Biochemical Pharmacology, Vol. 42, No. 7, pp. 1403–1410, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00
Pergamon Press plc

SYNERGISTIC AND ANTAGONISTIC EFFECTS OF COMBINATIONS OF CYCLOSPORINE A AND ITS METABOLITES ON INHIBITION OF PHYTOHEMAGGLUTININ-INDUCED LYMPHOCYTE TRANSFORMATION *IN VITRO*

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(*Received* 18 *January* 1991; *accepted* 1 *May* 1991)

Abstract—Cyclosporine A (CsA) and purified CsA metabolites were tested alone and in combination in cell culture to determine their effects on phytohemagglutinin (PHA)-induced lymphocyte proliferation. CsA was significantly more inhibitory than its metabolites at all concentrations tested (0–1000 ng/mL). CsA exerted maximum inhibition (70% decrease in [*methyl*-^{3}H]thymidine incorporation) at concentrations of 300 ng/mL or greater; metabolites M1, M17, and M21 depressed the response 46, 39, and 23%, respectively, at 300 ng/mL. Metabolites M8, M18, M26, M25, M13, and M203-218 were non-inhibitory. When combinations of M17 and CsA were tested for the effects on PHA-induced lymphocyte transformation, a synergistic effect occurred at combinations of low concentrations of M17 and CsA and an antagonistic effect at the higher concentrations. Of the 49 combinations of CsA and M17 tested, 30 were antagonistic, 16 synergistic and 3 undecided (approaching additivism). When 49 combinations of CsA and the non-immunosuppressive metabolite M8 were tested, 29 of the 49 combinations were synergistic, 17 antagonistic, 1 additive and 2 undecided (approaching additivism). Of the 29 synergistic combinations, 14 were strongly synergistic. The importance of the interaction of CsA and metabolites to the immunopharmacology of CsA therapy is discussed.

Cyclosporine A (CsA)§, a cyclic undecapeptide isolated from several species of fungi imperfecti [1–3], is a potent immunosuppressive agent used clinically to reduce the incidence of graft rejection in human organ transplantation and to suppress graft-versus-host disease after allogeneic bone marrow transplantation [4]. CsA has also been shown to be effective in producing significant improvement in patients with autoimmune diseases [5]. Recent studies suggest that CsA inhibits helper T cell activation both *in vivo* [6–8] and *in vitro* [9–11]. Addition of CsA to cultures of T cells inhibits their response to mitogenic and allogeneic stimuli [9, 12, 13] by altering lymphokine production by T helper cells [14, 15] and generation of cytotoxic T lymphocytes [9]. CsA blocks the expression of the gene for interleukin-2 (IL-2) production at the level of mRNA transcription [16, 17] and interrupts ongoing IL-2 mRNA synthesis [18]. The inhibition of IL-2 production may prevent clonal expansion of activated T and natural killer cells, as well as generation of cytotoxic effector cells.

CsA is metabolized in the liver to 20 or more compounds [19]. In many human transplant recipients, the concentrations of these metabolites in "trough" whole blood specimens and some solid tissue samples usually exceed levels of parent CsA [20–22]. The role of the metabolites in nephro- and hepatotoxicity associated with CsA therapy is uncertain. Purified metabolites have been tested separately *in vitro* for their effects on mitogen-induced lymphocyte transformation, mixed lymphocyte reactions, IL-2 production by stimulated peripheral blood mononuclear cells [23] and specific clones [24], and the generation of cytotoxic T cells [9]. The measured amount of immunosuppressive activity of various CsA metabolites has varied from study to study, but has always been less for each metabolite than for the parent drug [23, 25]. Disagreement in the immunosuppressive activity of metabolites has arisen, possibly due to the uncertain purity and quantitation of the metabolites [26, 27], differences in inter-individual sensitivity to CsA and metabolites [28], and variation in the *in vitro* assays used to assess immunosuppressive activity. [For a review of the available information about the immunosuppressive activity of CsA metabolites as found in the literature see Refs. 23 and 31.]

