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IN VITRO IMMUNOSUPPRESSIVE PROPERTIES OF CYCLOSPORINE METABOLITES[1, 2]

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The in vitro biological activity of cyclosporine (CsA) and four of its metabolites (M1, M8, M17, and M21) was determined. M1, M17, and M21 are primary metabolites, while M8 is a secondary metabolite derived from either M1 or M17. The order of inhibitory activity in phytohemagglutinin (PHA), concanavalin A (ConA), mixed lymphocyte culture (MLC), and interleukin-2 (IL-2) production assays was CsA > M17 > M1 > M21 ≫ M8. In the PHA assay, CsA was significantly more inhibitory than M17, but in Con A and MLC assays, the inhibitory activity of M17 approached that of CsA. More importantly, M17 and M1 inhibited the production of IL-2 in the MLC to the same extent as CsA. M21 was significantly less inhibitory than either M17 or M1, and M8 appeared to be largely devoid of biological activity. These experiments demonstrate that single hydroxylations of amino acids 1 (M17) and 9 (M1) do not significantly affect the ability of the molecule to block IL-2 production, but hydroxylation of both amino acids renders the molecule virtually inactive. In addition, the presence of the N-methyl group on amino acid 4 appears to be very important, since removal of this group (M21) greatly diminishes the immunosuppressive activity.

Cyclosporine (CsA)* is a lipophilic cyclic peptide that is known to have potent immunosuppressive properties. CsA has been shown to prolong the survival of organ allografts and inhibit cell-mediated immunity (*1–3*). The therapeutic efficacy of the drug is hampered by the fact that it also causes nephrotoxicity in vivo (*4, 5*). CsA is metabolized in vivo to produce a large number of biotransformation products (*6, 7*), but there are few studies to indicate to what extent CsA or the metabolites contribute to immunosuppression or toxicity. Cunningham et al. (*8*) reported that enhancing the activity of the rat cytochrome P_{450} system with Aroclor 1254 resulted in decreased nephrotoxicity without altering the level of immunosuppression. Although the authors did not identify which metabolites were produced, they suggested that the parent compound was the causative agent of nephrotoxicity and that some metabolites might possess immunosuppressive activity. Other investigators have reported immunosuppressive activity in the serum of

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* Abbreviations used: AGHS, agamma human serum; Con A, concanavalin A; CsA, cyclosporine A; CsC, cyclosporine C; CTL, cytotoxic T lymphocytes; HBSS, Hank's balanced salt solution; ^{3}H-thymidine, tritiated thymidine; HPLC, high performance liquid chromatography; IL-2, interleukin-2; 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; PHA, phytohemmaglutinin; PBMC, peripheral blood mononuclear cells.

patients treated with CsA, even in the absence of detectable parent compound (*9, 10*).

We recently reported that renal allograft recipients treated with oral CsA produce three primary metabolites, called M17, M1, and M21 (*11*). When the purified metabolites were compared with CsA for in vitro biological activity, we noted that all three exhibited inhibitory activity in the MLC. We therefore undertook the present studies to determine the immunosuppressive activity of these metabolites in more detail.

MATERIALS AND METHODS

CsA and metabolites. Purified CsA and metabolites M1, M8, M17, and M21 were a generous gift of G. Maurer, Ph.D., of Sandoz Ltd., Basle, Switzerland. The identities of these cyclosporines[5] were confirmed by spiking them into mobile phase and assaying them by high-performance liquid chromatography (HPLC). Each of the molecules chromatographed as a single homogeneous peak. The cyclosporines were prepared as previously described (*6*) and evaporated to dryness. For in vitro biological assays, these samples were reconstituted in absolute ethanol and diluted 210–20,000-fold in media. Ethanol diluted in the same manner was used as a control. For calibration of the HPLC assay, CsA and the metabolites were added quantitatively to medication-free blood.

CsA and metabolite measurement. The levels of CsA and the metabolites in ether-extracts of whole blood were determined by HPLC using a 4.6-mm × 25-cm cyanopropyl column (DuPont Instruments, Wilmington, DE) maintained at 65°C. The mobile phase consisted of 43% acetonitrile/57% water. A flow rate of 0.5 ml/min was used with UV-detection at 210 nm. CsA and metabolites were quantitated by a peak-height ratio with cyclosporine C (CsC) as an internal standard.

Cells. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of normal donors as previously described (*11*) and resuspended in RPMI-1640 (MA Bioproducts, Walkersville, MD) supplemented with 10–20% heat-inactivated human AB serum (Pel-Freez, Rogers, AR).

