Cyclosporine and Metabolites in Blood From Renal Allograft Recipients With Nephrotoxicity, Rejection, or Good Renal Function: Comparative High-Performance Liquid Chromatography and Monoclonal Radioimmunoassay Studies

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CYCLOSPORINE (CsA) metabolites, which were originally recognized as interferences in therapeutic monitoring of CsA by radioimmunoassay (RIA), have been shown using high-performance liquid chromatography (HPLC) to be present in high concentration in blood and are now being investigated for possible involvement in immunosuppression and toxicity. Of the more than 12 metabolites isolated and characterized so far,[1-3] attention has focused on the primary metabolites M1 and M17. HPLC measurements have demonstrated high concentrations of M17 and M1 in blood and tissue[4-5] and in vitro immunologic studies in our laboratory have revealed significant immunosuppressive activity by both metabolites.[6-8] An additional primary metabolite, M21, is present at low or undetectable levels in blood and tissue and showed only minimal immunosuppressive potency. Knowledge of the circulating concentrations of secondary metabolites has been limited to reports of in vivo isotopic studies in human volunteers showing that M8 constitutes less than 8% of the cyclosporines in blood and that M10, M13, and M18 each account for less than 2% of the cyclosporines.[3] It is currently not known whether primary or secondary metabolite levels are altered during states of nephrotoxicity and rejection.

The lack of methods for clinical monitoring of CsA metabolites has been a major obstacle in determining the immunosuppressive or toxic contribution of metabolism in CsA therapy. The widely used polyclonal RIA method of monitoring does not accurately quantitate metabolites due to the low and variable cross-reactivity (4% to 32%) with primary and, especially, secondary metabolites.[9] HPLC methods have been developed for the accurate measurement of CsA and the primary metabolites[4,5] but are technically demanding and do not currently quantitate other metabolites that may be present in blood. Monoclonal antibodies for use in RIA methods may provide a selective and widely usable assessment of CsA and its metabolites. Recent reports of two monoclonal antibodies by Quesniaux et al[10,11] show the selective binding of these antibodies to different epitopes of the CsA molecule. One of the monoclonal antibodies reported by these investigators interacts with CsA's biodegradation sites on amino acid residues 1, 4, 6, and 9, and therefore selectively binds native CsA. The other monoclonal antibody reacts weakly or not at all with residues 1, 4, 6, and 9, and therefore binds nonselectively with CsA and its metabolites. The development of RIA methods using these antibodies allows both a specific measure of CsA and a measure of the combined concentration of CsA and its metabolites. These methods should lead to a greater understanding of the

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contribution of metabolites to CsA-induced immunosuppression and nephrotoxicity.

In this study we compare CsA and metabolite profiles by HPLC and monoclonal RIA methods in serial blood samples from renal allograft recipients with good renal function, nephrotoxicity, and graft rejection. The specific aim of the investigation was to determine the contribution of CsA and primary metabolites to the monoclonal RIA measurements in clinical samples. In addition, HPLC fractions were analyzed by the nonselective monoclonal RIA method in order to identify additional metabolites that may be present in blood under different clinical conditions.

MATERIALS AND METHODS

RIA analysis of whole blood samples was performed by the polyclonal RIA (RIA-POLY)[9] and by the more recently developed specific (RIA-SP) and nonspecific (RIA-NS) RIA methods using monoclonal antibodies developed by Quesniaux et al.[10] All RIA reagents were obtained from Sandoz Ltd (Basel, Switzerland) and the manufacturer's protocols were followed without modification. Analysis by monoclonal RIA methods used sample pretreatment with methanol. The precision and recovery of the monoclonal RIAs have been evaluated and shown to be equivalent to the polyclonal RIA method.[12]

