve except that of 1 patient, lower CN activity was observed at T2 than at T0. Interestingly, on day 5 we observed significant changes in sample composition by time after drug intake that, at least theoretically, could have caused the measured decrease in leukocyte CN activity. However, since no changes in leukocyte composition are observed within day -1, when a decrease in CN was also observed, and as no relationship between leukocyte fraction and CN activity was found by linear regression, we conclude that our CN activity measurements reflect CN inhibition by CsA. The inhibition of CN by CsA in renal transplantation patients is partial and has a temporary duration, as has been observed by other groups(25,33). We show that trough CsA concentrations in these patients are sufficient to cause CN inhibition.

The effect of sample composition changes in renal transplantation patients could also affect other pharmacodynamic monitoring strategies that are used for monitoring CNI therapy, such as cytokine messenger RNA monitoring(34). Also, monitoring of other therapies such as inhibition of inosine monophosphate dehydrogenase activity by mycophenolic acid in these patients could be affected by sample composition variation(35). Investigation of sample composition, cell specific activities, and drug sensitivities could therefore be very useful for optimization of the clinical sensitivity and specificity of these methodologies.

In conclusion, we have shown that large inter- and intrapatient variation in peripheral blood leukocyte subset cell counts results in a large variation in sample composition, which depends on sample choice. In vitro measurement of cell-specific CN activities indicate that this variation could affect CN activity measurement in renal transplantation patients. No relationship was observed between sample composition and leukocyte CN activity, and leukocyte CN activity can therefore be used for monitoring CN activity in renal transplantation patients. Whether measurement of CN inhibition is useful to define and maintain the
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therapeutic index of CNI therapy is still unknown and requires investigation by adequate analytical methods. In this respect further insight in analytical confounders is very helpful for optimization of clinical specificity. In future studies, larger populations should be monitored for leukocyte CN activity to define the role of this activity in maintaining the therapeutic index of CNI therapy after organ transplantation.

Acknowledgments

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