Proliferation assays. The effect of CsA and metabolites on the mitogenic response of PBMC in phytohemagglutinin (PHA), concanavalin A (ConA), and mixed leukocyte culture (MLC) assays was determined as previously described (*11*). Briefly, 2×10^5 PBMC in RPMI-1640 with 10% AB serum were plated in 96-well flat-bottomed microtiter plates (Corning) with 250 μg/ml of PHA-M (Burroughs Wellcome, Wellcome, UK) or 10 μg/ml Con A (Sigma Chemical Co., St. Louis, MO) and incubated for 2 days at 37°C in 5% CO_2/95% air. The plates were pulsed with 0.6 μCi/well of tritiated thymidine (^{3}H-thymidine) and harvested 18 hr later. The MLC was performed by coculturing 10^5 responder and 10^5 irradiated-stimulator PBMC in RPMI-1640 supplemented with 20% AB serum. The wells were pulsed with ^{3}H-thymidine on day 5 and harvested 18 hr later. The cyclosporines were added at the initiation of the cultures. Complete dissolution of CsA in media was verified by high-performance liquid chromatography (*11*).

Generation and assay of cytotoxic T cells. The generation and assay of cytotoxic T cells were performed as previously reported (*12*). Briefly, unidirectional MLCs were performed by coculturing 10^6 responder PBMC with 10^6 irradiated allogeneic PBMC in a final volume of 2 ml of RPMI-1640 supplemented with 20% AB serum. The plates were incubated at 37°C for 10 days. PHA blasts to be used as target cells were prepared by culturing 2×10^6 PBMC (same cells used to stimulate CTL generation) in RPMI-1640 supplemented with 10% agamma human serum (AGHS, KC Biologicals, Lenexa, KS) and 250 μg/ml of PHA-M. The blasts were isolated 3 days later, washed 3 times in HBSS, and resuspended to 5×10^5 cells/ml in RPMI-1640 with 10% AGHS and 10% interleukin-2 (IL-2, Cellular Products, Buffalo, NY). The cells were grown in the presence of IL-2 until they were used in the CTL assay. These target cells were then washed and labeled with 250 μCi of ^{51}Cr (sodium chromate, NEN Products, Boston, MA). The CTL were then harvested from the MLC, washed, and incubated with the labeled target cells at a 50:1 ratio in V-bottomed 96-well plates. The plates were incubated for 4 hr and the supernatants counted in a gamma counter. The data are expressed as the percent of the control response.

Production and assay of IL-2. The effect of CsA and metabolites on the production of IL-2 in the MLC was performed as previously described (*13*), except that the supernatants were harvested on day 4. The supernatants were assayed for IL-2 activity using the IL-2-dependent cell line HT-2. Briefly, 5×10^3 HT-2 cells (0.1 ml) were plated in flat-bottomed microtiter wells in RPMI-1640 supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (Sterile Systems, Logan, UT). The MLC supernatants (0.1 ml) were added to each well and the plates were incubated for 18 hr at 37°C, then pulsed for 4 hr with 0.6 μCi of ^{3}H-thymidine. Dilutions of human IL-2 were used as positive controls in all assays and the proliferative response of these cells was found to be proportional to the concentration of IL-2.

RESULTS

The presence of CsA and metabolites in the peripheral blood. The structures of CsA and the metabolites used in this study were reported by G. Maurer (*7*) and are shown in Figure 1. M1, M17, and M21 are primary metabolites of cyclosporine, while M8 is a secondary metabolite derived from M1 or M17. The trough levels of these compounds in the whole blood of renal allograft recipients are shown in Table 1. In over 90% of the trough samples assayed, the concentration of M17 (36–566 ng/ml) exceeded that of CsA (<20–305 ng/ml). M1 has been found in somewhat lower levels (<20–274 ng/ml), but M21 appeared only in trace quantities (<20–56 ng/ml). Due to the presence of coeluting compounds, we cannot yet measure M8 in the peripheral blood.

Effects of CsA and metabolites on PHA and Con A responses. In PHA, Con A, and MLC assays, the absolute amount of inhibition by CsA and the metabolites varied as much as 2-fold, depending on the responder cells. Thus, in all experiments, CsA and the metabolites were tested simultaneously. The inhibitory effects of CsA and the metabolites on mitogen responsiveness are shown in Figure 2. CsA was significantly more suppressive than M17 or the other metabolites in the PHA assay, but CsA and M17 produced similar degrees of suppression in the Con A assay. M1 and M21 showed progressively less activity in both assays, and M8 appeared to lack inhibitory activity entirely.