What is consistent among the studies is that metabolites with single modifications, such as M17 (demethylation), possess the most immunosuppressive activity, and that metabolites with two or more modifications possess substantially less. For example, hydroxylation of both amino acids 1 and 9 results in a secondary metabolite (M8) which is

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§ Abbreviations: CsA, cyclosporine A; PHA, phytohemagglutinin; [^{3}H]TdR,[*methyl*-^{3}H]thymidine; IL-2, interleukin-2; Con A, concanavalin A; and MLC, mixed-lymphocyte culture.

largely devoid of immunosuppressive activity *in vitro*.

The immunosuppressive effect of combinations of CsA and its metabolites on lymphocyte reactivity *in vitro* has been described only recently. Zeevi *et al.* [29] reported a synergistic effect between CsA and M17 and have suggested that the effectiveness of CsA therapy may depend on the immunosuppressive effects of combinations of parent compound and individual metabolites. In their study, the addition of CsA or M17 separately to cultures at concentrations of 25 and 250 ng/mL, respectively, had little inhibitory effect on the primed lymphocyte test response of human heart biopsy lymphocytes. However, combinations of these doses of CsA and M17 were immunosuppressive (77% inhibition); a similar response was observed when the concentration of M17 in the mixture was reduced 10-fold. These studies, however, were limited to testing of three combinations [one concentration of CsA (25 ng/mL) in combination with 25, 50, and 250 ng/mL of M17, respectively]. This *in vitro* synergistic effect may have clinical significance in that lower doses of drug may be effective in increasing transplant survival, thus reducing the CsA dosage and toxic side effects.

Sewing *et al.* [30] have shown inhibition of proliferation of rat mesangial cells and of concanavalin A (Con A)-induced rat peripheral blood lymphocyte transformation by the metabolites H320 (M13), H355, and H350 (M203-218). In both assays the combination of the metabolites had a far greater effect than the addition of their single effects, especially at low concentrations. It was shown that even at ineffective concentrations of the single compounds, metabolites can potentiate (synergize) each other and gain biologically significant activity. Copeland *et al.* [31] examined the immunosuppressive activity of CsA and M17, alone and in combination, in the unidirectional mixed lymphocyte culture assay system in order to determine whether synergism occurs between the parent drug and metabolite. Two concentrations of M17 were chosen for the study: one that exhibited negligible immunosuppression (28 μg/L) and one that exhibited 50% immunosuppression (56 μg/L). Three concentrations of CsA (7, 14 and 28 μg/L) were tested with each of the M17 concentrations. The authors concluded that the overall suppression was additive, i.e. the total immunosuppression observed was close to what would be predicted from the sum of the two compounds. No synergism appeared to exist between the two.

The investigation of synergy, additivism, and antagonism in immunosuppression has been shown to be confusing and misleading [32]. The basic difficulty is that most investigators use improper criteria for determining the nature of drug interactions, i.e. they compare the effects of the agents used in combination with the sum of their effects when used alone. This type of comparison is based on assumptions that are incorrect, and conclusions based on it are generally valueless [32]. For this reason, we have re-examined the literature reports described above dealing with interactions of CsA and metabolite combinations and/or combinations of metabolites using the appropriate criteria for determining drug interactions as described by Berenbaum [32]. In addition, we describe the relative inhibitory effects of CsA and purified metabolites, tested separately at concentrations detected in patients' whole blood, on PHA-induced human peripheral blood lymphocyte transformation and mixed-lymphocyte culture (MLC) responses. We also examined the interaction of 49 combinations of CsA and the immunosuppressive metabolite M17, and 49 combinations of CsA and metabolite M8, a major metabolite which is not immunosuppressive. Examining the effect of combinations of CsA and its metabolites is an approach that better represents the situation prevailing *in vivo* and one from which more meaningful conclusions can be drawn.

