Validation of a T cell specific calcineurin activity assay for monitoring cyclosporine therapy

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Abstract

To further improve calcineurin (CN) inhibition therapy, pharmacodynamic monitoring is currently investigated as a novel approach. Although clear relations with pharmacokinetics parameters have been observed, its clinical usefulness has not be demonstrated thus far. In order to increase target cell specificity, a T cell specific CN activity assay was developed. T-cells were isolated from whole blood using anti-CD3 coated paramagnetic beads and the isolated cells were analyzed for purity, yield, recovery, cell death and CD4+ and CD8+ subpopulations. T cell isolates were 95.5% ± 1.5% pure and no differences were observed for CD4+ and CD8+ population distribution before and after isolation, illustrating that representative T cell preparations were taken from whole blood samples. The CN assay showed linearity and reproducibility and no significant washout of CsA during the isolation procedure was noted. CN inhibition was observed when the protocol was used to monitor CsA-based immunosuppressive therapy in six stable renal allograft recipients.

The described T cell isolation protocol isolates a representative, highly pure T cell sample from whole blood. Adequate analytical performance was observed and the method was found to be suitable to monitor T cell specific CN inhibition in stable renal allograft recipients receiving CsA.
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Introduction

Pharmacodynamic monitoring of immunosuppressive therapy offers new potential tools to further improve the therapeutic index of critical dose drugs such as the calcineurin (CN) inhibitors cyclosporine (CsA) or tacrolimus. These drugs share a large inter-individual variability in pharmacokinetic parameters and are associated with considerable adverse effects such as nephrotoxicity, hypertension and diabetes mellitus(1). Advanced pharmacokinetics such as intracellular drug concentrations, pharmacogenetics and pharmacodynamics are currently investigated to increase monitoring specificity and clinical performance of calcineurin inhibitors(2-4).

Several pharmacodynamic monitoring strategies have been described, but only a few have been tested in solid organ allograft recipients. Although definite correlations between drug concentrations and pharmacodynamics have been described(5-7), no clear clinical associations have been reported and further insight in analytical variables may lead to identification of more accurate markers(8). We have recently reported that matrix composition directly confounds calcineurin activity measurement(8). This notion and the generally accepted view that T cells are the major target cells of calcineurin inhibitor therapy led us to the development of a T cell specific calcineurin activity assay(9).

Materials and Methods

Materials

The following materials were used: cyclosporin A, tacrolimus, dithiothreitol (DTT), ascorbic acid, NP-40, and soybean trypsin inhibitor from Sigma-Aldrich, phenylmethylsulfonyl from Merck, leupeptin from Roche, aprotinin from Bayer, phosphate standard from Fluka, biomol green cellular calcineurin assay kit plus from Biomol, fluorescein isothiocyanate (FITC)-labeled CD4 (clone SK3), phycoerythrin (PE)-labeled anti-CD8 (clone SK1), APC-labeled anti-CD3 (clone SK7), APC-labeled anti-CD2 (clone S5.2), PerCP-labeled anti-CD45 (clone 2D1), APC-labeled anti-CD19 (clone SJ25Cl), FITC-labeled anti-CD3, PE-labeled anti-CD16 and anti-CD56 and anti-CD3 coated paramagnetic beads, all from BD Biosciences and RPMI 1640, iFCS, penicillin/streptomycin and paramagnetic anti-CD3-beads from Invitrogen.
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Patients and healthy volunteers
EDTA-anticoagulated blood from the anticubal veins of healthy volunteers and renal allograft recipients receiving a CsA based immunosuppressive regimen was used for validation of the T cell calcineurin activity assay. Formal approval from the institutional ethics committee was obtained, and informed consent for blood sampling was obtained from all participants. The renal allograft recipient group underwent graft implantation 12 to 24 months prior to sample date and received dual immunosuppressive therapy including prednisolone (10 mg daily) and CsA. CsA dosage was controlled by estimated min-AUC(0,2,3 h) aiming at a target AUC(0-12 h) of 3250 μg/L*h(10).

