A competitive protein-binding assay for cyclosporine (CsA) and its bioactive metabolites was developed. The assay was based on the intracellular cyclosporine-binding protein cyclophilin (CYP) and its metabolites. Cyclosporine from cytoplasmic extracts or erythrocyte lysates was measured by the binding assay with use of [3H]CsA as a tracer. Binding affinities of various CsA analogs and metabolites were compared, and their reported in vitro immunosuppressive activities were determined. The assay detected CsA at 50 μg/L (1 g = 0.832 mmol of CsA), with an analytical recovery of >80%, and CVs were <8% for intra-assay and <11% for interassay precision in the range of 150–1000 μg/L. We used this assay to measure CsA concentrations in blood and compared the results with those measured by HPLC or by CsA-specific (monoclonal) and CsA-nonspecific (polyclonal) radioimmunoassays. Binding assay results were in nearly all cases, less than those measured by the nonspecific RIA and frequently >20% above the values determined by the CsA-specific assays. Individual patients had pronounced differences in the relative proportions of CsA, CYP-binding (bioactive) metabolites, and cross-reacting CsA metabolites. Because the presence of bioactive metabolites may considerably contribute to the immunosuppressive activity of CsA, we consider the binding assay clinically useful for measuring CsA in biological fluids.

Additional Keyphrases: erythrocytes • cytoplasmic lysates • HPLC, radioimmunoassay compared • binding proteins • monitoring therapy

Cyclosporin A (CsA; cyclosporine), a hydrophobic cyclic undecapeptide of fungal origin with potent immunosuppressive properties, is used clinically to prevent rejection of allografts and to treat autoimmune diseases. Despite its effectiveness, the drug has a narrow therapeutic range, and the pharmacokinetics vary markedly within and between patients (1, 2). Therefore, concentrations of CsA must be monitored daily in patients during treatment. In addition to the parent drug, several metabolites of CsA have immunosuppressive activities, although their potencies relative to CsA are still being debated (3–12). Recently, Roesel et al. (13) isolated and characterized a novel immunosuppressive CsA metabolite (M-E), the effects of which, in cellular assays, were similar to those of CsA. Their studies therefore suggest that formerly unidentified metabolites may play an important role in addition to the immunosuppression exerted by CsA. Because some metabolites reach trough concentrations similar to or even greater than those of CsA (14–16), the contribution of these metabolites to the total immunosuppressive effects of CsA should be quantified.

CsA concentrations in blood can be measured by HPLC or specific radioimmunoassays, the former method being also capable of quantifying CsA metabolites (17–19). However, most of the published data relating concentrations of circulating CsA to clinical outcome have been derived from specimens of renal transplant patients, quantified with the polyclonal Sandoz (Basel, Switzerland) radioimmunoassay kit. The antiserum of this kit cross-reacts to various extents with CsA metabolites generated in vivo, some of which may have immunosuppressive activity (20). Despite controversies regarding the merits and limitations of each assay methodology, apparently comparable clinical significance has so far resulted in the coexistence of these analytical approaches (1, 2, 21, 22).

The CsA-binding protein cyclophilin (CYP), described by Handschumacher et al. (23, 24), shows a remarkable ability to discriminate between CsA analogs and metabolites with different immunosuppressive activities. Studies performed by Quesniaux et al. (25, 26), involving a large number of CsA analogs and metabolites, revealed the stereospecificity of the binding reaction. CYP seems to be present in large amounts in the cytoplasm of T cells, erythrocytes, thymus, kidney, and brain cells and in lower concentrations in most other tissues (27, 28). Two independent groups have demonstrated that CYP is identical with the enzyme peptidyl-prolyl cis–trans isomerase (29, 30). It is not yet clear whether the effects of CsA on T cells can be explained by its ability to inhibit the activity of this enzyme.

Recently, we developed a competitive CYP-binding assay for measuring CsA and its CYP-binding metabolites in hemolyzed blood (31). However, the assay was not sensitive enough (detection limit >300 μg/L) to reliably measure CsA concentrations in patients' samples. Here we describe an improved version of this assay with a detection limit near 50 μg/L. In addition we present data on the pharmacokinetics of CsA in end-stage renal-failure patients and on daily trough concentrations in kidney allograft recipients. If we assume a strong correlation between affinity of CsA metabolites
to CYP and their immunosuppressive activity ("physiological cross-reactivity"), this method offers a rational approach to quantifying the total immunosuppressive potency of CsA in patients' samples.