MATERIALS AND METHODS

Pure CsA was obtained from Sandoz Pharmaceuticals, East Hanover, NJ. Metabolites M1, M8, M13, M17, M18, M21, M25, M26, and M203-218 were purified from human bile through extensive isolation procedures as described previously [33]. The identity and purity of the isolated compounds were verified on three chemically different high performance liquid chromatography columns and through radioimmunoassay cross-reactivity studies [33] with authentic metabolites characterized by spectrochemical analyses [34] and provided by Dr. G. Maurer, Sandoz Pharmaceutical, Basel, Switzerland. Phytohemagglutinin (PHA, Product No. L-9132) was obtained from the Sigma Chemical Co. (St. Louis, MO). Cell culture medium (RPMI-1640), antibiotics, and fetal bovine serum were obtained from the Grand Island Biological Co. (Grand Island, NY) and [*methyl*-^{3}H]thymidine ([^{3}H]-TdR) from New England Nuclear (Boston, MA).

Preparation of CsA and metabolites for cell culture. Standard solutions of each cyclosporine were prepared in acetonitrile. The acetonitrile was removed under reduced pressure and the dry CsA (or metabolite) dissolved in 2% ethanol. Dilutions of CsA and metabolites were also made in 2% ethanol. The concentrations of the diluted CsA and metabolites were determined prior to use in culture by high pressure liquid chromatography.

Preparation of cells. Peripheral blood from a healthy donor was collected in preservative-free heparin (20 units/mL, final concentration). Mononuclear cells were obtained as described by Böyum [35]. The lymphocyte-rich mononuclear cells were harvested from the interface, washed by resuspension in phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}, centrifuged, and counted in a hemocytometer.

PHA assay. Cultures were performed in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). Each well contained 2.5×10^5 cells suspended in RPMI-1640 supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), 10% fetal bovine serum, and PHA (2.5 μg/mL culture). CsA and metabolites were introduced separately into culture, each in a 25-μL volume of 2% ethanol, prior to PHA addition. Control cultures (no metabolite or CsA) were adjusted for the amount

of ethanol added to cultures by addition of CsA and/or metabolite by addition of 2% ethanol. Cultures were incubated at 37° in humidified 5% CO_2/95% air. After 66 hr of incubation, 25 μL of [^{3}H]TdR (1.0 μCi; 6.7 Ci/mmol) was added to each well. After an additional 6 hr of incubation, cells were harvested onto glass-fiber filter paper (Reeve Angel 934AH, Whatman Inc., Clifton, NJ) with a semi-automated microharvester. The filters were washed six times with 0.25-mL aliquots of 0.15 M NaCl, air dried, placed in a vial containing 2 mL of Ecosint (National Diagnostics, Manville, NJ) and counted in a liquid scintillation counter with counting efficiency of 43%.

Effects of combinations of metabolites and CsA on PHA-stimulated lymphocyte transformation. The nature of metabolite and CsA interaction was evaluated as described by Berenbaum [32] using the equation:

$$\frac{\text{Metabolite concentration}}{\text{Equi-effective concentration of metabolite used alone}} + \frac{\text{CsA concentration}}{\text{Equi-effective concentration of CsA used alone}} \equiv \begin{matrix} <1 \text{ for synergism} \\ 1 \text{ for additivism} \\ >1 \text{ for antagonism} \end{matrix} \quad (1)$$

Metabolite concentration is the concentration present in the combination. CsA concentration is the concentration present in the combination. The equi-effective concentrations of the agents used alone to produce the observed effect of the combination were found by interpolation in the respective concentration–response curves. Effects that all the constituents of a combination have in common are termed homergic. The combination of two immunosuppressive agents such as M17 and CsA is a homergic combination, whereas the combination of an immunosuppressive agent (CsA) and a non-immunosuppressive metabolite, such as M8, is termed a heterergic combination. A heterergic combination can also be described as an interaction in which one agent (in this case M8) increases or decreases the immunosuppressive effect of another (CsA) without itself being able to produce that effect. Claims than an effect is heterergic rather than homergic require that the agent lacking the specific effect under consideration (immunosuppressibility) does not do so because of inadequate dosage. We have found M8 to be without effect on PHA responses at concentrations up to 1000 ng/mL. Higher concentrations were not tested since they would not be present in the patient. Reports of inhibition of PHA and MLC responses *in vitro* by M8 have been described; the concentration of M8 required for 50% inhibition of PHA response was, however, extremely high (>20,000 ng/mL). Heterergic combinations, like homergic, may show synergy (when the immunologically ineffective agent increases the effect of the immunosuppressive agent), antagonism (when it reduces its effect) or additivism (when it has no modifying effect). The fraction corresponding to the immunologically ineffective agent in equation 1 is zero as its equi-effective concentration is theoretically infinite, and isoboles for such combinations do not intersect the concentration-axis of the ineffective agent [32].

