0041-1337/86/4203-0262$02.00/0
TRANSPLANTATION

Vol. 42, No. 3
Printed in U.S.A.

IMMUNOSUPPRESSIVE METABOLITES OF CYCLOSPORINE IN THE BLOOD OF RENAL ALLOGRAFT RECIPIENTS[1]

THOMAS G. ROSANO,[2] BRIAN M. FREED,[3] JAMES CERILLI,[4] AND NEIL LEMPERT[4]

Departments of Clinical Chemistry, Biochemistry, and Surgery, Albany Medical College and Albany Medical Center Hospital, Albany, New York 12208

Cyclosporine levels by radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC) were monitored in serial blood samples (n=177) from 11 renal allograft recipients. HPLC analysis revealed three primary metabolites of CsA (M17, M1, and M21) in peak and trough blood samples; M17 was the preponderant metabolite. In 4 patients on whom serial metabolite assays were performed, M17 was found in the blood at 86–2004 ng/ml; M1 and M21 were found at up to 100 ng/ml. The immunosuppressive properties of purified metabolites M1, M17, M21, and M8 (which was not detected in the blood) were compared with CsA. M17—and, to a lesser extent, M1 and M21—were found to inhibit the in vitro response of human mononuclear cells in the mixed leukocyte culture and in mitogen (phytohemagglutinin [PHA], concanavalin A [Con A], and pokeweed mitogen [PWM]) assays at 1000 ng/ml. M8 exhibited no in vitro inhibitory activity. M17 was further tested at 10–1000 ng/ml in PHA and mixed lymphocyte culture (MLC) assays. M17 had considerably less inhibitory activity (12–43%) than CsA (18–70%) in the PHA assay. However, in MLC experiments M17 blocked the proliferative response by 39–72% at 100–800 ng/ml, which approached the degree of inhibition exhibited by CsA (63–87%). In 34 of 37 (92%) patient blood samples, the level of metabolite M17 was found to exceed the parent drug level and could not be measured accurately by RIA. The observed in vitro immunosuppressive activity of metabolites (particularly M17) and their presence in the blood of renal allograft recipients suggest a possible role for these metabolites in the immunopharmacology of CsA.

Cyclosporine (CsA)* is a potent immunosuppressive agent that selectively inhibits T cell responses. CsA reduces the incidence of rejection episodes in renal allograft recipients (*1–3*), decreases the requirement for adjunctive steroid therapy (*2, 4, 5*), and has resulted in a significant improvement in graft survival (*2, 6–8*). The major drawback to the use of CsA is the relatively high incidence of nephrotoxicity, which has necessitated the careful monitoring of CsA levels in the blood. Unfortunately, it has not been possible to establish a well-defined therapeutic window for CsA because nephrotoxicity may occur over a wide range of CsA levels (*9*).

Very little is known about the molecular mechanisms by which CsA induces immunosuppression or nephrotoxicity. However, it has recently been shown that induction of cytochrome P-450 with Aroclor 1254, which presumably causes an increase in metabolism of CsA, results in a decrease in nephrotoxicity without affecting the level of immunosuppression (*10–12*)—the authors suggest that renal tubular damage is due to the parent CsA molecule rather than the metabolites. It is therefore possible that the inability to establish a therapeutic window for CsA may be due to the fact that the relative contributions of CsA metabolites in immunosuppression and nephrotoxicity are not known.

It is often assumed that the radioimmunoassay (RIA) method measures the amount of CsA and metabolites in the blood (*13–15*). Several investigators have attempted to use the difference between the CsA level determined by high-performance liquid chromatography (HPLC), which is specific for CsA, and RIA as an indication of the level of metabolites in the blood (*16, 17*). However, of the nine CsA metabolites isolated from human urine (*18*), only one (M17) has been shown to exhibit significant crossreactivity with the antibody used in the RIA (*19*). Furthermore, the immunosuppressive properties of these metabolites and their presence in the blood of renal allograft recipients have not been demonstrated. We report here on the HPLC detection of three primary metabolites of CsA in the blood of renal allograft recipients, and the degree to which these metabolites suppress human T cell responses in vitro.