The whole blood concentrations of CsA, M17, and M1 were determined by a previously reported HPLC method.[4] Briefly, whole blood was extracted with ether under base and acid conditions. After an additional ether and heptane clean-up, the extract was chromatographed on a cyanopropyl column (DuPont Instruments, Wilmington, DE) at 65°C. The mobile phase (43% acetonitrile/57% H_2O) was pumped at a flow rate of 0.5 mL/min and the column effluent was continuously monitored by ultraviolet detection at 215 nm. Purified CsA, M1, M8, M17, M18, M21, and the internal standard, dihydrocyclosporine C (CsC), were obtained from Sandoz Ltd. The concentrations of M21 were low or undetectable in all of the patient samples analyzed in this study and were therefore not included in the tables and figures. To determine whether M18 may be interfering with our measurement of M1 as suggested by other investigators,[13] we also measured the concentration of M18 in blood by chromatographing the HPLC extracts of blood on a Supelcosil (Supelco Inc, Bellefontee, PA) octyl silica column (column temperature 65°C; 45% acetonitrile/25% methanol/30% H_2O mobile phase; 0.5 mL/min mobile phase). This method, which chromatographically resolved M18 and M1, did not detect M18 (detection limit of 25 ng/mL) in trough blood from the patient in this study. The concentration of M8 could not be determined by either method due to coeluting compounds present in blood, but purified M8 was chromatographed to determine its retention time under our chromatographic conditions. Other secondary metabolites of CsA were not available for chromatographic retention studies.

HPLC fractionation studies were performed to determine the relative RIA-NS reactivity of CsA and metabolites found in blood. Approximately 25 fractions (1 mL) from the HPLC column effluent were collected during the analysis of CsA and primary metabolites, and replicate aliquots (100 μL) were dried for subsequent RIA-NS analysis. The dried aliquots were redissolved in 1 mL of methanol and a 50 μL sample was added to the RIA-NS reaction mixture used in the analysis of methanol supernatants from whole blood. The binding depression was compared to CsA standards and the reactivity of fractions was expressed in CsA-equivalent nanograms per fraction.

We analyzed serial whole blood samples (N = 289) from 23 renal allograft recipients (13 men, nine women) during the first 3 months after transplantation. The patients ranged in age from 22 to 58 years. Patients were treated with CsA, azathioprine, and methylprednisolone as previously described.[4] Briefly, patients were initially treated with 2 to 5 mg/kg of CsA (at 12-hour intervals) as soon as they had achieved good renal function. The dose of CsA was then adjusted based upon therapeutic monitoring of trough whole blood by the RIA-POLY method. Methylprednisolone was given initially as a 300-mg bolus and then adjusted to 20 mg/d by 30 days posttransplant and 10 mg/day by 60 days. Azathioprine (4 mg/kg) was initiated preoperatively and immediately tapered to 0.75 to 1.0 mg/kg and adjusted if the white count fell to <5,000 cells/mL. Good renal function categorized patients with a stable serum creatinine concentration of less than 2.5 mg/dL throughout the 3-month study period. Classification of nephrotoxicity was based on a significant rise in creatinine that reversed in response to a reduction in the CsA dose without antirejection treatment. Rejection was defined as a significant rise in serum creatinine with a reversal in renal dysfunction as a result of antirejection therapy without a change in the CsA dosage. Using this classification system, ten patients were clearly categorized by good function (8), rejection (1) or nephrotoxicity (1). The other 13 patients experienced an episode of renal dysfunction that could not be categorized because both antirejection medication and a reduction in the CsA dose were used in reversing the dysfunction. In addition to serial trough blood monitoring, a pharmacokinetic study was also performed in blood samples collected during a dosing interval for one of the patients with good renal function. Twelve days after transplantation, blood was collected prior to the morning dose and at one, two, four, six, eight, and 12 hours after the dose. All blood samples from the 23 patients were analyzed by the

