Specific $^3$H Radioimmunoassay with a Monoclonal Antibody for Monitoring Cyclosporine in Blood

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A specific radioimmunoassay involving a mouse monoclonal antibody to cyclosporine has been developed for monitoring the parent drug in blood. Pretreatment with methanol removes cyclosporine from the erythrocytes. The limit of detection is about 12 μg/L, sample volume is 50 μL of blood, and within- and between-assay CVs are <7%. Assay results correlated well with those obtained by "high-performance" liquid chromatography (HPLC) for liver (n = 42), for heart (n = 84), for bone-marrow (n = 36), and for kidney (n = 140). For blood specimens obtained from patients treated with cyclosporine postoperatively for as long as 65 months, the mean RIA/HPLC ratio in all with transplant indications was close to 1. Therefore, the specific radioimmunoassay apparently can be used instead of HPLC to measure the parent drug in blood.

Cyclosporine, the active ingredient of Sandimmune* (Sandoz Ltd., Basel, Switzerland), is an immunosuppressive, hydrophobic cyclic undecapeptide of fungal origin. It is clinically effective in the treatment of allograft rejection (2–5) and has recently been used to treat autoimmune diseases (6, 7). Because cyclosporine has a narrow therapeutic range and displays large within- and between-patient differences in its pharmacokinetics, concentrations of the drug must be monitored during therapy (8).

Clearly, a therapeutic range that is universally accepted would be of clinical value in guiding therapy with cyclosporine. However, two major problems prevent the establishment of a single therapeutic range for cyclosporine. First, some medical centers monitoring the drug choose to assay whole-blood specimens, while others prefer plasma or serum. This makes comparisons difficult, particularly because cyclosporine exhibits a temperature- and time-dependent binding to erythrocytes (9). Therefore, we recommend that only whole blood be used for monitoring cyclosporine. The second problem concerns the choice of assay for monitoring the drug. Cyclosporine can be measured in blood by either "high-performance" liquid chromatography (HPLC) (10, 11) or radioimmunoassay (RIA) (12). In the past, HPLC was the only specific method available to measure the parent drug, whereas the RIA measured both the parent drug and some of its metabolites. Therefore, concentrations of cyclosporine measured by RIA in blood were always about two- to fourfold (13) higher than the corresponding HPLC results. Nonetheless, most medical centers prefer to monitor cyclosporine by RIA, despite the lack of specificity, because it is more easily performed.

Here, we describe a RIA based on the use of a mouse monoclonal antibody (14) for measuring parent drug in whole blood. A simple pretreatment with methanol removes cyclosporine from the erythrocytes. The RIA provides assay values for cyclosporine in whole blood that are very similar to those found by HPLC.

Materials and Methods

Radioimmunoassay reagents. All the reagents used in the RIA were from the Sandimmune kit (Sandoz Ltd., Basel, Switzerland): Tris buffer concentrate (pH 7.5), charcoal-suspension, standard (16 mg/L), lyophilized specific monoclonal antibody, $[^{3}H]$dihydrcyclosporine tracer (1.8 μCi or 66.6 KBq in 1 mL of aqueous ethanol). All the reagents were prepared for use according to the manufacturer's instructions. The cyclosporine-free whole blood used to prepare the standards and quality-control samples was obtained from the Red Cross Blood Bank, Basel. Analytical-grade methanol (Merck no. 6009) was used to pretreat the whole-blood specimens.

Cyclosporine metabolites. Metabolites 1, 8, 9, 10, 13, 16, 17, 18, 21, 25, and 26 were obtained as described previously (15, 16). The concentration of each metabolite was measured in ethanol (spectroscopic grade) at 215 nm, with cyclosporine as the reference standard.

Clinical blood specimens. Whole-blood specimens were taken from patients who had received immunosuppressive therapy with cyclosporine after transplantation of either a kidney, heart, liver, or bone marrow. Blood specimens came from patients who were being treated with cyclosporine postoperatively for various intervals ranging from three days to 65 months. Except for the bone-marrow group, cyclosporine was given orally, and all blood specimens assayed were collected before the next dose. Some patients in the bone-marrow group had received cyclosporine by intravenous infusion and only some of the blood specimens were pre-dose.

Each whole-blood specimen was collected into tubes containing EDTA as anti-coagulant, then stored at −20 °C.

Cyclosporine standards for RIA. The ethanol solution of cyclosporine (16 mg/L) supplied in the Sandimmune kit was diluted in drug-free whole blood to obtain a set of assay standards with concentrations ranging from 25 to 1600 μg/L. The zero standard was cyclosporine-free whole blood.

Pretreatment of whole-blood samples. The cyclosporine standards, quality-control samples, and clinical specimens are treated identically and assayed in triplicate. Add 50 μL of whole blood from each type of sample to 950 μL of methanol; cap the tubes, vortex-mix for 10 s, then centrifuge for 5 min at 1600 × g. Be careful not to let any of the methanolic supernate evaporate.