MATERIALS AND METHODS

Patients. From January 1984 to January 1985, 42 end-stage renal disease patients received cadaveric renal allografts with CsA as the primary immunosuppressive agent. From January to September 1984, CsA (5 mg/kg/day in two doses given at 12-hr intervals) was initiated when the patient had achieved good renal function. Prednisone was given initially as a 300-mg bolus and then adjusted to give the patient 30 mg/day during the first month posttransplant and 20 mg/day during the second month. Patients with good renal function were then maintained on 10 mg prednisone/day. Whole-blood RIA for CsA was performed daily while the patient was in the hospital, then thrice-weekly on an outpatient basis during the next month. The frequency of CsA monitoring was decreased to

[1] This work was supported by the James Hilton Manning and Emma Austin Manning Foundation, and by the National Kidney Foundation of Northeastern New York.

[2] Address correspondence to: Thomas G. Rosano, Ph.D., Department of Clinical Chemistry, Albany Medical Center Hospital, Albany, NY 12208.

[3] From the Transplantation Immunology Laboratory, Department of Surgery, Albany Medical College, Albany, New York 12208.

[4] From the Division of Renal Transplantation, Department of Surgery, Albany Medical College, Albany, New York 12208.

* Abbreviations used: Con A, concanavalin A; CsA, cyclosporine A; CsC, cyclosporine C; CsD, cyclosporine D; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; ^{3}H-thymidine, tritiated thymidine; M1, (4-hydroxy-N-methyl-L-leucine^9) cyclosporine; M8, ([N-methyl-]2-amino-3,8-dihydroxy-6,7-dehydro-4-methyl-octenoic acid^1)(4-hydroxy-N-methyl-L-leucine^9)cyclosporine; M17, ([N-methyl-]2-amino-3,8-dihydroxy-6,7-dehydro-4-methyl-octenoic acid^1) cyclosporine; M21, (N-demethylated leucine^4) cyclosporine; MLC, mixed leukocyte culture; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RIA, radioimmunoassay.

weekly, biweekly, and then to monthly in the absence of complications. Patients transplanted between September 1984 and January 1985 also received a 4 mg/kg of azathioprine preoperatively, which was immediately tapered to 1 mg/kg and adjusted when needed to maintain a WBC count of >5,000 cells/cc.

RIA analysis of CsA. RIA analysis (*19*) of whole-blood hemolysates was performed with reagents and methods from Sandoz Ltd., Basel, Switzerland. All analyses were performed in duplicate. The coefficient of variation for CsA analysis by this method ranged from 4 to 11%.

HPLC analysis of CsA. Quantitative analysis of CsA levels by HPLC was performed as previously described (*15*). Briefly, isocratic separation of CsA and the internal standard (CsD) was performed on a Zorbax CN column (DuPont Instruments, Wilmington, DE) with a mobile phase containing acetonitrile/methanol/water (34/18/48 by volume). A column flow rate of 0.75 ml/min at 60°C was maintained. Sample extraction was performed by the method of Carruthers et al. (*14*). The coefficient of variation for CsA analysis by this method ranged from 4% to 6%.

HPLC analysis of CsA metabolites. The HPLC analysis of CsA described above did not allow adequate separation of CsA metabolites. To identify and quantitate metabolites, the following HPLC method was developed. Sample preparation was performed by a modification of the ether extraction procedure reported by Carruthers et al. (*14*). Modifications included the substitution of CsC as the internal standard and ether extraction under alkaline conditions prior to acid conditions. HPLC-grade diethyl ether was necessary to eliminate components in the ether that coelute with CsA metabolites. Recovery of the metabolites was >90% under these conditions. Chromatographic separation of these metabolites was performed with a reverse-phase CN column (DuPont Instruments, Wilmington, DE) maintained at 70°C. The mobile phase consisted of 35% (v/v) acetonitrile/water and its flow rate was 1.5 ml/min. UV-detection was set at 210 nm with 0.02 attenuation units full scale. The peak height ratio method was used for quantitation. Purified M1, M17, and M21 (*18*) used in identification and quantitation of CsA metabolites were a generous gift of G. Maurer, Ph.D., and W. Neiderberger, Ph.D., (Biopharmaceutical Dept, Sandoz Ltd, Basel, Switzerland). The metabolites were reconstituted in absolute methanol and the concentrations determined gravimetrically with an automatic electrobalance (Cahn Model 4700, Cerritos, CA). For standard material, blood from nonmedicated volunteers was spiked with purified M1, M17, and M21.

Crossreactivity of M17 in the RIA. Crossreactivity of M17 in the RIA was determined by dilution studies with hemolyzed blood samples containing pure CsA or M17. Blood used in the study was obtained from healthy nonmedicated volunteers. The initial concentrations of CsA and M17 used in the dilution studies were 1900 ng/ml and 2390 ng/ml, respectively.