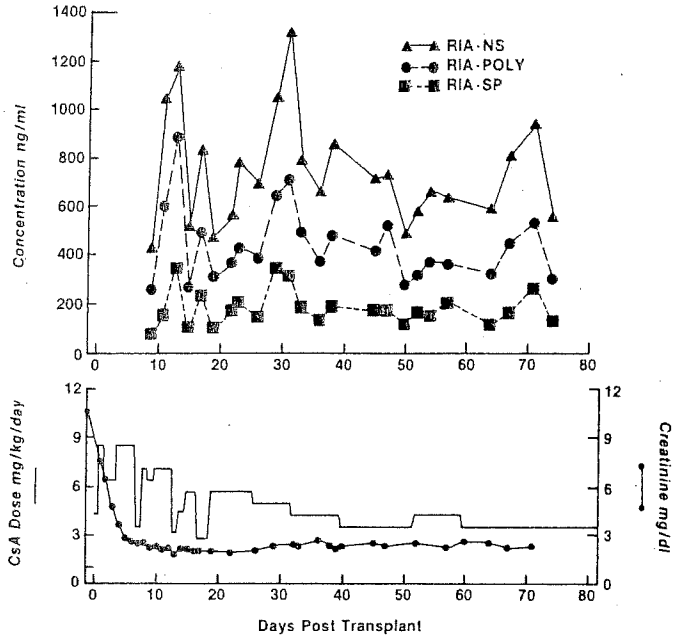

Fig 1. Comparison of RIA methods of monitoring CsA therapy in trough blood obtained during a 3-month course of therapy in a renal allograft recipient with good renal function. The lower panel displays the CsA dose and serum creatinine concentration during this period.

quantitative HPLC and RIA methods described above. HPLC fractionation studies were performed on selected blood samples from patients with good renal function, rejection, or nephrotoxicity.

RESULTS

A comparison of the RIA methods for monitoring CsA therapy in a renal allograft recipient experiencing good renal function is shown in Fig 1. The RIA-NS and RIA-POLY methods result in higher estimates of the CsA concentration than the RIA-SP method, but a comparison of drug monitoring profiles indicates a good correlation between the methods. Studies have also been performed in seven additional patients with good renal function and a similar profile of comparative RIA data was observed for all patients. A statistical summary of both RIA and HPLC measurements for these patients is shown in Table 1. The mean concentrations by RIA followed the order RIA-NS>RIA-POLY>RIA-SP. In paired *t* test comparisons of RIA data, the mean concentrations for each of the methods differed significantly ($P < .005$) from each other. A comparison to the CsA concentration determined by HPLC showed that metabolites constitute an average of 51% and 69% of the concentration determined by the RIA-POLY and RIA-NS methods, respectively. There was, however, no difference between CsA measurements determined by the HPLC and RIA-SP methods, indicating that the RIA-SP method is specific for CsA. The concentration of primary metabolites was measured directly by the HPLC method and showed that the average trough blood concentration of M17 exceeded that of CsA and that

Table 1. Comparison of RIA and HPLC Monitoring of CsA Therapy in Trough Whole Blood From Renal Allograft Recipients With Good Renal Function

Method	Whole Blood Trough Level (ng/mL) Mean ± SD
RIA-NS	514 ± 251
RIA-POLY	320 ± 151
RIA-SP	146 ± 75
HPLC-CsA	158 ± 92
HPLC-M17	260 ± 147
HPLC-M1	86 ± 51

NOTE. Statistical summary for the analysis of 118 trough blood samples from eight renal allograft recipients. The paired *t* test revealed a significant difference ($P < .05$) between each of the RIA methods but no difference in the RIA-SP and HPLC-CsA measurements.

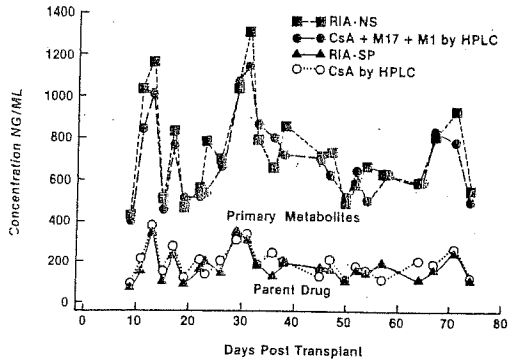

Fig 2. Comparison of RIA and HPLC methods of monitoring CsA and metabolites in trough blood from a renal allograft recipient with good renal function. The concentrations determined by the RIA-SP and HPLC-CsA methods both assess the level of parent drug and the screened area represents the total concentration of M1 and M17 determined by HPLC.