MLC assay. CsA and metabolites contained in RPMI-1640 supplemented with 10% heat-inactivated human serum were added in 50-μL aliquots to 96-well microtiter plates. Responder cells (1×10^5) and 1×10^5 irradiated (3000 rads) stimulator cells were then added to each well and the volume was adjusted to 200 μL with RPMI-1640. Cultures were incubated at 37° in 5% CO_2/95% air for 5 days, then pulsed with 50 μL of [^{3}H]TdR (1 μCi; 6.7 Ci/mmol) and allowed to incubate for an additional 18 hr. Cells were collected onto glass-fiber filter paper, were washed and amounts of radioactivity associated with cellular DNA were measured with a beta–scintillation counter.

RESULTS

The effects of CsA and nine of its metabolites, each tested separately, on the PHA-induced transformation of human peripheral blood lymphocytes are illustrated in Fig. 1. All compounds were tested simultaneously and under identical conditions. CsA was significantly more immunosuppressive than any of its metabolites which confirms previous results [23]. Parent CsA displayed nearly 50% inhibition at 50 ng/mL; maximum inhibition (70% decrease in [^{3}H]TdR uptake) was observed at concentrations of 300 ng/mL or greater. Metabolites M17, M1, and M21 inhibited the response by less than 15% at 50 ng/mL, but caused 43, 52, and 48% inhibition, respectively, at 1000 ng/mL; M8, M18, M26, M25, M13, and M203-218 were

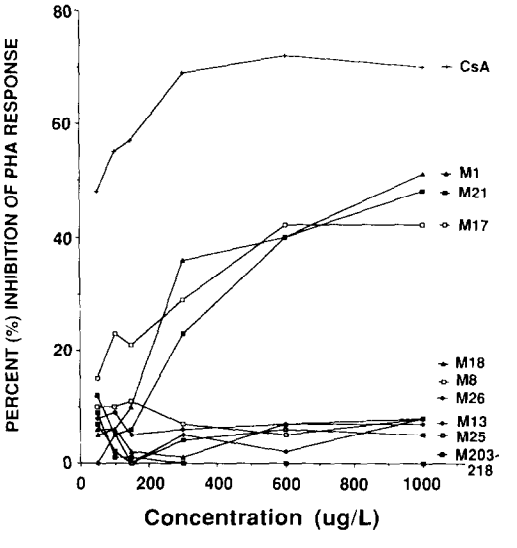

Fig. 1. Inhibitory effects of CsA and purified metabolites on PHA-induced lymphocyte transformation. Mean ± SD for control culture (no CsA or metabolite) was 108,403 ± 5,978 cpm. The overall average coefficient of variation for data points (N = 3) was 8.1 ± 5.6%.

Table 1. Combined effects of metabolite M17 and CsA on PHA-induced lymphocyte transformation

(A) Percent activity of control PHA-induced [^{3}H]TdR incorporation for the various combinations

M17 (ng/ml of culture) \ CsA (ng/ml of culture)	0	4	7	19	32	64	166	276
0	1 100	2 100	3 100	4 68.7	5 54.1	6 33.4	7 25.4	8 25.2
10	9 95.4	78.7	71.1	51.5	59.3	32.3	28.2	27.4
21	17 91.2	76.8	61.3	36.7	54.0	33.1	25.8	26.1
47	25 71.7	43.4	57.7	47.9	58.7	45.4	36.9	32.0
66	33 72.4	79.6	75.3	58.0	54.4	38.8	25.6	27.7
215	41 60.9	55.1	48.2	41.3	54.1	37.1	28.1	32.6
372	49 54.7	54.6	48.1	47.9	59.3	44.2	35.3	31.0
620	57 56.2	66.2	53.8	55.8	61.5	56.2	46.7	42.0

The value in each box represents the mean percent activity of PHA-induced [^{3}H]TdR incorporation obtained in the presence of the cyclosporine combinations indicated taking the value obtained in the absence of cyclosporines as 100%. Cells cultured in the absence of PHA and cyclosporines incorporated 328 ± 70 cpm compared to 142,617 ± 12,032 cpm in the presence of PHA and the absence of cyclosporines.