Renal transplant patient samples. Serial CsA levels were analyzed in whole-blood hemolysates from 11 patients who had received cadaveric renal allografts. CsA levels were monitored by RIA and HPLC for each patient over a period ranging from 12 to 83 days. Except for the peak blood sample (drawn 4 hr after the oral dose) all bloods were drawn just prior to the subsequent dose and are referred to as trough samples. Serial samples from patients 2, 3, 10, and 11 were also analyzed for the level of CsA metabolites. All samples were stored frozen until analyzed.

Assays for in vitro immunosuppressive activity. Whole blood (20 ml) was collected in heparinized syringes from normal volunteers and diluted with an equal volume of Hanks' balanced salt solution (HBSS). The blood was then layered over 10 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at 400 ×g for 40 min. The peripheral blood mononuclear cells (PBMC) were removed from the Ficoll interface and washed three times in HBSS. The cells were resuspended to 10^6 cells/ml in RPMI-1640 (MA Bioproducts, Walkersville, MD) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. In mitogen assays, 2×10^5 cells (0.2 ml) in media supplemented with 10% agamma human serum (KC Biologicals, Lenexa, KS) were plated in 96-well flat-bottomed plates (Costar). The cells were stimulated with phytohemagglutinin (PHA, Burroughs-Wellcome HA-15, 250 μg/ml), concanavalin A (Con A, Sigma, 10 μg/ml), or pokeweed mitogen (PWM, Gibco, 100 μg/ml) and incubated at 37°C in humidified 6% CO_2/94% air. Then 48 hr later (6 days later in the PWM assay) the wells were pulsed with 0.6 μCi of ^{3}H-thymidine (NEN Research Products, Boston, MA; specific activity 1.9 Ci/mmol) for 18 hr and harvested onto glass fiber filters using a Mash II cell harvester (MA Bioproducts). The disks were placed into scintillation vials with 3 ml of scintillation fluid (Econofluor, NEN) and counted in a liquid scintillation counter (Beckman).

The mixed leukocyte culture (MLC) was performed by culturing 10^5 responder PBMC with 10^5 irradiated (3000 rads, cesium source) stimulator cells in media supplemented with 20% autologous plasma in flat-bottomed tissue culture plates. The plates were incubated for 5 days at 37°C, then pulsed and harvested as described for the mitogen assays.

The effects of CsA and its metabolites on proliferation in the in vitro assays were determined by adding the agents to the cultures prior to the addition of the mitogens or stimulator cells. CsA, M1, M8, M17, and M21 were reconstituted in methanol and diluted in RPMI-1640 to the concentrations shown in the Figures 1–6. Methanol diluted in the same manner (300–500 fold) exhibited no inhibitory activity in any of the assays.

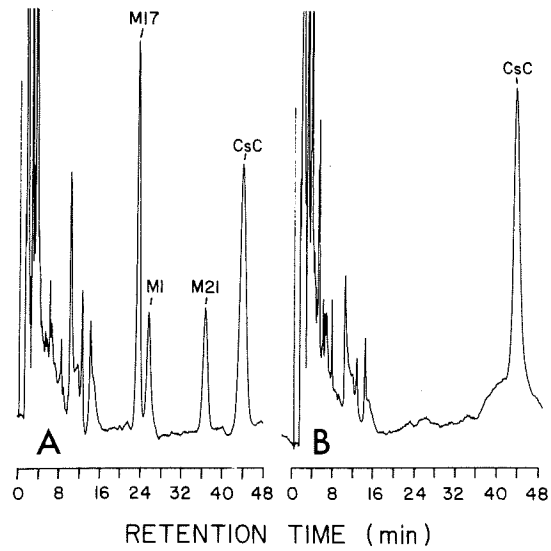

FIGURE 1. Separation of CsA metabolites by HPLC. Chromatogram (A) is from blood spiked with purified M17 (870 ng/ml), M1 (290 ng/ml), and M21 (790 ng/ml). Chromatogram (B) is a control blood obtained from a nonmedicated volunteer. CsC was included in both samples as the internal standard.