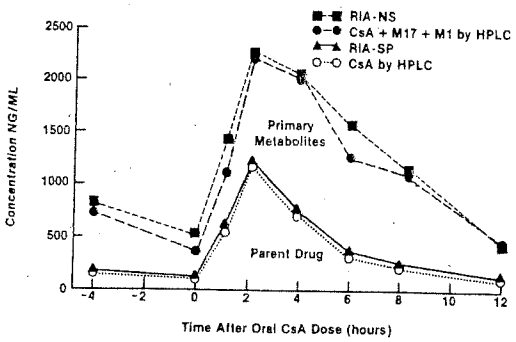

Fig 3. Pharmacokinetic profile of monoclonal RIA and HPLC measurements of CsA and metabolites during a 12-hour period after oral administration of CsA to a renal allograft recipient with good renal function. The screened area represents the total concentration of M1 and M17 determined by HPLC.

M1 was present, but at lower concentrations than M17.

Parallel RIA and HPLC measurements also allowed an assessment of the levels of parent drug, primary metabolites, and other metabolites in blood. In Fig 2 the trough blood concentration of parent drug is represented by both the RIA-SP or HPLC-CsA measurement. The total concentration of CsA+M17+M1 by HPLC correlated well with the concentration determined by the RIA-NS method. The screened area thus represents the amount of primary metabolites, M17 and M1, in the patient's blood. The primary metabolites accounted for all of the difference between the RIA-NS measurement and the specific measurement of parent drug, indicating that M17 and M1 were the only significant contributors to the crossreactivity observed in the RIA-NS measurement in this period. A close quantitative relationship between the RIA-NS and CsA+M17+M1 measurements was observed in blood of all patients with good renal function. The mean concentration of CsA+M17+M1 for 118 samples from the eight patients with good renal function was 504 ± 245 ng/mL compared to 514 ± 251 ng/mL for the RIA-NS measurement (correlation coefficient = 0.90).

The close correlation between RIA-NS and CsA+M17+M1 measurements was also determined in serial blood samples obtained during a dosing interval for one of the patients. The pharmacokinetic profile (Fig 3) again showed that the primary metabolites accounted for most or all of the metabolite crossreactivity in the RIA-NS assay, indicating that secondary metabolites which are measured by the RIA-NS method did not accumulate in blood to an appreciable extent during the dosing interval.

To determine whether additional metabolites are present in blood during CsA therapy, trough blood samples from several patients with good renal function were fractionated by HPLC and the fractions were assayed by the RIA-NS method. A representative study is shown in Fig 4. Ultraviolet monitoring (215 nm) revealed chromatographic peaks for CsA, the primary metabolites and the exogenously added internal standard (CsC). The corresponding RIA-NS reactivity of the HPLC fractions is displayed in the lower panel and shows that fractions containing CsA, M17, and M1 accounted for over 95% of the RIA-NS reactivity in trough blood. Reactivity in fractions 20 and 21 was due to CsC, which was added during the sample preparation procedure. Only a minor amount of RIA-NS