(B) Type of interaction for the various combinations of CsA and M17 shown in panel A

M17 (ng/ml of culture) \ CsA (ng/ml of culture)	0	4	7	19	32	64	166	276
0	1	2	3	4	5	6	7	8
10	9 0.49 S	0.39 S	0.53 S	1.20 A	0.95 S or Add	1.90 A	2.74 A
21	17 0.68 S	0.34 S	0.35 S	1.07 Add or A	1.11 A	1.48 A	2.21 A
47	25 0.09 S	0.38 S	0.47 S	1.33 A	1.39 A	3.02 A	2.34 A
66	33 1.89 A	0.96 S or Add	0.90 S	1.18 A	1.25 A	1.33 A	3.15 A
215	41 0.78 S	0.16 S	0.39 S	1.57 A	1.16 A	1.90 A	4.31 A
372	49 1.14 A	0.16 S	0.47 S	2.56 A	1.42 A	2.91 A	3.94 A
620	57 3.60 A	0.20 S	2.00 A	4.28 A	3.51 A	4.05 A	5.81 A

The number appearing in the box indicates the numerical value obtained in equation 1 using the values shown in panel A. The letter indicates the type of interaction—synergism (S), antagonism (A), or additivism (Add)—for the specific combination. The synergistic combinations appear in the shaded areas. The small numbers appearing in the upper left corner of some boxes indicate the reaction number.

not significantly inhibitory over this concentration range.

Homergic combinations. Forty-nine combinations of M17 and CsA were examined for their effects on PHA-induced mitogenesis. M17 was tested at concentrations of 10, 21, 47, 66, 215, 372, and 620 ng/mL and CsA at 4, 7, 19, 32, 64, 166, and 276 ng/mL of culture. The equi-effective concentrations of M17 and CsA used alone to produce the observed effect of the combination were determined by interpolation in the respective concentration–response curves constructed from the data shown in Table 1 (reactions 1 through 8 for CsA and 1, 9, 17, 25, 33, 41, 49, and 57 for M17). Synergism between M17 and CsA was found in 16 of the 49 combinations, antagonism in 30 combinations, and in 3 combinations the interaction was additive or closely approached additivism (values of 0.95, 0.96, and 1.07 in equation 1). For approximately one-fourth of the synergistic combinations (4 of 16), the sum of the fractions in equation 1 was ≤ 0.2 indicating a high degree of synergism, i.e. where the effect of the combination was greater than that of M17 or CsA used alone at higher concentrations.

As seen in Table 1, synergism between M17 and CsA occurred with combinations of the lower CsA concentrations tested (4, 7, and 19 ng/mL) and most concentrations of M17. At the higher CsA concentrations tested (32, 64, 166, and 276 ng/mL), the presence of M17 in cultures along with CsA resulted in an antagonistic effect (reduction of the effectiveness of the two immunosuppressive agents).

Heterergic combinations. The effects of 49 combinations of CsA and metabolite M8 were examined using peripheral blood mononuclear cells from the same donor used in the M17/CsA combination study described above so as to eliminate differences in sensitivity to CsA which varies among individuals. M8 was tested at concentrations of 27, 41, 108, 162, 340, 678, and 1080 ng/mL and CsA at 5, 10, 20, 43, 86, 126, and 270 ng/mL of culture. The equi-effective concentrations of M8 and CsA used alone to produce the observed effects of the combinations were found by interpolation in the respective concentration–response curves constructed from the data appearing in Table 2, i.e. reactions 1 through 8 for CsA and 1, 9, 17, 25, 33, 41, 49, and 57 for M8. Synergism between M8 and CsA was observed in 29 of the 49 combinations, antagonism in 17 combinations, additivism in 1 combination, and in 2 treatments the interaction was very close to additivism (values of 0.96 and 1.07 in equation 1). For approximately one-half (14 of 29) of the synergistic combinations, the sum of the fractions in equation 1 was ≤ 0.2 indicating a high degree of synergism. At M8 concentrations of 27 and 41 ng/mL, the combinations of M8 with CsA (5–270 ng/mL) resulted in a synergistic effect. At M8 concentrations greater than 108 ng/mL, certain combinations resulted in synergistic effects while others had an antagonistic effect.