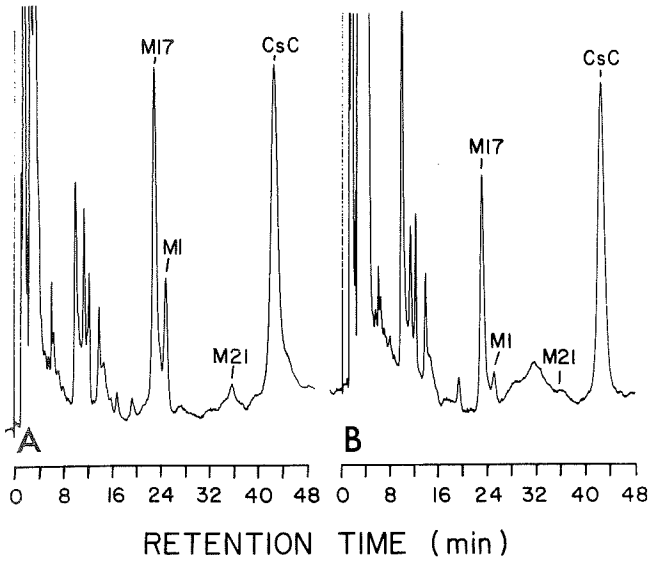

FIGURE 2. HPLC analysis of CsA metabolites in peak and trough samples from a renal allograft recipient. Samples were drawn 4 hr (A, peak) and 24 h (B, trough) after an oral dose of CsA. The peak sample contained M17 (620 ng/ml), M1 (260 ng/ml), and M21 (80 ng/ml). The trough sample contained M17 (464 ng/ml) and M1 (53 ng/ml), but no detectable amount of M21. The CsA peak elutes at approximately 60 min and is not shown in these chromatograms.

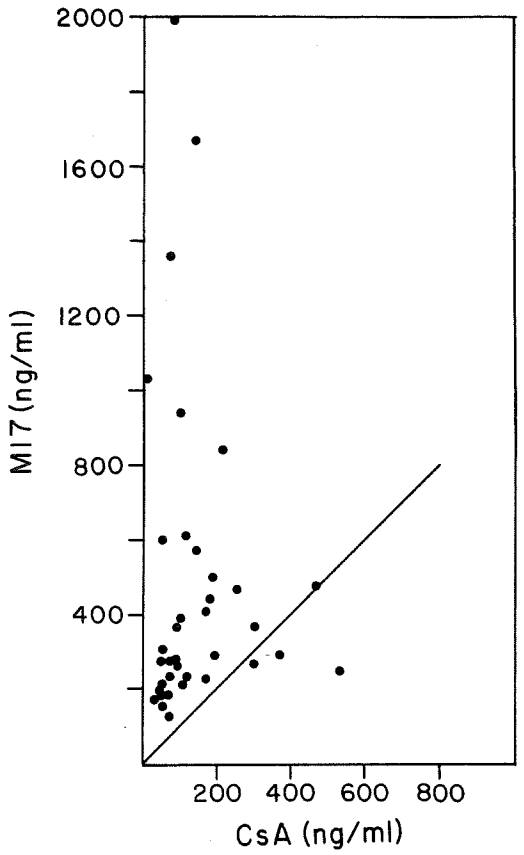

FIGURE 3. Comparison of CsA and M17 levels in renal allograft recipients. Serial trough blood samples (n=37) were obtained from 4 patients and analyzed for both CsA and metabolites by HPLC. The diagonal line represents equivalence between CsA and M17.

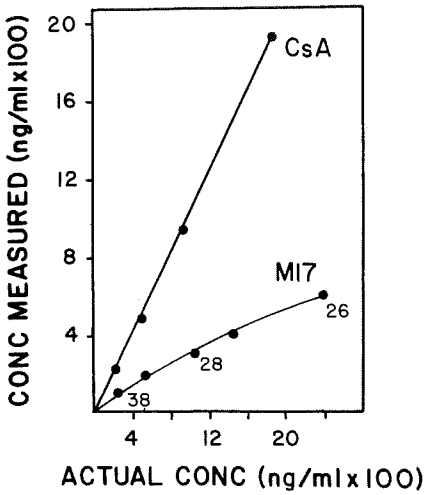

FIGURE 4. Crossreactivity of M17 in the CsA radioimmunoassay. CsA and M17 were added to whole blood in the concentrations shown. The blood samples were analyzed by RIA for apparent CsA concentration. The numbers adjacent to the points represent the percentage of the actual M17 concentration detected by RIA.