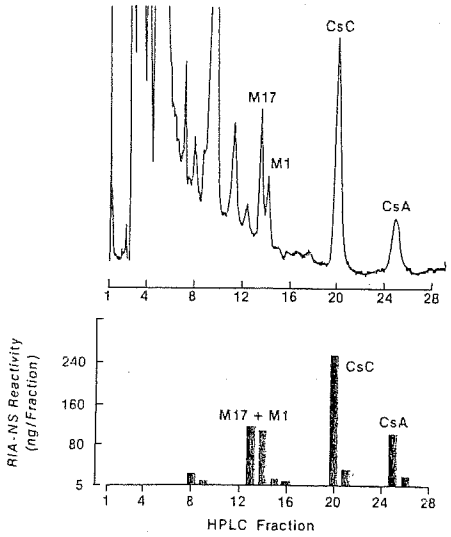

Fig 4. HPLC chromatogram and fractionation study of trough blood from a renal allograft recipient with good renal function. The upper panel shows the column effluent monitoring by ultraviolet detection at 215 nm. Concentrations of CsA (118 ng/mL), M17 (320 ng/mL), and M1 (165 ng/mL) were determined by the HPLC method. The corresponding RIA-NS reactivity of fractions collected during the chromatographic run is displayed in the lower panel.

reactivity was detected in fractions 8 and 9, suggesting that a low level of a more polar, secondary metabolite may have been present in the blood. CsA, M17, and M1 were therefore the major source of the RIA-NS reactivity in blood from patients with good renal function.

RIA and HPLC analyses were also performed on serial blood samples from a renal allograft recipient with CsA-related nephrotoxicity. As seen in Fig 5, the rise in serum creatinine from 1.2 to 3.3 mg/dL was proceeded by an elevation in cyclosporines measured by the RIA methods. A reduction in the CsA dose from 11.9 to 4.8 mg/kg/d without concomitant antirejection therapy resulted in a decrease in the serum creatinine to prenephrotoxic levels. The upper panel, which displays RIA monitoring results during the same period, shows little change in the parent drug level (RIA-SP) during the period of nephrotoxicity. A more significant rise was observed in the RIA-POLY data, but the most dramatic increase was observed in the RIA-NS determined concentrations, which returned to normal after a reduction in the CsA dose. Com-

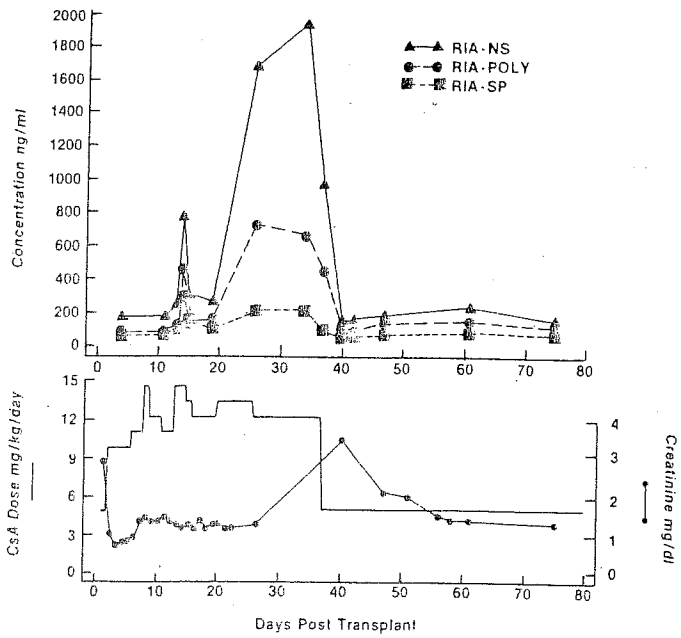

Fig 5. Comparison of RIA methods of monitoring CsA therapy in a renal allograft recipient with an episode of CsA-related nephrotoxicity. The CsA dose and serum creatinine concentrations are displayed in the lower panel.