Immunosuppressive effects of CsA and metabolites on MLC. In the MLC assay, CsA was also more inhibitory than its metabolites (Fig. 2). CsA exerted maximum inhibition (>95%) at 250 ng/mL, while M17, M1, and M21 depressed the response 82, 63, and 50%, respectively, at the same concentration. A 25 ng/mL concentration of CsA was required to decrease the MLC response by 50% compared to 64 ng/mL for M17. Metabolites M8 and M18 were only slightly inhibitory (<30%) at 250 ng/mL. M26, M13, M25, and M203-218 were inactive even when concentrations up to 1000 ng/mL were used (not shown). These studies are in agreement with the findings of others [24, 29, 31, 36–41] that metabolites of CsA not inhibitory to the PHA response of cells are also without a significant inhibitory effect on

Table 2. Combined effects of metabolite M8 and CsA on PHA-induced lymphocyte transformation

(A) Percent activity of control PHA-induced [^{3}H]TdR incorporation for the various combinations

M8 (ng/ml of culture) \ CsA (ng/ml of culture)	0	5	10	20	43	86	126	270
0	1 100	2 72.0	3 70.1	4 68.7	5 63.3	6 53.7	7 48.2	8 45.2
27	9 83.0	69.2	55.5	58.9	55.9	41.8	37.2	37.5
41	17 76.3	57.9	48.2	54.8	54.1	42.2	46.8	37.7
108	25 76.6	59.7	64.2	61.4	61.4	55.1	53.1	43.1
162	33 97.1	76.3	74.1	65.8	62.7	55.1	47.7	40.9
340	41 89.5	81.8	72.1	57.8	65.8	52.1	45.9	40.7
678	49 78.6	48.1	58.5	56.4	63.9	47.6	49.2	38.4
1080	57 88.1	75.3	71.8	61.0	72.7	65.1	59.9	46.4

The value in each box represents the mean percent activity of PHA-induced [^{3}H]TdR incorporation obtained in the presence of the cyclosporine combinations indicated taking the value obtained in the absence of cyclosporines as 100%. Cells cultured in the absence of PHA and cyclosporines incorporated 316 ± 122 cpm compared to 48,710 ± 4,528 cpm in the presence of PHA and the absence of cyclosporines.

(B) Type of interaction for the various combinations of CsA and M8 shown in panel A

M8 (ng/ml of culture) \ CsA (ng/ml of culture)	0	5	10	20	43	86	126	270
0	1	2	3	4	5	6	7	8
27	9	0.33 S	0.14 S	0.40 S	0.66 S	0.34 S	0.00 S	0.00 S
41	17	0.09 S	0.08 S	0.28 S	0.57 S	0.00 S	0.86 S	0.00 S
108	25	0.11 S	0.33 S	0.50 S	1.07 A or Add	1.14 A	1.58 A	0.00 S
162	33	2.00 A	3.30 A	0.80 S	1.22 A	1.22 A	1.01 Add	0.00 S
340	41	2.50 A	2.00 A	0.40 S	1.72 A	0.96 Add or S	0.76 S	0.00 S
678	49	0.04 S	0.18 S	0.31 S	1.43 A	0.69 S	1.44 A	0.00 S
1080	57	2.00 A	4.00 A	0.50 S	8.60 A	2.90 A	2.80 A	1.80 A

The number appearing in the box indicates the numerical value obtained in equation 1 using the values shown in panel A. The letter indicates the type of interaction—synergism (S), antagonism (A), or additivism (Add)—for the specific combination. The synergistic combinations appear in the shaded areas. The small numbers appearing in the upper left corner of some boxes indicate the reaction number.