T cell isolation, sample preparation and calcineurin assay
EDTA-anticoagulated blood (1.5 mL) was incubated with 60 μL of pre-washed (0.1 % BSA, 10 mM Hapes, 9.0 g/L NaCl, pH 7.5) anti-CD3 paramagnetic bead suspension for 25 minutes at 4°C with continuous tilting and rotation. After incubation, samples were placed in a magnet (DynaMac-2, Invitrogen) for 1 min. and supernatant was discarded. T cells were washed 4 times with 1 mL erythrocyte lysis buffer (8.4 g/L NH₄Cl, 1.0 g/L KHCO₃, pH 7.3). During this washing procedure samples and washing solution were kept on ice. Isolated and washed T cells were directly lysed by addition of 100 μL lysis buffer (50 mM Tris-HCl (pH 7.4), 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 three freeze-thaw cycles (liquid N₂ / 30°C). Cell debris and beads were spun down (10.000g, 10 min, 4°C), supernatant was collected and snap frozen in liquid N₂ before storage at -80°C until CN activity measurement.

The calcineurin activity assay used, was previously described, with one adjustment; a 0-1.5 nmol phosphate calibration curve was used for phosphate quantification(11). Calcineurin activity was expressed as nmol phosphate released per minute per μg protein. Protein concentrations of lysates were determined using the Coomassie Plus protein assay (Thermo Scientific, Rockford, IL, USA).

Cellular analysis
T-cell recovery, purity and yield were calculated from hematological analyses before and after T-cell isolation. T-cell concentrations of the starting material (whole blood) were calculated from leukocyte differentiates, performed on a Sysmex XE2100, and lymphocyte T-cell differentiations obtained using flow-cytometry. Lymphocytes gated in the side-scatter / CD45 dot-plot, were analyzed for CD3+, CD3+ /CD4+ and CD3+/CD8+ populations.
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For analyzes of the isolated T-cell population, the paramagnetic beads were detached by resuspension in RPMI culture medium containing 10 % inactivated fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin and overnight incubation in a humidified environment (5%, CO₂). Next day, the soaked off beads were removed by use of magnet. From the supernatant, manual erythrocyte and leukocyte counts were performed. Cell death was determined by trypan blue staining and leukocyte differentiate were determined after May-Grunwald/Giemsa staining of cytospins (Sysmex XE2100).

Determination of the T-cell purity within the lymphocyte population was performed by flow-cytometric analysis of B-, NK- and T-cells. Since the CD3 epitope was absent after detachment of T cells from the CD3-coated paramagnetic beads, we used the CD2 epitope as T cell marker for the isolated lymphocytes. Here, lymphocytes gated in the side-scatter / CD45 dot-plot were analyzed for CD19+, CD16/56+ populations in one panel and CD2+, CD2+/CD4+ and CD2+/CD8+ populations in a second panel.

Data analysis

Data are presented as mean ± SD. One-way ANOVA was used for statistical analysis of the multiple groups in the storage experiments. Statistical difference between two groups was determined by student t-test and statistical difference was defined as p<0.05.

Results

The final T cell purity isolated from healthy volunteer blood (n=15) was 95.5% ± 1.5% with 4 ± 1.2% of erythrocytes present (table 6.1).

<table>
<thead>
<tr>
<th>erythrocytes (%)</th>
<th>granulocytes (%)</th>
<th>monocytes (%)</th>
<th>CD19+ (%)</th>
<th>CD15/56+ (%)</th>
<th>CD2+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 (1.2)</td>
<td>n.d</td>
<td>n.d</td>
<td>0.09 (0.06)</td>
<td>0.3 (0.2)</td>
<td>95.5 (1.5)</td>
</tr>
</tbody>
</table>

Table 6.1. T cell sample composition. Purity of T cell isolate was determined for 15 healthy volunteers. Data are presented as mean % (SD) of contaminants present in isolate. n.d.; non detected.

A yield of 0.86 ± 0.44 \( \cdot 10^6 \) T cells and a recovery of 42% ± 14 % was found. Since the recovery was relatively low the CD3 coated beads were up-titrated, but did not increase T cell recovery. Significant cell death after isolation was excluded by trypan blue staining, being only 7% ± 2%. No changes were observed in CD4+ and
CD8+ T cell subpopulations before and after the isolation procedure (p=0.89 and 0.84 respectively, n=5).

Blood was stored at 4°C for 0, 4, 8 and 24 hours (n=3) after collection and no decrease in activity was detected (p=0.78). Stability of calcineurin activity during lysate storage was investigated for 0, 1, 2, 6 and 15 days and a decrease in calcineurin activity was observed after 2 days (13%, p=0.046). Subsequently, sample lysates were stored for maximal 1 day before calcineurin activity measurement.

Linearity was observed when T cell-beads fractions isolated from healthy volunteers were serial diluted with beads without T cells, before T cell lysis (figure 6.1).