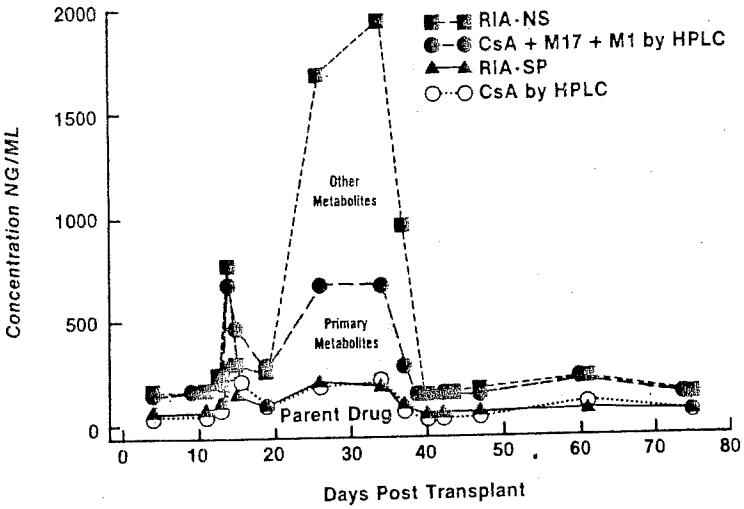

Fig 6. Comparison of monoclonal RIA and HPLC measurements of CsA and metabolites in trough blood from a renal allograft recipient with CsA-related nephrotoxicity. The screened area represents the total concentration of the M17 and M1 determined by HPLC.

parative monoclonal RIA and HPLC measurements displayed in Fig 6 showed further that the primary metabolites (screened area) increased during the nephrotoxic episode but did not fully account for the concentration of crossreacting metabolites measured by the RIA-NS method. To verify whether secondary metabolites accumulated during the nephrotoxic episode, the blood was fractionated by HPLC and the fractions assayed by RIA-NS. Figure 7 displays the results of trough blood samples collected prior to and during nephrotoxicity. The RIA-NS reactivity in blood taken prior to nephrotoxicity again showed a profile similar to that seen in patients with good renal function, where CsA, M17, and M1 were the major contributors to the RIA-NS reactivity. During nephrotoxicity,

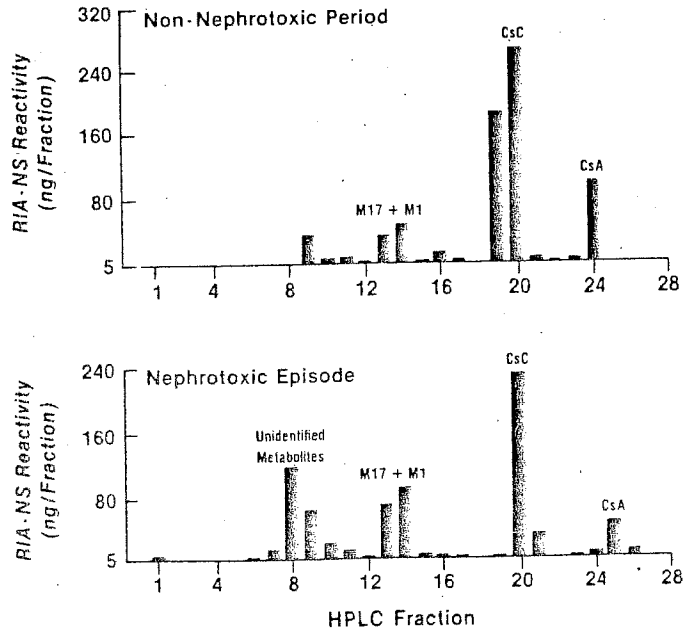

Fig 7. RIA-NS reactivity of HPLC fractions of blood during nephrotoxic and non-nephrotoxic states. The upper panel displays a study of trough blood obtained on day 13 with concentrations of CsA (76 ng/mL), M17 (85 ng/mL), and M1 (68 ng/mL) determined by HPLC. Analysis of trough blood obtained 34 days after the transplant is shown in the lower panel with CsA (223 ng/mL), M17 (348 ng/mL), and M1 (102 ng/mL) concentrations determined by HPLC.

however, 53% of the RIA-NS reactivity was measured in fractions 7 to 10. The earlier retention time of these metabolites indicates that these metabolites are more polar than M17. These polar metabolites have not been structurally identified, but HPLC studies using purified M8 show similar chromatographic retention times for M8 and the unidentified polar metabolites. The disproportionate increase in polar metabolites was found in HPLC fractionation studies of blood samples collected throughout the nephrotoxic period prior to the reduction in CsA dose. An index of this accumulation of polar metabolites in these samples was the high RIA-NS/RIA-SP concentration ratio for whole blood. During nephrotoxicity this ratio ranged from 8.3 to 14.3 while prior to or following nephrotoxicity the ratio ranged from 2.0 to 3.2. This latter ratio range was similar to the ratios (1.6-3.4) in 118 blood samples from the eight patients with good renal function. Therefore, the fractionation studies confirm the presence of polar metabolites during nephrotoxicity and the RIA-NS/RIA-SP ratio provided an index of the nephrotoxic event in this patient.