**Materials and Methods**

**Reagents**

Lyphocheck whole-blood control (human) Levels I and II were obtained from Bio-Rad Laboratories, Hercules, CA 94547. Blood cell concentrates (buffy coats) and a 200 g/L solution of human serum albumin (HSA) were purchased from Immuno AG, Vienna, Austria. HeLa Ohio cells were a kind gift from the Institute of Biochemistry, University of Vienna.

Acetonitrile, ammonium sulfate, Tris, Tween 20, dimethyl sulfoxide, dithioerythritol (DTE), and charcoal were all analytical-reagent grade from Merck, Darmstadt, F.R.G. Dextran T-70, Sephadex G-50, Sephacryl S-200, and diethylaminoethyl (DEAE)-Sephacel were from Pharmacia, Upsala, Sweden; Blue Trisacryl M was from IBF Biotechnics, Toulouse, France.

The CsA standard stock solution was taken from the Sandimmune RIA kit (Sandoz); the CsA analogs CsC, CsD, CaG, CaH, and O-acetyl-CsA were kindly supplied by Dr. T. G. Payne (Sandoz); the CsA metabolites M1, M8, M17, M18, M26, and M203–218 were a generous gift from Dr. K. Sewing, Hannover, F.R.G. (17). The two CsA radioimmunoassay kits used were Cyclo-Trac and Cyclo-Trac SP (Incstar, Stillwater, MN). [{3H}CSA (code TRK 904, specific activity 10.5 kCi/mol) was from Amersham International, Amersham, Bucks., U.K.

**Procedures**

**Preparation of cyclophillin.** Suspend 2 × 10⁹ HeLa Ohio cells in phosphate-buffered saline (10 mmol of phosphate buffer and 140 mmol of NaCl per liter, pH 7.2) and disrupt by homogenization in a glass homogenizer. Centrifuge the lysate for 30 min at 20 000 × g to remove large cell organelles and membranes, and subject the turbid supernate to ultracentrifugation (12 h at 50 000 × g). Precipitate the clear supernate by adding ammonium sulfate, 500 g/L; dissolve the precipitate in a small volume of Tris-DTE buffer (10 mmol of Tris·HCl and 1 mmol of DTE per liter, pH 7.5), and dialyze for 48 h against the same buffer. Layer the CYP-containing solution onto a 3.5 × 2 cm Blue Trisacryl M column equilibrated with Tris-DTE buffer. Wash until protein is no longer detectable in the eluate, and elute CYP with Tris-DTE buffer containing 100 mmol of NaCl per liter. Check the purity of the eluate by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (24), staining with Coomassie Brilliant Blue G-250 and silver. Store the fractions with the greatest CsA-binding capacity at −20 °C.

Alternatively, one can derive CYP from whole blood, as follows: Collect blood cells by centrifugation and rinse once with isotonic saline (NaCl, 150 mmol/L). Lyse the cells by freezing and thawing, and dilute with an equal volume of Tris-DTE buffer. Purify 10 mL of the extract by gel filtration on Sephadex G-50, followed by chromatography on Sephacryl S-200. Apply to a Blue Trisacryl M column (3.5 × 2 cm) the pooled CsA-binding activity eluting after the hemoglobin peak, and isolate the CYP-containing fraction as described above for cytoplasmic extracts. Still better purification can be achieved by using anion-exchange chromatography on DEAE-Sephacel instead of Sephacryl S-200: CYP elutes in the flow-through volume, whereas hemoglobin is retained on the column.

**Sample preparation.** Collect blood by venipuncture into evacuated collection tubes containing EDTA, then hemolyze by freezing and thawing; keep samples at −20 °C until assayed. Before assay, add 0.1 mL of acetonitrile to 0.5 mL of hemolysate, mix, and add a further 1 mL of acetonitrile with constant shaking. Centrifuge at 4000 × g for 10 min and dry 1 mL of supernatant solution under a stream of nitrogen at 37 °C. Redissolve the residue in 0.3 mL of ethanol/water (60/40 by vol).