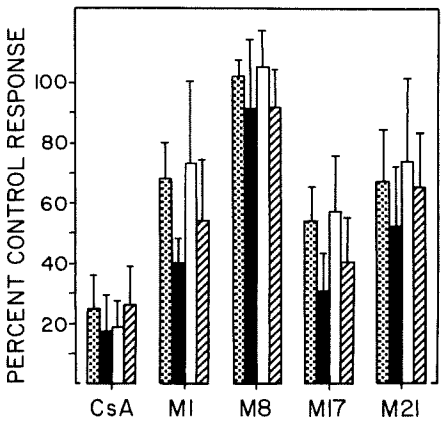

FIGURE 5. Immunosuppressive activity of CsA and metabolites in vitro. Data are expressed as the mean ±SD. The compounds were reconstituted in absolute methanol and diluted to a final concentration of 1000 ng/ml in the culture wells. Methanol diluted appropriately exhibited no inhibitory activity (control response). Data are presented as the percentage of control response in PHA (checkered boxes, n=6), Con A (black boxes, n=6), PWM (open boxes, n=4), and MLC (slashed boxes, n=12) assays. Individual assays were performed in triplicate.

Statistics. The MLC data were analyzed by Student's *t* test for unpaired data.

RESULTS

Comparison of CsA levels by RIA and HPLC. A comparison of CsA levels measured by HPLC, and RIA in 177 blood samples from 11 renal allograft recipients was performed. In all the samples, the level of CsA measured by RIA was greater than the level measured by HPLC (data not shown). A similar comparison with CsA-spiked blood from nonmedicated volunteers resulted in equivalent levels by RIA and HPLC, indicating a high degree of correlation between the two assays in the absence of metabolites. The percentage of the RIA levels that was actually due to CsA is shown in Table 1. From these studies, we determined that the actual amount of CsA in the blood varied from 6% to 81% of the level measured by RIA.

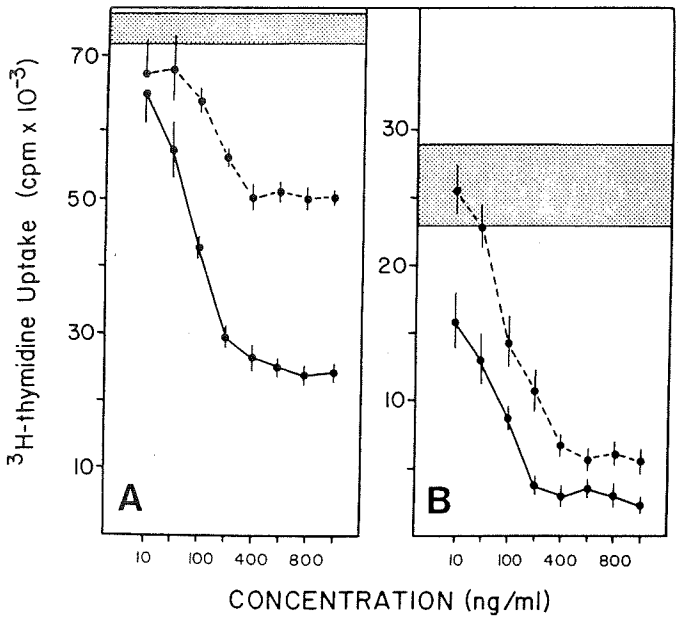

FIGURE 6. Effect of CsA and M17 on PHA and MLC responses. CsA and M17 were added to the cultures at 10–1000 ng/ml. The cells were then stimulated with PHA (A) or allogeneic cells (B). The mean ±SD of ^{3}H-thymidine uptake for cells treated with CsA (●——●) and M17 (●- - -●) are shown in reference to 1 SD of the control response (shaded area).

TABLE 1. Percentage of RIA level resulting from CsA

Patient	n	[CsA (HPLC)/CsA (RIA)] × 100		Days posttransplant
		Mean	Range	
1	21	38	(23–51)	2–30
2	10	14	(6–24)	10–20
3	6	62	(34–78)	3–12
4	22	44	(31–70)	3–24
5	24	36	(23–60)	1–24
6	13	48	(34–64)	107–186
7	32	59	(36–81)	3–83
8	13	36	(22–44)	244–272
9	16	37	(28–54)	5–124
10	14	33	(12–59)	239–256
11	6	39	(28–60)	158–179
Control[a]	20	98	(91–105)	—

[a] Control data were obtained from 20 analyses of a CsA-supplemented blood pool from nonmedicated volunteers.

Variations in this percentage were observed among the patients and even in blood samples from the same patient drawn on different days.