Assay procedure. All reagents are added to polystyrene assay tubes according to the pipetting scheme in the leaflet accompanying the Sandimmune kit. In brief, add 50 μL of the methanol supernate to 500 μL of the diluted Tris buffer-concentrate (now 50 mmol/L, pH 7.5). Then add 100 μL of the $[^{3}H]$dihydrcyclosporine tracer and 50 μL of the solution containing the specific monoclonal antibody, vortex-mix, and incubate the mixture for 16 h at 4 °C. Place all the samples in an ice-bath (0–4 °C) for 15 min. Thereafter, to each assay tube, except the total radioactivity tube(s), add...
500 μL of the stirred charcoal suspension; keep the samples cool (0–2 °C). After 2 min, vortex-mix, and leave the samples in the ice-bath for a fixed time (12 to 15 min). Then centrifuge (1600 × g, 5 min, 4 °C) and count the radioactivity in the supernate by liquid scintillation counting, correcting for quenching.

Assay calculation. We used an RIA curve-fitting program (Betamic II RIA-Option; Kontron, Zürich, Switzerland) to fit the standard curve and interpolate the concentration of cyclosporine in the quality-control samples and clinical specimens.

Accuracy and precision. We added cyclosporine to drug-free whole blood to give three quality-control samples with cyclosporine concentrations of 100, 400, and 1000 μg/L. We assayed each of these quality-control samples in sextuplicate on eight different occasions during two weeks, to assess within-run and between-run variability (17).

Specificity. The specificity of the RIA for cyclosporine was tested by determining the cross reactivity of metabolites 1, 8, 9, 10, 13, 16, 17, 18, 21, 25, and 26 in the assay (18).

HPLC procedure. We used a modified HPLC procedure to measure cyclosporine in whole blood (11). Briefly, the procedure was as follows. We mixed 0.5 mL of each blood sample with 0.2 mL of methanol, followed by 1.2 mL of acetonitrile/water (97.5/2.5 by vol) containing cyclosporin D, 240 μg/L, as the internal standard. The sample was centrifuged and 1.2 mL of the supernate was diluted with 0.3 mL of distilled water and washed with 2 mL of n-hexane. We injected 0.9 mL of the lower phase into a pre-column (Spherosorb C-6, 5-μm particles, 50 × 4.6 mm; Phase Separations, Ltd., Queensferry, Clwcy, CH5 2NU, U.K.) to “clean up” the sample, before measuring the cyclosporine on the analytical column (Supelcosil LC-18, 3-μm particles, 150 × 4.6 mm; Supelco, Bellefonte, PA 16823-0048). Both columns were run at 70 °C, and detection was by ultraviolet absorption at 205 nm. Typically, the unknowns were analyzed in singlicate. Quantification was by the internal standard method based on the peak-area ratios. Included in each analysis batch were the quality-control samples we had used for the RIA determinations. The corresponding between-assay means (n = 10) and CVs for the quality-control samples were 98.3 (11.0%), 936.9 (10%), and 991.4 (9.2%) μg/L.

Regression analysis. We analyzed the RIA and HPLC data by a nonparametric linear regression, assuming that the variables were from arbitrary continuous distribution and that both variables were subject to error (19). With this procedure the regression line parameters, slope and intercept, were estimated together with their confidence interval at the 95% significance level.

Results

RIA Performance

The range of the standard curve for cyclosporine was 25 to 1600 μg/L; the limit of detection was about 12 μg/L (Figure 1). The accuracy and reproducibility of the RIA are summarized in Table 1. Analytical recovery of cyclosporine added to whole blood (100, 400, and 1000 μg/L) was between 99 and 100%. Within- and between-assay CVs for the quality-control samples were always <7%.

The cross reaction of cyclosporine metabolites 8, 9, 13, 16, 17, 18, 25, and 26 in the RIA was <1% each. Metabolites 1, 21, and 10 cross reacted by 3 to 4% (Table 2).

Measurement of Cyclosporine in Clinical Blood Specimens

Measurement of cyclosporine concentrations in blood, by the specific RIA and by HPLC, were linearly correlated (P <0.05) in all the transplant groups (Figure 2). The liver, heart, bone-marrow, and kidney assay data (RIA vs HPLC) clustered closely around the line of identity, giving the following regression with the 95% confidence interval for slope (a), intercept (b), and correlation coefficients (r):

liver:

\[ y = 0.98x + 7.4 \text{ (n = 42); } r = 0.979 \]

a: 1.06 > 0.98 > 0.89; b: 21.6 > 7.4 > −5.9

heart:

\[ y = 1.19x - 9.8 \text{ (n = 64); } r = 0.976 \]