**Competitive protein-binding assay.** Perform the assay in polypropylene mini-tubes with conical bottoms (Sarstedt, Numbrecht, F.R.G.). Prepare a working solution of [{3H}CSA by diluting 0.03 mL of the stock solution in 10 mL of assay buffer (Tris·HCl 30 mmol/L, DTE 1.5 mmol/L, ammonium sulfate 0.3 mol/L, human serum albumin 30 g/L, Tween 20 0.2 mL/L, and sodium azide 0.2 g/L) containing ethanol (100 mL/L). Mix 0.05 mL of redissolved extract or CsA standard with 0.05 mL of tracer (~30 000 dpm) on ice, add 0.1 mL of assay buffer—containing an amount of CYP sufficient to obtain a maximal binding of ~30%—and incubate on ice for 30 min. Estimate nonspecific binding by substituting assay buffer for CYP.

Separate bound and free [{3H}CSA by adding 0.1 mL of ice-cold charcoal (20 g/L) suspended in Tris-DTE buffer containing 0.5 g of Dextran T70 per liter. Mix the contents of each tube and incubate for 5 min on ice, then centrifuge without delay (4000 × g, 5 min). Mix 0.2–mL aliquots of the supernates with 3 mL of scintillation fluid and measure the radioactivity in a scintillation counter.

**Patients**

To determine the concentration of CsA at trough, we collected blood samples from five kidney allograft recipients daily, from the day of transplantation until dismissal from the hospital. To study the pharmacokinetics and metabolism of CsA, we collected blood samples from 10 volunteers (both sexes) with renal failure before administration of a single oral dose of CsA (8 mg/kg) and at 30 min (six samples), 1-h (eight samples), and 2-h (six samples) intervals thereafter for 24 h. Blood samples from the United Kingdom Quality Assessment Scheme were obtained from D. Holt, Charing Cross Hospital, London, U.K.

**Results**

**Components of the Assay Buffer**

**Purification of CYP.** Purification was important to improve the sensitivity of the assay. Thus, the more
than 100-fold enrichment of CYP achieved by the purification scheme improved the assay sensitivity (detection limit) from 400 \( \mu g/L \) (crude cytosolic extract) to 50 \( \mu g/L \). The detection limit is defined as 85\% of the binding of the zero-concentration sample (B\textsubscript{0}). (A further increase to 20 \( \mu g/L \) was achieved when CYP was separated from hemoglobin by anion-exchange chromatography.) When purity of the CYP preparation was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, staining the gel with Coomassie Blue G-250 revealed a single band. However, silver staining showed several additional bands, demonstrating minor contamination with proteins in the molecular mass range between 20 and 40 kDa.

**Ethanol concentration.** Because it was necessary to reconstitute the extracted and evaporated samples in ethanol (600 mL/L), we studied the influence of the solvent concentration on assay performance. A pronounced decrease of B\textsubscript{0} was observed at ethanol concentrations >200 mL/L; therefore, we regarded a solvent concentration of 180 mL/L as the upper limit of ethanol concentration. Neither acetonitrile nor dimethyl sulfoxide could be used at higher concentrations.

**Ammonium sulfate concentration.** High ionic strength may sometimes stabilize protein–ligand interactions. Because binding of CsA to CYP is reportedly stable at high salt concentrations (27), we investigated the effects of various concentrations of ammonium sulfate on binding characteristics. B\textsubscript{0} increased slightly at ammonium sulfate concentrations >0.1 mol/L and was greatest at 0.32 mol/L (40\%; vs 32\% in the absence of salt); nonspecific binding was not affected. Further increases in ionic strength markedly decreased B\textsubscript{0}. An ammonium sulfate concentration of 0.32 mol/L had no effect on the selective binding of other cyclosporines. In addition, assay of 20 patients’ samples in the presence and absence of ammonium sulfate, 0.32 mol/L, gave comparable results (data not shown).

**Tween 20 and HSA.** The presence of 0.2 mL of Tween 20 and 20 g of HSA per liter was indispensable to prevent nonspecific adhesion of CsA to the wall of the reaction vessel.

**Incubation temperature.** Maintenance of incubation temperature was critical. Unbound CsA was removed by charcoal adsorption, which should be performed at low temperature. Incubation at room temperature resulted in poor reproducibility and high nonspecific binding. Although the use of charcoal separation instead of the Sephadex LH-20 column assay described by Handschumacher et al. (23) reduced assay sensitivity by ~50\%, the LH-20 assay turned out not to be suitable for measurements in biological samples, probably owing to interferences of the blood extract with the column matrix.