Identification and quantitation of CsA metabolites in blood. Analysis of blood samples using the HPLC method for CsA revealed additional unresolved peaks with retention times similar to those of the primary metabolites of CsA (M1, M17, M21). A chromatographic method was therefore developed for the measurement of primary metabolites. This procedure allowed the separation of M1, M17, and M21 in blood spiked with purified metabolites (Fig. 1A). A typical analysis of blood for nonmedicated volunteers demonstrated the specificity of the assay (Fig. 1B). The primary metabolites of CsA were identified in blood from renal allograft recipients treated with CsA. Typical metabolite patterns in peak and trough blood samples are shown in Figure 2. M17 was the preponderant metabolite in peak and trough blood samples, followed by lesser amounts of M1. M21 was found only in blood samples containing high levels of M17. The concentration of metabolites was determined in serial trough samples (n=37) in 4 renal allograft recipients. The level of M17 ranged from 80 ng/ml to 2004 ng/ml. Levels of M17 above 800 ng/ml were observed in blood samples from only one of the four patients. M1 and M21 were found at concentrations below 100 ng/ml. No significant correlation was observed between the level of M17 and CsA as measured by HPLC (Fig. 3). In 34 of 37 samples, the level of M17 exceeded the level of CsA.

Immunocrossreactivity of CsA metabolite 17. To determine the extent of RIA crossreactivity by M17, we performed a series of RIA analyses on medication-free blood spiked with pure CsA or M17. In a comparative dilution experiment, the measurement of CsA was linear over the entire dilution range while dilutions of M17 showed a nonlinear response (Fig. 4). The absolute concentration of M17 was greatly underestimated by the RIA method. RIA crossreactivity ranged from 26% to 38%, and it decreased as the concentration of M17 increased. The extent of error in the RIA estimation of M17 levels therefore increased with increasing concentrations of the metabolite.

In vitro immunosuppressive activity of CsA and metabolites. CsA and four metabolites (M1, M8, M17, and M21) were assayed initially at 1000 ng/ml for in vitro inhibition of the response of PBMC to PHA, Con A, PWM, and allogeneic cells (MLC). As can be seen in Figure 5, CsA inhibited all responses to 17–26% of the control. M17 inhibited PHA and PWM responses to only 54–57% of control, but inhibition in the Con A (30.7±12.7%) and MLC (39.7±15.8%) assays approached the level of inhibition exhibited by CsA. M1 and M21 exhibited significant immunosuppressive activity in the PHA, Con A, and MLC assays, but were considerably less inhibitory than either CsA or M17. M8 exhibited virtually no suppressive activity.

The MLC is of particular interest in these studies in that it is an in vitro model of the immune response to allografts. CsA exhibited significantly more suppressive activity (25.9±12.6% of the control) than M17 (39.7±15.8%; P=0.02). However, M17 was significantly more suppressive than either M1 (54.2±17.5%; P=0.04) or M21 (65.1±17.7%; P=0.001). Neither CsA nor any of the metabolites were toxic to the cells at the concentrations tested (data not shown).

CsA and M17 were then assayed in PHA and MLC experiments to determine their inhibitory effects over a range of therapeutic concentrations. As can be seen in Figure 6, CsA was considerably more suppressive than M17 in the PHA assay. However, in the MLC, M17 was only slightly less inhibitory than CsA in the 100–1000 ng/ml range.

DISCUSSION

The importance of CsA metabolites in the immunopharmacology of CsA therapy is largely unknown. Based on findings of MLC-inhibitory activity in the serum of CsA-treated renal allograft recipients when only low levels of the parent drug were detected, it has been suggested that metabolites of CsA may contribute significant immunosuppressive activity (*20, 21*). Freeman et al. (*22*) have recently identified metabolites M17 and M18 in the blood of normal individuals treated with CsA and phenytoin, but the metabolism of CsA in the absence of phenytoin was not studied. Maurer identified metabolites

M1, M8, M10, M17, M18, and M21 in the blood of normal volunteers given oral doses of CsA (*23*). We now report the identification of three CsA metabolites (M1, M17, and M21) in the blood of renal allograft recipients treated with CsA; all three are primary metabolites according to the pathways proposed by Maurer et al. (*18*). M17, formed by hydroxylation of the side chain of amino acid 1, appeared to be the preponderant metabolite. This finding is consistent with data reported by Maurer on the metabolism of ^{3}H-cyclosporine by human subjects following an oral administration of the drug (*23*). M1, formed by hydroxylation of the methyl leucine in position 8, was present in lower concentrations than M17—and M21, a demethylated derivative of CsA, was detectable only when high levels of M17 were found. Our chromatographic analysis demonstrated that the level of metabolites usually exceeded the level of the parent compound in patients on CsA therapy. A significant amount of metabolites appeared within 4 hr of oral administration of CsA, suggesting the drug was metabolized rapidly.

