Effects of Cyclosporine Metabolites M17 and M18 on Proliferation and Interleukin 2 Production in the Mixed Lymphocyte Culture

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CYCLOSPORINE A (CsA) is a potent immunosuppressive agent that is widely used to prevent graft rejection. Although the drug has been tested extensively in vivo, most of the known biological effects of CsA have been demonstrated in vitro. For example, CsA has been shown to block the proliferative response of T cells to mitogens and alloantigens,[1,2] to inhibit the synthesis of interleukin 2 (IL 2)[3] and gamma interferon,[4] and to block the generation of cytotoxic T lymphocytes (CTL).[1] Thus, while the exact mechanism by which CsA prevents graft rejection in vivo is not known, these in vitro tests clearly demonstrate the compound's potent immunosuppressive activity.

The major drawback to the use of in vitro assays for estimating the therapeutic potential of CsA is that they do not account for the possible contribution of metabolites. CsA is metabolized by the cytochrome P_{450} system of the liver[5] and nine cyclic metabolites have been identified.[6] We previously demonstrated the presence of three of these metabolites (M17, M1, and M21) in the blood of renal allograft recipients treated with oral CsA.[7] The trough levels of M17 were found to exceed those of the parent compound in >90% of the whole blood samples tested. Using purified metabolites obtained from Dr Gerard Maurer at Sandoz Pharmaceuticals (Basel, Switzerland), we further demonstrated that M17 and M1 inhibit proliferation and IL 2 production in the human mixed lymphocyte culture (MLC), while metabolites M21 and M8 were essentially inactive.[8] In the present study, the in vitro immunosuppressive activity of M17 was compared with the activity of the secondary metabolite, M18, which is a tetrahydrofuran derivative of M17. The purpose of this study was to determine if a small change in the structure of this metabolite significantly affected its biological activity.

MATERIALS AND METHODS

Cells

Peripheral blood mononuclear cells (PBMC) were obtained from normal donors by density-gradient centrifugation of whole blood on Ficoll-Paque (Pharmacia, Piscataway, NJ). The PBMC were washed three times in Hank's balanced salt solution (HBSS) and resuspended to 10^6 cells/mL in RPMI-1640 (MA Bioproducts, Walkersville, MD) supplemented 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% to 20% heat-inactivated human AB serum (Pel-Freeze, Rogers, AZ).

In Vitro Assays

CsA, M17, and M18 were generous gifts of Dr G. Maurer, Sandoz, Switzerland. The cyclosporines were resuspended in absolute ethanol and diluted in HBSS, so that the final concentration of ethanol was <0.4% in the assays. The effects of CsA and metabolites on the in vitro proliferative responses to phytohemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogen (PWM), and allogeneic cells (MLC) were tested as previously described.[6,7] Briefly, PBMC were plated at 2×10^5 cells/well in medium with 10% AB serum and stimulated with 250 μg/mL PHA-M (Burroughs-Wellcome, Research Triangle Park, NC), 10 μg/mL ConA (Sigma, St Louis),

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Supported by the James Hilton Manning and Emma Austin Manning Foundation and the Sandoz Research Institute.

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0041-1345/87/1901-0437$03.00/0

or 100 μg/mL PWM (Gibco, Grand Island, NY). PHA and ConA assays were incubated for 48 hours, while PWM assays were incubated for five days. The plates were then pulsed for 18 hours with tritiated thymidine (^{3}H-TdR) and harvested with a MASH II cell harvester (MA Bioproducts, Walkersville, MD). The MLC was performed by culturing 10^5 PBMC with 10^5 irradiated (3,000 rad) allogeneic PBMC in medium plus 20% AB serum. Five days later, the plates were pulsed with 0.6 μCi of ^{3}H-TdR and harvested as described for the mitogen assays. In all experiments, CsA or the metabolites were added at the initiation of the cultures. Controls were performed using ethanol diluted in the same manner.

In order to measure the production of IL 2, culture medium was collected on day 4 from allogeneic MLCs, centrifuged at 1,000 × g, and the supernatants stored at 4 °C until they could be assayed. The samples were assayed for the presence of IL 2, using the murine HT-2 cell line as previously described.[8,9]

RESULTS

The effect of metabolites M17 and M18 on the in vitro proliferative responses to PHA, ConA, PWM, and in the MLC are shown in Fig 1. As previously shown, M17 is considerably less suppressive than CsA in the PHA assay. M17 exhibited 20% to 30% inhibition of the PHA and ConA responses, but M18 exhibited <12% inhibition in both assays. None of the cyclosporines inhibited the response of PBMC to PWM, and M17 and M18 actually enhanced this response. In the MLC, M17 inhibited the proliferative response by 94%, but M18 inhibited the response by only 60%. CsA inhibited the MLC by 99%.

We previously demonstrated that the production of IL 2 in the MLC is more sensitive than the proliferative response to inhibition by CsA and its metabolites.[8] As can be seen in Table 1, M18 exhibited a maximum of 45% inhibition of proliferation at 600 to 800 ng/mL, but completely blocked IL 2 production at 400 to 800 ng/mL. However, M18 appeared to be significantly less active than M17, which suppressed the proliferative response by 89% at 800 ng/mL and inhibited IL 2 production by 47% to 52% at 100 to 200 ng/mL (compared with <10% suppression for M18).