Effect of CsA and metabolites on proliferation and IL-2 production in the MLC. As can be seen in Table 2, 800 ng/ml of CsA, M17, and M1 completely blocked IL-2 production and

[5] The term "cyclosporines" refers to all of the metabolites and derivatives of CsA.

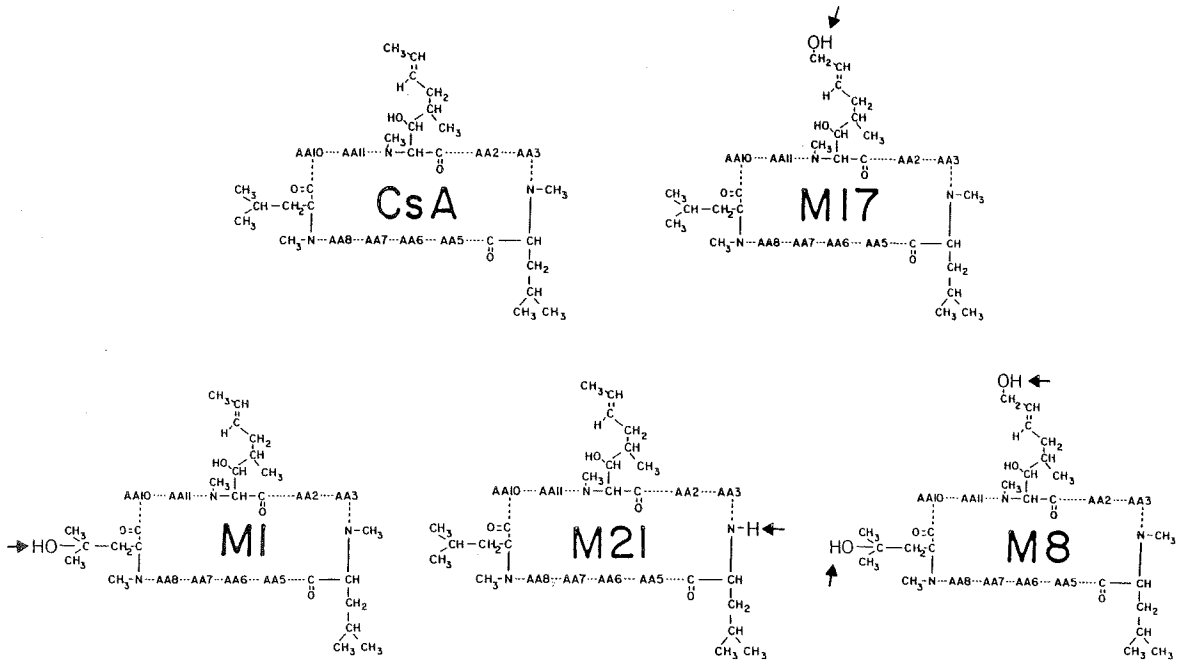

FIGURE 1. Structures of CsA and metabolites. The structures are drawn according to the scheme proposed by Maurer (*7*). Only the amino acids that are altered by metabolism have been drawn. The arrows point to the hydroxylated or demethylated side groups.

TABLE 1. Levels of CsA and metabolites in blood of renal allograft recipients

Compound	Whole blood trough level (ng/ml)[a]	
	Range	Mean ± SD
CsA	<20–305	135±77
M17	36–566	244±152
M1	<20–274	116±135
M21	<20–56	18±15
M8	Not measured	

[a] Patients (n = 8) were treated with 5 mg/kg of CsA in two equal doses at 12-hr intervals as previously described (*11*). Trough levels were measured in 33 whole-blood samples collected immediately prior to the next dose. The HPLC assay used to quantitate CsA and metabolite levels does not measure M8, so the concentration of this metabolite in the blood is presently unknown.