The trough blood concentrations of CsA and metabolites were also studied in serial samples from a patient with renal dysfunction due to a graft rejection episode. Figure 8 shows monoclonal RIA and HPLC monitoring profiles for a renal allograft recipient who had a rise in the level of creatinine from 2.0 to 4.4 mg/dL. During treatment with antilymphoblast globulin without any adjustment in the CsA dose, the serum creatinine level fell to 2.3 mg/dL and stabilized. The primary metabolites were the major contributors to the crossreactivity in the RIA-NS assay throughout the course of therapy. Polar metabolites did not accumulate at any time during the clinical course, suggesting that renal dysfunction induced by rejection does not result in the appearance of polar metabolites. Polar metabolites were also not detected by comparative HPLC and RIA measurements in serial blood samples (N = 144) from an additional 13 patients treated with antilymphoblast globulin, but the episodes of renal dysfunction in

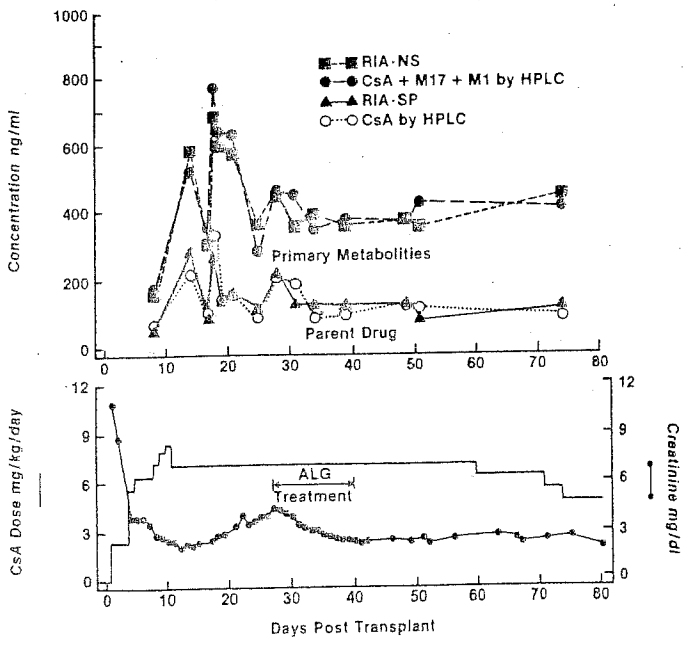

Fig 8. Comparison of monoclonal RIA and HPLC measurements of CsA and its metabolites in trough blood from a renal allograft recipient with a rejection episode. The screened area represents the total concentration of M1 and M17 determined by HPLC.

these patients could not be clearly defined as rejection because the CsA dose was reduced during treatment of the renal dysfunction.

DISCUSSION

Efforts to optimize immunosuppression and minimize toxicity during CsA therapy have been complicated by the extensive bioconversion of CsA to metabolites and by the lack of specificity of the widely used polyclonal RIA method. Monoclonal RIA methods now provide selective monitoring techniques for both clinical practice and research. The RIA-SP method allows a specific measure of native CsA and can replace the labor-intensive HPLC methods that have been used in therapeutic monitoring and research by some investigators. The RIA-NS analysis offers an additional quantitation of metabolites, which may be important in the therapy of the patient. We know from prior HPLC studies that the primary metabolites M17 and M1 are present in high concentrations in blood and tissue during therapy.[4,5] HPLC methods have not, however, allowed a determination of more polar metabolites that may be circulating in the blood of CsA-treated patients. HPLC separation of CsA and its metabolites combined with RIA-NS monitoring of fractions provides a sensitive technique for detecting other metabolites that may accumulate under differing clinical conditions. Application of this technique in the current study shows that M17 and M1 are the major metabolites in blood in patients with either good renal function or rejection. This finding along with the previous demonstration of in vitro immunosuppressive properties of these metabolites leads us to conclude that CsA, M17, and M1 contribute to immunosuppressive therapy in these patients.