**Competitive CYP-Binding Assay**

**Standard curve and accuracy.** Figure 1 illustrates the standard curve obtained for 10 analytical runs performed in duplicate. The mean ± 2 SD for the binding capacity was 28.5\% ± 2.9\%, and for nonspecific binding 5.7\% ± 1.2\%. The assay detection limit was 50 \( \mu g/L \) and the useful range was 50–800 \( \mu g/L \). We tested within-run precision by assaying three samples containing high, medium, and low concentrations of CsA in eight replicates each. The results were as follows: 50 \( \mu g/L \), CV = 14.1\%; 200 \( \mu g/L \), CV = 7.8\%; 800 \( \mu g/L \), CV = 5.1\%. Between-day precision was calculated by measuring low- and high-concentration CsA samples (Lyphocheck I and II) in duplicate each day for 10 consecutive days. The mean (and CV) for each duplicate was 164 (11.4\%) and 1002 \( \mu g/L \) (7.9\%).

**Recovery studies.** The recovery of the extraction procedure was checked either by using the Sandoz RIA to assay patients’ samples and CsA-supplemented controls, or by measuring the radioactivity of the extract of CsA-free control blood supplemented with tracer. Recovery of the extraction step was always >95\%, but after drying the extracts with nitrogen, only about 90\%–95\% could be redissolved in 0.3 mL of ethanol/water solution (60/40 by vol). Thus, this recovery was 87\%–97\% for radioactive samples, 84\%–98\% for samples supplemented with CsA at 360 or 720 \( \mu g/L \), and 84\%–97\% for patients’ samples.

Analytical recovery was studied with CsA-free blood samples supplemented with either 360 or 720 \( \mu g \) of CsA per liter, which were run as controls in parallel with patients’ samples through the entire analytical process: 325 ± 41 (90\% ± 13\%) and 632 ± 43 \( \mu g/L \) (88\% ± 7\%), respectively (mean ± SD, \( n = 16 \)). Moreover, the CsA concentrations in Lyphocheck I and II CsA samples (which were used as controls in most experiments) were nearly always within the concentration range indicated by the supplier.

**Cross-reactivities.** We investigated the binding of CYP to CsA, CsC, CsD, CsG, CsH, O-acetyl CsA, and the metabolites M1, M8, M17, M18, M26, and M203-218 (acid metabolite). Apart from CsA, none of the analogs and metabolites were available in a radioactively labeled form. Therefore, we measured the binding of the CsA analogs by the competitive-binding assay and determined binding affinities at various concentrations (Table 1). As expected, CsA had the greatest affinity,
Table 1. Binding of CsA Analogs and Metabolites to CYP in the Competitive Binding Assay

<table>
<thead>
<tr>
<th>CsA analog and metabolite concn, µg/L</th>
<th>250</th>
<th>500</th>
<th>1000</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA</td>
<td>60*</td>
<td>35</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>M17</td>
<td>68</td>
<td>52</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td>CsG</td>
<td>90</td>
<td>70</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td>CsC</td>
<td>93</td>
<td>75</td>
<td>54</td>
<td>40</td>
</tr>
<tr>
<td>M1</td>
<td>100</td>
<td>90</td>
<td>73</td>
<td>54</td>
</tr>
<tr>
<td>M8</td>
<td>100</td>
<td>95</td>
<td>85</td>
<td>65</td>
</tr>
<tr>
<td>CSD</td>
<td>100</td>
<td>98</td>
<td>90</td>
<td>72</td>
</tr>
<tr>
<td>M203-218</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>70</td>
</tr>
<tr>
<td>CsH, M18, M26, O-acetyl CsA</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Percent of B<sub>B</sub> n = 3 each.

followed by M17, CsG, and CsC. Weak binding was observed with M1, M8, CsD, M203-218, and no binding could be detected with CsH, O-acetyl CsA, M18, and M26. The order of CYP-binding affinity was therefore CsA > M17 > CsG, CsC > M1 > M8 > CsD, M203-218.

Measurement of CsA in Whole-Blood Specimens: Intermethod Comparison

Samples from the U.K. Quality Assessment Scheme. To compare our binding assay with measurements by polyclonal RIA and HPLC performed in other European laboratories, we obtained samples from the U.K. Quality Assessment Scheme. An example is given in Table 2; all three samples were whole-blood pools from patients receiving CsA. In samples A and B the values obtained by the CYP-binding assay were lower than the Sandoz RIA values, whereas in sample C the CYP-binding assay gave a value almost twice that of the RIA. One might assume that this sample contained a relatively high proportion of CYP-binding metabolites not detectable by the RIAs. On the other hand, an interference with the assay cannot be excluded. However, in analytical recovery experiments performed with CsA-supplemented blood samples, only minor interferences were seen, most of which slightly reduced the CsA values (see above).