Other investigators (*13, 14*) have demonstrated crossreactive interference in the RIA, presumed to be due to CsA metabolites. Our data are consistent with these reports—and, in addition, demonstrate significant interindividual and intraindividual variation in the amount of crossreactants. Donatsch et al. (*19*) have shown that, of the major metabolites, M17 has by far the greatest degree of crossreactivity with the antibody used in the RIA. However, the RIA greatly underestimated the actual concentration of M17, and the extent of the error was directly proportional to the concentration of the metabolite. When the metabolites in the blood of renal allograft recipients were below the limit of detection by HPLC, the correlation between CsA levels measured by RIA and HPLC was good. When the level of metabolites was high, a significant discrepancy was observed between CsA levels measured by the two assays. For example, a blood sample from a renal allograft recipient had 294 ng/ml CsA when measured by RIA. When analyzed by HPLC methods, the blood contained 84 ng/ml CsA and 2004 ng/ml of M17. It is clear from these studies that the RIA is not an accurate method by which to estimate the level of metabolites in the blood.

We have also demonstrated that M17 exhibits a considerable amount of immunosuppressive activity in vitro. Although M17 was not as inhibitory as CsA, we have also shown that the concentration of M17 in the blood may exceed that of CsA by greater than 20-fold. M17 may therefore be contributing a significant amount of immunosuppressive activity in the CsA-treated patient. It is interesting to note that the hydroxylation of amino acid 1 (M17) or amino acid 9 (M1) in the CsA molecule did not completely abolish its immunosuppressive activity, but hydroxylation of both amino acids results in a secondary metabolite (M8) that exhibits no inhibitory activity in vitro.

It has recently been demonstrated that enhancing the activity of the cytochrome P-450 system with Aroclor 1254 resulted in decreased nephrotoxicity without altering the level of immunosuppression (*10–12*). These studies did not identify specific metabolites of CsA, so it is not possible to determine which were immunosuppressive or nephrotoxic. However, it is clear that it will be important to determine the extent to which CsA metabolites are nephrotoxic, since the answer to this question may have far-reaching implications for the future of CsA therapy. Indeed, recent reports have suggested that nephrotoxicity is an irreversible chronic condition that threatens the use of CsA as an immunosuppressive agent (*24*). Protocols that reduce CsA nephrotoxicity without drastically altering the level of immunosuppression will therefore greatly improve the therapeutic efficacy of this drug.

We have not been able to study the in vivo immunosuppressive activity of M17 or any of the other metabolites because only small quantities have been available to us. Nevertheless, the high concentration of M17 in the blood of renal allograft recipients indicates the potential importance of this metabolite in patient therapy.

The practical implications for patient monitoring and for further studies into the biological importance of CsA metabolites are evident from our findings. The apparent immunosuppressive activity of M17 suggests that monitoring the level of M17 in the blood of renal allograft recipients may be important. Recent studies by Rogers et al. (*21*) have demonstrated the presence of an immunosuppressive factor, or factors, in the serum of patients treated with CsA. Patients who generated this factor, the characteristics of which appear to be similar to those of CsA, exhibited significantly fewer rejection episodes than patients without the factor. It is possible that the factor discovered by these investigators is metabolite M17. However, Freeman et al. (*22*) reported that only a small portion of the total M17 could be detected in plasma. Furthermore, using purified M17 spiked into whole blood, we observed that >90% of the M17 was distributed into the cell compartment (data not shown). These findings suggest that the factor reported by Rogers et al. may be yet another immunosuppressive metabolite of the parent compound. Further studies are needed to define the in vivo immunosuppressive properties of M17 and other metabolites, as well as the roles of these metabolites in CsA therapy.

Acknowledgments. We gratefully acknowledge the technical assistance of Mrs. Priscilla Yadegari and Ms. Diane Hayes.