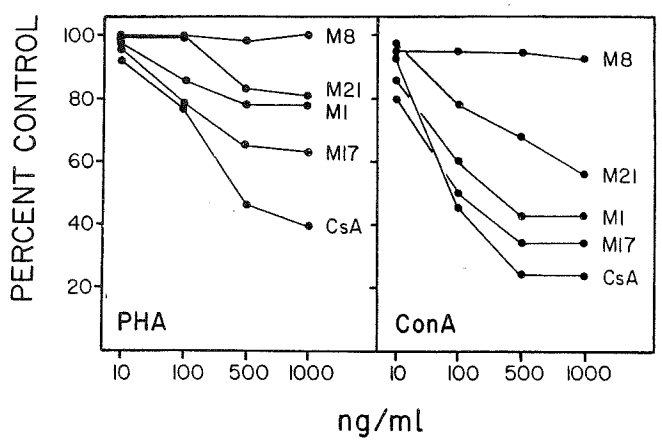

FIGURE 2. Effect of CsA and metabolites on PHA and Con A responses of human T cells. The data are presented as the percent of the control response at various concentrations of CsA or metabolites.

TABLE 2. Effect of CsA and metabolites on IL-2 production

Compound	Concentration (ng/ml)	Percent inhibition[a]	
		MLC	IL-2
CsA	800	85	100
	600	67	100
	400	19	98
	200	0	70
	50	0	18
M17	800	89	100
	600	66	100
	400	37	93
	200	0	47
	50	0	27
M1	800	85	100
	600	60	100
	400	9	94
	200	0	57
	50	0	3
M21	800	32	51
	600	2	43
	400	0	0
	200	0	0
	50	0	0
M8	800	4	40
	600	0	0
	400	0	0
	200	0	0
	50	0	0

[a] The data represent inhibition of IL-2 production and the proliferative response in the same MLC.

inhibited the proliferative response by 85–89%. However, 600 ng/ml of CsA, M17, or M1 also blocked IL-2 production by 100%, but the proliferative response in all cases was inhibited by only 60–67%. Similarly, 400 ng/ml of CsA, M17, or M1 inhibited production of IL-2 by 93–98%, but produced less than

TABLE 3. Effect of CsA and M17 on the generation of CTL

Compound	Percent inhibition[a]			
	PHA	ConA	MLC	CTL
CsA	59	58	99	99
M17	25	32	94	100

[a] The responses were determined using the same responder cells in all assays. CsA and M17 were tested at 1000 ng/ml.

40% inhibition of the proliferative response. At 800 ng/ml, M21 inhibited IL-2 production by only 51% and M8 inhibited production by only 40%. Consistent with the data derived using CsA and the other metabolites, inhibition of IL-2 production by M21 and M8 was associated with significantly less inhibition of the proliferative response in the MLC.

Effect of CsA and M17 on the generation of CTL. Due to the relatively large quantities of metabolites needed to assay for inhibition of CTL generation, only M17 was tested in this study. As can be seen in Table 3, 1000 ng/ml of M17 blocked the generation of CTL as effectively as CsA. This level of inhibition correlated well with inhibition of the MLC proliferative response, but M17 was significantly less inhibitory than CsA in PHA and Con A assays using the same responder cells.

DISCUSSION

Our previous report (*11*) indicated that M17 had considerably less inhibitory activity than CsA in the PHA assay, but that the activity of M17 approached that of CsA when assayed in the MLC. The experiments presented here confirm those findings and provide some insight into the effect of hydroxylation and demethylation of CsA on its biological activity.

CsA is thought to be metabolized by the cytochrome P_{450} system of the liver by sequential hydroxylation or demethylation (*7*). As a result of these processes, the metabolites are more polar than the parent compound. Such changes in the structure of CsA could, in theory, alter the immunosuppressive or nephrotoxic properties of the molecule. For example, Wenger (*14*) reported that amino acids 1,2,3, and 11 are important in the biological activity of CsA, and that substitution of these amino acids diminishes immunosuppressive activity. In addition, a carboxylic acid metabolite of CsA has been found and reported to be inactive (*15*). However, both of these studies assessed the immunosuppressive properties of the cyclosporine analogues solely by inhibition of PHA responsiveness. We have clearly shown that the PHA assay is a poor method for determining biological activity. For example, 1000 ng/ml of M17 blocked IL-2 production and the generation of CTLs in the MLC by >99%, but inhibited the proliferative response to PHA by <40%. Similarly, M1 at 800–1000 ng/ml blocked IL-2 production in the MLC by 100%, but inhibited PHA responsiveness by only 20%. It is apparent from these studies that cyclosporine analogues and metabolites should be tested more rigorously in order to identify those that possess in vitro immunosuppressive activity. Clearly, inhibition of IL-2 production in the MLC is far more sensitive to CsA and the metabolites than is the proliferative response. We noted that 400 ng/ml of CsA, M17, and M1 inhibited IL-2 production by >90%, but inhibited the corresponding MLC responses by only 9–37%. Furthermore, 600 ng/ml of each of these cyclosporines apparently blocked IL-2 production by 100%, but inhibited the MLC response by only 60–67%. Thus, even the most sensitive proliferation assay (MLC) did not appear to be a good indicator of the potential biological activity of the metabolites.