Daily trough concentrations in kidney allograft recipients. Daily trough concentrations of CsA were measured in the blood of five kidney allograft recipients over at least 10 days, starting with the day of transplantation. In all patients, day-to-day CsA concentrations measured by the polyclonal RIA and the CYP-binding assay varied considerably more than the concentration of CsA alone (as determined by HPLC). A typical result is given in Figure 2. In general, CsA concentrations measured by the CYP-binding assay were lower than RIA concentrations; nevertheless, on some days CYP-binding assay concentrations exceeded the RIA values. Regression analyses performed for two patients from whom data had been obtained for more than two weeks revealed pronounced interindividual differences. Thus, there was no correlation between CYP-binding assay and polyclonal RIA in patient 1 (slope = 0.19, intercept = -195 µg/L, r = 0.37), and only weak correlation between CYP-binding assay and HPLC (slope = 0.86, intercept = 178 µg/L, r = 0.73), whereas in patient 2 a much better correlation was observed both for CYP-binding assay/polyclonal RIA (slope = 0.37, intercept = -103 µg/L, r = 0.83) and CYP-binding assay/HPLC (slope = 1.14, intercept = 155 µg/L, r = 0.90).

Pharmacokinetics of CsA in hemodialysis patients. To investigate CsA pharmacokinetics and metabolism, we gave 10 pretransplant end-stage renal-failure patients a single oral dose of CsA, 8 mg/kg of body weight. CsA concentrations were determined over the next 24 h by polyclonal and monoclonal RIA and by the CYP-binding assay. Figure 3 represents the 24-h pharmacokinetic profiles of CsA in two selected patients who showed pronounced differences in their individual metabolisms. In one patient (Figure 3a), a single concentration peak after 2 h was detected with all three assay systems. This patient produced only low concentrations of CYP-binding metabolites, as deduced by comparing the areas under the concentration/time curves for the monoclonal RIA and the CYP-binding assay. After the peak in cyclosporine concentration, a rapid decrease was measured by all three assay systems. In another patient (Figure 3b), a first concentration peak after 2 h was followed by a second peak 2 h later, which was most pronounced in the CYP-binding assay. In contrast to the first patient, this patient had much higher concentrations of CYP-binding metabolites. The area under the curve ratios of CYP-binding assay/polyclonal RIA and of CYP-binding assay/polyclonal RIA for these two pa-
patients were pronouncedly different, but only slightly different for the polyclonal/monoclonal RIA ratio. The ratio of CYP-binding assay/monoclonal RIA was 1.06 and 1.68, the ratio of CYP-binding assay/polyclonal RIA was 0.62 and 1.05, and the ratio of polyclonal RIA/monoclonal RIA was 1.72 and 1.60 for patients a and b, respectively. Regression analysis of the 3-, 6-, and 12-h values showed, as expected, a continuous decrease in the correlation between the CYP-binding assay and the monoclonal RIA (r = 0.96, 0.88, 0.73) and the polyclonal RIA (r = 0.87, 0.85, and 0.77); the best correlation between polyclonal and monoclonal RIA was for measurements at 6 h (r = 0.88, 0.96, 0.84).

Discussion

CsA is metabolized in the liver by the cytochrome P450 system. So far, >20 metabolites have been identified, the immunopharmacological significances of which are largely unknown. On the basis of cellular in vitro experiments, several authors have concluded that some CsA metabolites make a significant contribution to the total immunosuppressive activity, whereas others consider the activity of metabolized CsA to be negligible (4–13). Although M17 is generally considered the most immunosuppressive CsA metabolite, most consider it to possess only 10%–25% of the activity of CsA (3, 8, 10, 12). In contrast, Freed et al. (4, 5) reported that M17 exerts biological activity comparable with that of the parent compound. These authors investigated the effects of various cyclosporins on [3H]thymidine incorporation and IL-2 production in various in vitro assay systems. In their assay systems, the effects on lymphokine production were more pronounced than the effects on cell proliferation, in agreement with our own observations (31, and unpublished data). Others found that M17 exerts 50%–70% of the activity of the parent compound (6, 7, 9, 11). Finally, the novel metabolite M-E recently described by Roesel et al. (13) reportedly exerts 80% of the immunosuppressive activity of CsA. Thus, the in vivo contribution of CsA metabolites to overall immunosuppression is unresolved.