Our observations in a single patient with nephrotoxicity show that other metabolites may accumulate in blood and suggest that metabolites may be an index or possibly a cause of nephrotoxicity. It is of particular interest that the accumulation of metabolites during nephrotoxicity in this patient was not accompanied by a proportional increase in the concentration of CsA. This would suggest that the parent drug monitoring may not correlate as well with nephrotoxicity, at least for this patient. Yee et al[14] have previously suggested that metabolites accumulate during nephrotoxicity and may be an index of the event. In a study of CsA-treated bone marrow recipients, these investigators found that the probability of nephrotoxicity was not significantly influenced by the CsA concentration measured by HPLC but did correlate with the RIA-POLY method that measures CsA and metabolites. It should be noted that these findings were based on plasma analyses and whole blood studies were not performed. Our study, however, does show that the metabolite pattern in the blood during nephrotoxicity can differ from the pattern in non-nephrotoxic patients. Further identification of the metabolites that may be associated with nephrotoxicity will be necessary and may be possible with recently developed HPLC methods that measure primary as well as secondary metabolites in blood.[15]

The actual role of metabolites in nephrotoxicity is currently unknown but has been studied to a limited extent in rats. Luke et al,[16] for instance, in studies with the perfused rat kidney, found that CsA did not significantly alter renal function and suggested that nephrotoxicity in vivo may be due to a toxic metabolite. More direct studies by Ryffel et al[17] showed that CsA administered to spontaneously hypertensive rats at 10 mg/kg/d for 21 days produced renal morphologic changes consistent with nephrotoxicity, but that equal doses of M17 did not. Unfortunately, these studies were based on oral administration of M17, which they showed to be poorly absorbed. The major limitations of this study are that whole blood levels of CsA and M17 were not measured and that other metabolites were not evaluated. Other investigators have shown differences in nephrotoxicity between

the rat model and humans. Rats, for instance, require much higher doses of CsA in order to induce a nephrotoxic state that appears both functionally and histologically different from toxicity observed in man.[18] It is interesting to note that rats, in addition to their resistance to the nephrotoxic effect of CsA, also differ from humans in their blood and tissue levels of metabolites. In recent studies,[19] we have found that CsA is the predominant cyclosporine in blood, kidney, and liver of rats and that much lower concentrations of M17 and M1 are present. This pattern differs significantly from the pattern of metabolites and parent drug in human blood and tissue. In addition, HPLC fractionation studies did not show any evidence of the accumulation of polar metabolites in rat blood, even when rats were treated with 75 mg/kg/d for seven days.[19] Therefore CsA concentrations predominate over metabolite levels in rats whereas the opposite is true in humans. Further study of the differences in toxicity and metabolism between the rat and man is needed and may lead to important insights into the mechanism of nephrotoxicity.

Metabolites must also be studied further in a large series of nephrotoxic patients in order to identify metabolites that may be present during toxicity and to determine any causal relationship between metabolites and toxicity. An important goal of the human and animal studies will be to determine whether metabolic or synthetic modification of CsA's structure results in a change in toxic and immunosuppressive properties. We already know that M17 and M1 have somewhat less immunosuppressive potency than CsA yet much greater potency than M8, a secondary metabolite.[6] We must also determine whether CsA and its metabolites vary in their toxic potential and whether these differences are separable from their immunosuppressive properties. These studies may ultimately lead to the development of new drugs that are immunosuppressive, but less toxic to the patient.

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