Our data demonstrate that inhibition of IL-2 production and ^{3}H-thymidine uptake by cyclosporines do not correlate very well. One might therefore ask which in vitro assay is more representative of the drug's biological activity. It should be noted that DNA synthesis in the MLC is simply related to the induction of the cell cycle by newly activated lymphocytes. This event apparently requires very little IL-2, since inhibition of IL-2 production by 90% had little effect on ^{3}H-thymidine uptake. However, the proliferation of large numbers of helper and cytotoxic T cells necessary for allograft rejection might be greatly impaired by such a reduction in IL-2 production. Thus, although inhibition of IL-2 synthesis by 90% does not inhibit the MLC proliferative response, it might have profound effects on the generation of CTL in vivo. These data may explain why in vitro proliferation assays using PBMC from patients do not correlate with clinical evidence of immunosuppression (unpublished observations).

It should also be noted that the IL-2 assay used in this study detects free IL-2 in the culture supernatants. Thus, the T cells in the MLC can synthesize DNA in the apparent absence of IL-2 because, in fact, low levels of IL-2 are probably being made and are immediately bound to IL-2 receptors on the cell surface. Only when IL-2 is made in excess of the amount needed to saturate the IL-2 receptors can free IL-2 be detected in the supernatants. With these observations in mind, it appears that CsA, M17, and M1 are approximately equal in their ability to inhibit IL-2 production. On the other hand, M21 and M8 are essentially inactive at concentrations below 400–600 ng/ml.

These findings allow use to draw some preliminary conclusions about the structure of the cyclosporines and their biological activity. Single hydroxylations at amino acids 1 (M17) or 9 (M1) do not drastically alter the ability of the metabolites to block IL-2 production, but hydroxylation of both amino acids (M8) renders the molecule virtually inactive. Demethylation at amino acid 4 appears to depress significantly the activity of this metabolite (M21). Thus, while the lipophilic nature of the cyclosporines is probably important for its immunosuppressive activity, differences in overall hydrophobicity cannot fully explain the observed differences in immunosuppressive activity. For example, based on the order of elution on HPLC, M21 is more hydrophobic than M17 or M1, yet it possesses considerably less biological activity. These data suggest that although cyclosporines are highly methylated, the particular N-methyl group on amino acid 4 is essential for immunosuppressive activity. We therefore conclude that the different domains of the cyclosporine molecule are independently involved in conferring biological activity.

Very little is known about the mechanism by which CsA blocks IL-2 production by helper T cells. It has been suggested that CsA interferes with T cell activation by binding to cellular molecules, such as cyclophilin (*16*) or calmodulin (*17*). It is therefore possible that specific changes in the structure of CsA may alter the interaction between CsA and the receptor/target. In support of this theory, Ryffel et al. (*18*) demonstrated that an inactive keto-derivative of CsA could not compete for the binding site of the immunosuppressive CsC, even at concentrations 100 times that of CsC. Ultimately, the CsA metabolites and other analogues should prove to be valuable tools with which to determine the site of action of CsA.

Four other cyclic peptide metabolites of CsA (M9, M10, M16, and M18) are known (*7*). Only M18, a tetrahydrofuran derivative of M17, has been tested for in vitro immunosuppressive activity (*19*). At 800 ng/ml, M18 exhibited <10% suppression of PHA and ConA responses, compared with 25–34% for M17 in the same experiment. In the MLC, M18 inhibited the proliferative response by 60%, compared with 94% for M17. Although further tests are needed, these preliminary findings suggest that the formation of a cyclic ether between the beta and epsilon carbons of amino acid 1 diminishes the immunosuppressive activity of M17. M9, which is a tertiary metabolite derived from M16 by N-demethylation of amino acid 4, would not be expected to be inhibitory because the methyl group on amino acid 4 is essential for biological activity. The in vitro immunosuppressive properties of metabolites M10 and M16, which are derived from M1 by secondary hydroxylations at amino acids 4 and 6, respectively, have not yet been studied. It should be very interesting to study these cyclosporines, since recent experiments by Rogers et al. (*10*) have provided strong evidence for the existence of immunosuppressive metabolites in the serum of patients treated with CsA. The identity of these metabolites is not known, but their presence in the serum makes it unlikely that they are either M17 or M1, both of which are >95% cell-associated (*20*). It is possible that the metabolites reported by Rogers and his colleagues may be either M10 or M16, both of which are more polar than M17 and therefore not detected by our current HPLC assay. It is therefore important that we determine the immunosuppressive activity of all of the major metabolites. It will then be possible to develop assays for the biologically active metabolites and monitor their levels in the patients treated with CsA.