The intracellular CsA-binding protein, CYP, described by Handschumacher et al. (23) is unique among CsA-binding proteins because of its ability to discriminate between CsA and CsA analogs corresponding to their in vitro immunosuppressive activity. Although in binding studies Quesniaux et al. (25, 26) observed some discrepancies between biological activity and binding to CYP, they concluded that binding of any cyclosporine to CYP was a necessary prerequisite for its biological activity. In these investigations a competitive ELISA was used in which microwell plates were coated with CsA conjugated to BSA, and bound CYP was detected by anti-CYP antiserum.

We have developed a competitive radiometric binding assay in which [3H]CsA was used as the tracer molecule to investigate binding of cyclosporins to (partially purified) CYP. In general, we obtained good agreement between the data reported by Quesniaux et al. and the binding affinities of 12 CsA analogs and metabolites determined in our assay. However, there were some differences: in the ELISA, CsC and CsD bound to CYP with almost the same affinity as to CsA, whereas in our assay the concentrations needed to inhibit [3H]CsA binding by 50% were 1000 μg/L for CsC and >4000 μg/L for CsD, compared with 300 μg/L for CsA. We were also unable to detect binding of M18, M26, and O-acetyl CsA in the concentration range investigated (125–4000 μg/L), and we observed lower affinities relative to CsA for both M1 and M8. One possible explanation for these discrepancies may be a fundamental difference between the two assay systems. Although both were competitive assays, immobilized CsA was used as the substrate for CYP in the ELISA, whereas in our assay both the CYP and the CsA tracer were in solution, which might better reflect the in vivo situation. On the other hand, the degree of purity of the CsA derivatives might also account for some of the quantitative differences observed; this is unlikely for CsC, CsD, CsG, CsH, and O-acetyl CsA because they were obtained in both cases from the same source (Sandoz). However, this possibility cannot be excluded for the metabolites investigated, because their purity was not checked in our laboratory. Nonetheless, the purification scheme in which metabolites from Sandoz were used as reference substances has been published in detail, and isolated metabolites were found to be chromatographically and spectroscopically pure (17); moreover, these metabolites showed the same cross-reactivity with the polyclonal Sandoz RIA as the reference metabolites.

Surprisingly, M17 displayed the highest binding affinity of all CsA analogs and metabolites tested, which was unexpected in light of the controversial reports concerning the biological activity of this metabolite (see above). This finding may be explained by the assumption that binding to CYP is only one prerequisite for immunosuppressive activity. On the other hand, a CYP-binding assay may give better and more objective information about immunosuppressive activity than the various and sometimes controversial in vitro systems. Nevertheless, because trough concentrations of M17 may exceed those of the parent compound several-fold (14–16), the presence of this metabolite should not be neglected in monitoring CsA. In this context, Kunzendorf et al. (32) recently reported significantly higher concentrations of M1 and M17 in nonrejecting kidney-transplant recipients as compared with those in patients undergoing frequent rejections.

The assay protocol of the CYP-binding assay can be optimized to allow measurements of CsA in the pharmacological range, despite the relatively low affinity constant of CYP. With use of a simple extraction procedure for CsA, measurements in biological specimens are possible. The method is suitable for large numbers of samples and may be considered an alternative to the direct measurements by HPLC of all relevant metabolites. In contrast to HPLC, an assay based on CYP as binding protein enables measurement of concentrations of CsA and its metabolites weighted according to their CYP affinities, i.e., immunosuppressive potencies (provided there is indeed a clear correlation between CYP affinity and biological activity). Therefore, such an assay would detect the total immunosuppressive activity in the sample, expressed in micrograms of CsA per liter. Polyclonal RIAs involving antisera with different reactivities for individual metabolites also reflect the patient's metabolism. However, because of various cross-reactivities of the antisera, results obtained by different polyclonal RIAs may differ considerably (33). The CYP-binding assay usually measured CsA concentrations lower than those measured by the polyclonal RIA (CycloTrac); however, in most cases the results with the CYP-binding assay exceeded those with the HPLC or monoclonal RIA by >20%. The ratios between the three sets of results varied considerably according to the relative proportion of parent drug, CYP-binding metabolites, and cross-reacting metabolites.