REFERENCES

1. Baldwin WM, Hutchinson LF, Meijer CJLM, Tilney NL. Immune responses to organ allografts. III. Marked decrease in medullary thymocytes and splenic T lymphocytes after cyclosporin A treatment. Transplantation 1981; 31: 117.
2. Najarian JS, Strand M, Fryd DS, et al. Comparison of cyclosporine versus azathioprine-antilymphocyte globulin in renal transplantation. Transplant Proc 1983; 15: 2463.
3. Bunzendahl H, Wonigeit K, Klempnauer J, Brolsh C, Pichlmayr R. Cyclosporine and steroids: effects on clinical course after renal allotransplantation. Transplant Proc 1983; 15: 2531.
4. Starzl TE, Hakala TR, Rosenthal JT, Iwatsuki S, Shaw BW. The Colorado-Pittsburgh cadaveric renal transplantation study with cyclosporine. Transplant Proc 1983; 15: 2459.
5. Squifflet JP, Pirson Y, Jamart J, Wallemacq P, Westelinck K, Alexandre GPJ. Cyclosporine in cadaver renal transplantation at a center with good results using conventional treatment. Transplant Proc 1985; 17: 1212.
6. Stiller C. The Canadian trial of cyclosporine. Cyclosporine therapy compared to standard immunosuppression in renal transplants: an exploration of nephrotoxicity. Transplant Proc 1983; 15: 2479.
7. Sells RA. A prospective randomized substitutive trial of cyclosporine as a prophylactic agent in human renal transplant rejection. Transplant Proc 1983; 15: 2495.
8. Johnson RWG, Wise WH, Bakram A, et al. A four-year prospective study of cyclosporine in cadaver renal transplantation. Transplant Proc 1985; 17: 1197.
9. Loertscher R, Abbud-Filho M, Strom TB. Cyclosporine: a promising immunosuppressive agent. Drug Ther 1984; (July): 16.

10. Cunningham C, Whiting PH, Burke MD, Wheatley DN, Simpson JG. Increasing the hepatic metabolism of cyclosporine abolishes nephrotoxicity. Transplant Proc 1983; 15: 2712.
11. Cunningham C, Gavin MP, Whiting PH, et al. Serum cyclosporine levels, hepatic drug metabolism, and renal tubulotoxicity. Biochem Pharmacol 1984; 33: 2857.
12. Thompson AW, Whiting PH, Simpson JG. Cyclosporine: immunology, toxicity, and pharmacology in experimental animals. Agents Actions 1984; 15: 306.
13. Abisch E, Beveridge T, Gratwohl A, et al. Cyclosporin A: correlation between HPLC and RIA serum levels. Pharm Weekbl (Sci) 1982; 4: 84.
14. Carruthers SG, Freeman DJ, Koegler JC, et al. Simplified liquid chromatographic analysis for cyclosporine A and comparison with radioimmunoassay. Clin Chem 1983; 29: 180.
15. Rosano TG. Effect of hematocrit on cyclosporine (cyclosporin A) in whole blood and plasma cyclosporine levels in renal transplant patients. Clin Chem 1985; 31: 410.
16. Robinson WT, Schran HF, Barry EP. Methods to measure cyclosporine levels: high pressure liquid chromatography, radioimmunoassay, and correlation. Transplant Proc 1983; 15: 2403.
17. Yee GC, Kennedy MS, Storb R, Thomas ED. Pharmacokinetics of intravenous cyclosporine in bone marrow transplant patients. Comparison of two methods. Transplantation 1984; 38: 511.
18. Maurer G, Loosli HR, Schreier E, Keller B. Disposition of cyclosporine in several animal species and man. I. Structural elucidation of its metabolites. Drug Metab Dispos 1984; 12: 120.
19. Donatsch P, Abisch E, Homberger M, Traber R, Trapp M, Voges R. A radioimmunoassay to measure cyclosporin A in plasma and serum samples. J Immunoassay 1981; 2: 19.
20. Fidelus RK, Ferguson RM. The immunosuppressive action of cyclosporine (CyA) as evaluated in 24-hour kinetic profiles. Transplant Proc 1983; 15: 1921.
21. Rogers AJ, Yoshimura N, Kerman RH, Kahan BD. Immunopharmacodynamic evaluation of cyclosporine-treated renal allograft recipients. Transplantation 1984; 38: 657.
22. Freeman DJ, Laupacis A, Keown PA, Stiller CR, Carruthers SG. Evaluation of cyclosporin-phenytoin interaction with observations on cyclosporin metabolites. Br J Clin Pharmacol 1984; 18: 887.
23. Maurer G. Metabolism of cyclosporine. Transplant Proc 1985; 17: 19.
24. Strom TB, Loertscher R. Cyclosporine-induced nephrotoxicity. Inevitable and intractable? N Engl J Med 1985; 311: 728.

Received 20 May 1985.
Accepted 9 December 1985.