The implications of this study for clinical transplantation are important. We have found high levels of M17 and M1 in the blood and tissue of renal allograft recipients treated with CsA, suggesting that they may represent a major contribution to the therapeutic effect (*20*). However, almost nothing is known about the contribution of these and other metabolites to nephrotoxicity. Clearly, the answer to this question will greatly assist the designing of treatment protocols in the future.

REFERENCES

1. Borel JF, Feurer C, Magnee C, Staehelin H. Effects of the new antilymphocyte peptide cyclosporin A in animals. Immunology 1977; 32: 1017.
2. Kostakis AJ, White DJG, Calne RY. Prolongation of rat heart allograft survival by cyclosporine A. IRCS Med Sci 1977; 5: 280.
3. Hess AD, Tutschka PJ. Effect of cyclosporin A on human lymphocyte responses in vitro. I. CsA allows for the expression of alloantigen-activated suppressor cells while preferentially inhibiting the induction of cytolytic effector lymphocytes in MLR. J Immunol 1980; 124: 2601.
4. Whiting PH, Thomson AW, Blair JT, Simpson JG. Experimental cyclosporin A nephrotoxicity. Br J Exp Pathol 1982; 63: 88.
5. Myers BD, Roses J, Newton L, Luetscher J, Perlroth M. Cyclosporine-associated chronic nephrotoxicity. N Engl J Med 1984; 311: 699.
6. Maurer G, Loosli HR, Shreier E, Keller B. Disposition of cyclosporine in several animal species and man. I. Structural elucidation of its metabolites. Drug Metab Dispos 1984; 12: 120.
7. Maurer G. Metabolism of cyclosporine. Transplant Proc 1985; 17: 19.
8. Cunningham C, Burke MD, Wheatley DN, Thomson AW, Simpson JG, Whiting PH. Amelioration of cyclosporin-induced nephrotoxicity in rats by induction of hepatic drug metabolism. Biochem Pharmacol 1985; 34: 573.
9. Fidelus RK, Ferguson RM. The immunosuppressive action of cyclosporine (CyA) as evaluated in 24-hour kinetic profiles. Transplant Proc 1983; 15: 1921.
10. Rogers AJ, Yoshimura N, Kerman RH, Kahan BD. Immunopharmacodynamic evaluation of cyclosporine-treated renal allograft recipients. Transplantation 1984; 38: 657.
11. Rosano TG, Freed BM, Cerilli J, Lempert N. Immunosuppressive metabolites of cyclosporine in the blood of renal allograft recipients. Transplantation 1986; 42: 262.
12. Freed BM, Mozayeni B, Lawrence DA, Wallach FR, Lempert N. Differential inhibition of human T lymphocyte activation by maleimide probes. Cell Immunol 1986; 101: 181.
13. Freed BM, Rapoport R, Lempert N. Inhibition of early events in the human T lymphocyte response to mitogens and alloantigens by hydrogen peroxide. Arch Surgery (in press).
14. Wenger R. Synthesis of cyclosporine and analogues: structure, activity, relationships of new cyclosporine derivatives. Transplant Proc 1983; 15: 2230.
15. Hartman NR, Trimble LA, Vederas JC, Jardine I. An acid metabolite of cyclosporine. Biochem Biophys Res Commun 1985; 133: 964.
16. Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DLV. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 1985; 226: 544.
17. Colombani PM, Robb A, Hess AD. Cyclosporin A binding to calmodulin: a possible site of action on T lymphocytes. Science 1985; 228: 337.
18. Ryffel B, Gotz U, Heuberger B. Cyclosporin receptors on human lymhocytes. J Immunol 1982; 129: 1978.
19. Freed BM, Rosano TG, Quick C, Lempert N. Effects of cyclosporine metabolites M17 and M18 on proliferation and IL-2 production in the mixed lymphocyte culture. Transplant Proc (in press).
20. Rosano TG, Freed BM, Pell M, Lempert N. Cyclosporine metabolites in human blood and renal tissue. Transplant Proc 1986; 18 (suppl 5): 35.

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