DISCUSSION

CsA is extensively metabolized by the liver and excreted via the feces and urine.[6] However, significant amounts of metabolites M17, M1, and M21 have been found in the peripheral blood of renal allograft recipients treated with oral cyclosporine.[6,7] In addition, other investigators have reported that metabolite M18 is also present in the peripheral blood.[5,10] In order to determine if these metabolites might contribute to the therapeutic potential of CsA, we have studied the in vitro biological activity of the major metabolites (M17, M1, M21). Metabolite M17 was the most suppres-

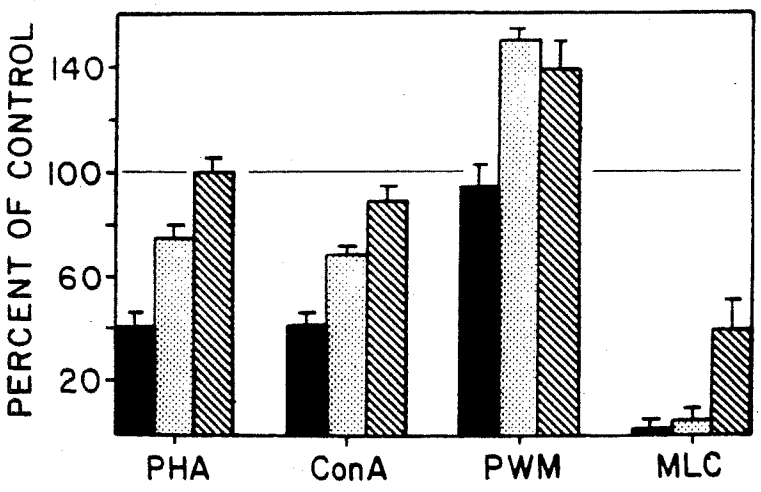

Fig 1. The effect of CsA, M17, and M18 on in vitro proliferative responses. The responses were all determined using the same responder cells and 800 ng/mL of CsA (solid boxes), M17 (shaded boxes), or M18 (striped boxes).

Table 1. The Effect of CsA and Metabolites M17 and M18 on Proliferation and IL2 Production in the MLC

Compound	ng/mL	MLR*	% Inhibition	IL-2†	% Inhibition
Control	—	44,213	—	5,661	—
CsA	800	4,941	89	609	100
	600	10,598	76	1,039	100
	400	25,823	42	1,586	98
	200	45,315	0	2,754	70
	100	38,811	17	5,147	12
	50	35,601	19	4,908	18
M17	800	3,460	92	543	100
	600	10,835	76	759	100
	400	20,055	55	1,823	93
	200	41,196	7	3,732	47
	100	42,434	4	3,518	52
	50	38,636	13	4,532	27
M18	800	17,619	60	1,436	100
	600	20,683	53	1,705	96
	400	32,603	26	1,315	100
	200	38,566	13	4,334	32
	100	47,789	0	4,136	37
	50	44,516	0	6,341	0

*Mixed lymphocyte response in cpm of ^{3}H-TdR uptake.

†IL2 was measured by the response of HT-2 cells. In the absence of IL2, the response was 1,518 cpm.

sive, although it appeared to be less active than CsA.[7] M1 was also inhibitory, but metabolites M21 and M8 (a secondary metabolite derived from either M1 or M17) were largely devoid of inhibitory activity except at very high concentrations (>800 ng/mL). We now report that M18 also has in vitro immunosuppressive activity, but it is significantly less active than M17 or CsA.

We previously reported, based on four independent experiments, that CsA and M17 inhibited the proliferative response of PBMC to the B cell mitogen, PWM. We have since performed an additional four experiments and have found that, depending on the responder cells, 800 ng/mL of CsA caused between 70% suppression and 30% stimulation of the proliferative response to PWM (data not shown). M17 caused between 40% suppression and 70% stimulation of this response. These finding have been confirmed with dose-response studies at 600 to 800 ng/mL of the cyclosporines (data not shown). Similar variability in the in vitro activity of CsA and the metabolites has been found in the human MLC,[8,11] but in no instance were these cyclosporines stimulatory. Other investigators have reported that 100 to 1,000 ng/mL of CsA markedly suppresses the blastogenic response of human PBMC to PWM,[12] but we could not confirm those findings in all instances.

Our data suggest that the immunosuppressive properties of CsA may depend on the general cyclic lipophilic nature of the molecule. However, the fact that amino acid substitutions and single hydroxylations of amino acids 1 or 9 do not abolish the inhibitory effects of CsA suggest that biological activity is not strictly dependent on the configuration of the parent compound. This theory is supported further by the finding that some synthetic analogues of CsA have in vitro immunosuppressive activity comparable to that of the parent compound.[13]

At present there is little data available on the in vivo immunosuppressive activity of M17 or M1. M17 is poorly absorbed from the gastrointestinal tract, so oral administration of this metabolite does not cause immunosuppression in the rat (B. Ryffel, Sandoz, personal communication). However, the fact that metabolites M17 and M1 are generally present in the blood and tissue of renal allograft recipients at levels equal to or greater than that of CsA[14] suggests that these metabolites may contribute significantly to the therapeutic efficacy of the drug.

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