The data from the pharmacokinetic study showed marked differences between individual patients. In the patient in Figure 3a, the ratio of polyclonal monoclonal RIA increased slowly during the first 12-h period, from 1.0 to 3.1, whereas the ratio of CYP-binding assay monoclonal RIA remained close to 1.0. In contrast, both the ratio of polyclonal monoclonal RIA and CYP-binding assay monoclonal RIA varied considerably in the patient in Figure 3b, indicating higher metabolic activity. Interestingly, a second peak of (bioactive) metabolites could be detected in this patient after 4 h. CsA concentrations calculated from the area under the time/concentration curve revealed pronounced differences between these two patients with respect to the ratios of CYP-binding assay monoclonal RIA and CYP-binding assay polyclonal RIA; however, the ratios of polyclonal monoclonal RIA were of comparable size. From these preliminary data, we hypothesize that patients may be divided in two groups: patients with relatively low concentrations of bioactive CsA metabolites and patients producing larger amounts of bioactive metabolites. This difference can be detected only by the CYP-binding assay and not by the polyclonal RIA. A more detailed analysis of this pharmacokinetic study has been published elsewhere (34).

When we compared daily CsA trough concentrations in kidney-transplant recipients, we observed a high day-to-day variability of concentrations determined by polyclonal RIA and CYP-binding assay, whereas those determined by HPLC varied less (Figure 2). This reflects daily differences in metabolic activity that are unpredictable and may depend on many factors (2). Interestingly, in two cases where regression analysis could be performed, a better correlation was seen between CYP-binding assay and HPLC than between CYP-binding assay and polyclonal RIA. Although, because of the small number of patients investigated, it is not yet possible to correlate these findings to any clinical outcome, we believe that neither monospecific nor polyclonal assays can give sufficient information about a patient's state of immunosuppression.

A similar approach to measuring CsA activity was recently reported by Donnelly and Soldin (35), with use of crude cytosolic extracts, which in addition to CYP contain several other CsA-binding proteins (36). It is difficult to compare their data with ours because the patients' samples used in their studies were not clearly defined. Moreover, the majority of our data originates from a pharmacokinetic study of non-immunosuppressed individuals, for whom clinical conditions differ from those in the post-transplant situation. Nevertheless, because the standard curves look similar in both radiometric assays, CYP is probably also the major binding protein in this receptor assay. Unfortunately, Donnelly and Soldin presented no data concerning the binding of CsA derivatives. Future studies involving larger numbers of well-defined patients' samples should reveal similarities and discrepancies between these two types of CsA receptor-binding assay.

CsA reportedly binds to several cytosolic proteins, including calmodulin (37). However, the importance of calmodulin has been seriously questioned because the results of Colombani et al. (37) have not been reproduced in other laboratories (26, 38, 39, and unpublished data). So far, CYP is the best characterized of all CsA receptors. Moreover, it is present in large quantities in the cytosol, and its biochemical function has recently been detailed (29, 30). Evidence has accumulated that binding to CYP and inhibition of its enzymatic activity may be a crucial part of the action of
CaA. Interestingly, the intracellular receptor for the novel immunosuppressive drug FK-506 exerts the same enzymatic activity as CYP, although it does not bind CaA (40, 41). Apparently, a class of peptidyl-prolyl cis-trans isomerases may be relevant to the transmission of intracellular signals during the process of cell activation. Both CaA and FK-506 share a number of cell-biological properties, and their effects on lymphocytes are frequently indistinguishable (40, 42–44). If indeed peptidyl cis-trans isomerases such as CYP or the FK-506 binding protein are involved in the immunosuppressive action of the corresponding drugs, the binding constants of analogs and metabolites should reflect their biological activity. Thus, assays that involve binding proteins instead of drug-specific antibodies may better enable us to determine the total immunosuppressive potency of their ligands and may be a clinically useful alternative to other current detection methods.

We thank Dr. K. Sewing for his generous gift of purified CaA metabolites, Dr. T. G. Payne for providing CaA analogs, Dr. J. Wolfsdorf for providing clinical samples, M. Horzumsky for her expert technical assistance, Dr. G. Hamilton (1. Chirurgische Universitätsklinik, University of Vienna) for measuring CaA by HPLC, and E. Lachkovics for her critical reading of the manuscript.

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