![Calibration curve of the calcineurin assay](image)

**Figure 6.1. Linearity of calcineurin assay.** Upper: a typical calibration curve of the calcineurin assay. Lower: linearity of calcineurin assay determined by dilution of T-cells. These results are presented as mean ± SD (n=3) and calculated from calibration curve shown in upper figure.

The detection limit of the phosphate measurement was 0.15 nmol phosphate when calculated as the mean of blank measurement plus 3 standard deviations (n=8).
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When blood from one healthy volunteer was taken 4 times over a 4 month period, a reproducibility including intra-individual variation of 7% was found. To investigate whether CsA is washed out during the isolation procedure, we incubated whole blood with CsA and tested whether doubling of the washing steps, required for T cell isolation, decreased calcineurin inhibition by CsA. We could detect a calcineurin inhibition of about 30% for final whole blood CsA concentrations of 1000 and 2500 μg/L (p=0.0011) and no loss of calcineurin activity (p=0.18) or washing out of CsA (p=0.57) during the isolation procedure (figure 6.2).

![Figure 6.2: Washing out of CsA.](image)

To test whether the T cell calcineurin activity protocol can be used for monitoring CsA therapy, we monitored T cell calcineurin activity in 6 renal allograft recipients that received CsA therapy. The observed T cell calcineurin activities and whole blood CsA concentrations of two representative patients are illustrated in figure 6.3. Significant inhibition of T cell calcineurin activity was observed (14%) when activities just before drug intake (T0) (23.3±9.6 pmol phosphate/min./μg protein) were compared to activities 2 hours after drug intake (T2) values (20.0±7.8 pmol phosphate/min./μg protein, p=0.026) with corresponding CsA concentrations of 105±18 μg/L (T0) and 736±303 μg/L (T2, p = 0.0015).
Discussion

One of the major issues associated with the current pharmacodynamic monitoring strategies for calcineurin inhibition therapy is the large inter-individual variability observed that does not necessarily correlates to clinical endpoints. Part of this variation may be ascribed to either sample choice, sample composition and/or accuracy of the analytical techniques used(8,9). A T cell specific protocol for measurement of calcineurin activity might overcome most of these issues. Final T cell isolates were of high purity, ensuring that calcineurin activity measured after isolation was not significantly confounded by the presence of other cells. For instance, erythrocytes have only low cellular calcineurin activity(12), but they do contain proteins and when calcineurin activity is expressed per protein, large variations in erythrocytes could seriously confound measurement outcome. In addition, no changes in the CD4+ and CD8+ population distribution were observed within the T cell fraction after isolation. This indicated that a representative T cell sample was taken from the blood.

Recovery of T cells was relatively low, but up-titration of the beads did not increase the recovery of T cells. The T cell yield from 1.5 mL of blood from healthy
volunteers as well as for renal allograft recipients was however sufficient to perform the calcineurin assay with a suitable analytical reproducibility. The most likely cause of the low recovery is cell loss during the bead/T cell washing procedure. This is confirmed by lower calcineurin measurement and lower protein concentration measurement after doubling of the washing steps. Storage of T cell lysate showed a decrease in calcineurin activity starting already at the 2nd day after initial storage. Previously is was observed that storage of leukocyte lysate using the same lysis buffer could be stored up to 3 weeks(11). The most likely explanation for this discrepancy is the presence of the paramagnetic beads or remainings thereof in the cell lysate. Its possible that remainings of the beads could for instance oxidize and subsequently inactivate calcineurin(13,14). Loss of intracellular CsA during T cell isolation was found to be of only limited relevance, since doubling of the washing steps did not affect calcineurin inhibition by CsA. This indicates that the cellular conditions are maintained throughout the T cell isolation procedure.

In this protocol we couple the T cell isolation protocol to a calcineurin assay for pharmacodynamic monitoring of CsA therapy. The isolation protocol could however also be followed by other enzymatic assays such as mTOR activity, inosine monophosphate dehydrogenase (IMPDH) or cellular drug concentration measurement that could be of interest for allograft recipients. Intracellular T cell analysis could also be of interest in other diagnostic fields; a wide range of other intracellular enzymes and markers can be analyzed in T cells isolated from 1.5 mL of blood.

In conclusion we present a validated protocol for the isolation of highly pure T cell fractions that can be used to monitor calcineurin therapy and other intracellular markers.

References

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