a: 1.30 > 1.19 > 1.12; b: 0.9 > −9.8 > −25.3

bone marrow:

\[ y = 1.00x + 17.8 \text{ (n = 36); } r = 0.948 \]

a: 1.16 > 1.00 > 0.81; b: 54.2 > 17.8 > −17.5

kidney:

\[ y = 0.95x + 15.5 \text{ (n = 140); } r = 0.935 \]

a: 1.04 > 0.95 > 0.87; b: 29.1 > 15.5 > 3.2

The RIA/HPLC assay ratios in all transplant indications were closely scattered around the line of unity and showed no tendency to increase or decrease during the 65 months of postoperative monitoring (Figure 3). For the liver-transplant patients the mean ± SD for the RIA/HPLC assay ratio was 1.00 ± 0.12 (n = 42), heart 1.13 ± 0.13 (n = 64), bone marrow 1.09 ± 0.19 (n = 36), and kidney 1.07 ± 0.20 (n = 157).

Discussion

The Sandimmune kit with the specific mouse monoclonal antibody clearly measures the parent drug in whole blood
Table 2. Cross Reactivity of Cyclosporine and Some of Its Metabolites with the Specific Monoclonal Antibody

| Compound      | IC<sub>50</sub> µg/L | Cross reactivity, %
<table>
<thead>
<tr>
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<tr>
<td>Cyclosporine</td>
<td>0.8</td>
<td>100</td>
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<tr>
<td>Metabolite no.</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>31.7</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>547.2</td>
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</tr>
<tr>
<td>9</td>
<td>9553.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>10</td>
<td>22.8</td>
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</tr>
<tr>
<td>13</td>
<td>1103.3</td>
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<tr>
<td>26</td>
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</tr>
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</table>

*The IC<sub>50</sub> is the concentration of cyclosporine or metabolite that decreases the specific binding of the tracer in the competitive RIA by 50%. °(IC<sub>50</sub> cyclosporine/IC<sub>50</sub> metabolite) × 100. *Numbered as in refs. 15, 16.

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Fig. 2. Relation between concentrations of cyclosporine in blood as measured by the specific RIA and HPLC in patients who received transplants of liver, heart, bone marrow, or kidney. The line of identity is drawn in each panel, for comparison.

without interference from its principal metabolites. Several authentic cyclosporine metabolites (15, 16), including the main one in humans, no. 17, cross react poorly in the assay. More importantly, in all the transplant indications that were tested, the RIA values correlated well with those obtained by HPLC.

Obviously, the ability to measure the parent drug with the Sandimmune kit may improve therapeutic drug-monitoring of transplant patients, particularly those who are undergoing liver transplantation (23). Patients who receive a liver transplant are often difficult to monitor, because they sometimes have high concentrations of cyclosporine metabolites in their blood, owing to diminished hepatic function (20). The metabolites cross react to a different degree with the polyclonal antiserum that is used in the cyclosporine RIA kit, and as a result, cause the HPLC/RIA ratio to increase considerably beyond 2 to 4. Hence, the method recommended for monitoring liver-transplant patients has been HPLC. However, with the Sandimmune kit, it should now be possible to adjust the dose of cyclosporine to maintain its efficacy and minimize its toxicity, without HPLC being needed.

Further evidence for the high specificity of the monoclonal antibody for the parent drug comes from the clinical and temporal stability of the RIA/HPLC assay ratio. The mean assay ratio in all transplant indications came close to 1 during the 65 months of drug monitoring. This observation alone demonstrates that, despite the expected changes in metabolite pattern that occur in patients over such an interval of time, the RIA retains its specificity.

The question of whether cyclosporine alone or its metabolites, or both, contribute to immunosuppression or toxicity can now be more easily investigated in vivo. The Sandimmune kit, in addition to the specific monoclonal antibody, contains a nonspecific monoclonal antibody (16) that measures both the parent drug and some of its metabolites. Thus, it will be possible to correlate changes in cyclosporine/metabolite concentrations with clinical events.

From a practical point of view, the Sandimmune kit is easy to use and shows better accuracy and reproducibility than the present cyclosporine kit (21). The methanol pretreatment step completely removes all cyclosporine from the erythrocytes and minimizes quenching by hemoglobin. Such a pretreatment step had already been recommended, although the authors used acetone instead of methanol (22). Because assay of cyclosporine in whole blood has been improved considerably, it is hoped that more centers monitoring the drug will convert to this body fluid as the sample of choice. Recently, there was a recommendation by the American Association for Clinical Chemistry to use whole blood for cyclosporine monitoring (23).

In conclusion, the specific monoclonal antibody in the Sandimmune kit provides an easy means of monitoring cyclosporine in patients who have received a kidney, liver, heart, or bone-marrow transplant. Monitoring centers that do not have access to HPLC will now be able to measure the parent drug in whole-blood specimens.
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References