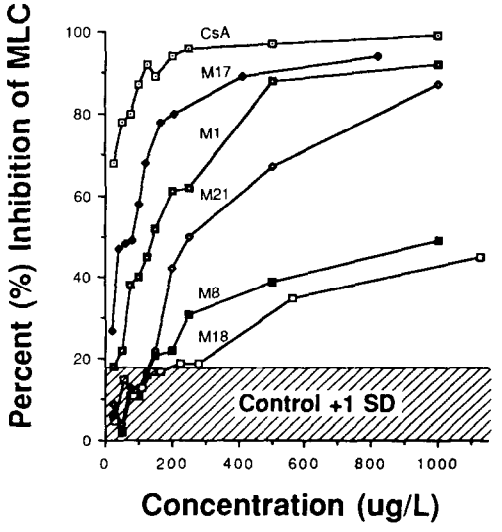

Fig. 2. Effects of CsA and purified metabolites on proliferation in the mixed-lymphocyte culture (MLC). Mean ± SD for control culture (no CsA or metabolite) was 31,388 ± 5,902 cpm. The overall average coefficient of variation for data points (N = 12) was 14.9 ± 13.2%.

MLC response, Con A- and anti-CD3 antibody-stimulation, and IL-2 production which are generally considered to be more sensitive assays to CsA and its metabolites. M8, M26, M13, and M203-218 were shown by Schitt *et al.* [37] to be inactive in the MLC even when concentrations up to 10,000 ng/mL were used.

The difference in sensitivity of the various *in vitro* proliferative assays to CsA and its immunosuppressive metabolites may depend on the mode of activition [23] and lymphocyte concentrations utilized. Increasing the cell number decreases the effectiveness of CsA tested *in vitro*. Typical numbers of lymphocytes utilized per assay for several of the *in vitro* tests are as follows: primed lymphocytes test (1×10^4), anti-CD3 stimulation (1×10^5), MLC ($1–2 \times 10^5$) and the lectin mitogen (PHA and Con A) assays ($2–3 \times 10^5$).

DISCUSSION

The importance of both immunosuppressive and non-immunosuppressive metabolites of CsA in the immunopharmacology of CsA therapy is largely unknown. The effectiveness of CsA therapy may depend highly upon the immunosuppressive effects of combinations of individual metabolites and the parent compound. It is possible that low levels of CsA in combination with CsA metabolites may be an adequate immunosuppressant when the concentrations of CsA or any of its metabolites alone would not be adequately immunosuppressive [29]. If some of the metabolites were active or acted synergistically with CsA, there could be a patient benefit in filling somewhat the time gap between the CsA concentration peaks after repetitive dosing of CsA.

In this report we have examined the effects of 49 combinations of CsA and M17 and the same number of combinations of CsA and M8 for their effects in the PHA-induced lymphocyte transformation assay. Sixteen combinations of M17 and CsA acted synergistically. Thirty combinations of CsA and M17 resulted in a lessening of the inhibitory effect (antagonistic effect). Antagonism between parent drug (CsA) and metabolite or between metabolites themselves is obviously not a favourable interaction because it results in a reduction of immuno-

suppressibility. Looking at the absolute changes in CsA inhibition exerted by the nonimmunosuppressive metabolite M8, the high degree of synergism would indicate a relatively strong effect of M8 on CsA immunosuppressiveness. M8 increased the inhibitory effect of CsA in 29 of the 49 combinations; 14 of the 29 synergistic combinations were strongly synergistic (≤ 0.2 in equation 1). Seventeen combinations were antagonistic (same receptor site but different affinities). In summary, specific homergic combinations (M17 and CsA) and heterergic combinations (M8 and CsA) can result in synergism while other combinations act antagonistically.

As seen in Tables 1 and 2, the type of interaction (antagonistic or synergistic) can change considerably, especially with high concentration combinations of M8 and CsA. This is the first detailed analysis of CsA/metabolite interaction and the first demonstration of antagonism between metabolite and parent drug. Our studies for analyzing the metabolite/CsA interactions were obviously more laborious and involved than the studies by others described above, but conclusions based on it may be relied upon.

The PHA-induced transformation of peripheral blood lymphocytes offers a well-defined system to assess relative immunosuppression of the cyclosporines, specifically on lymphocyte reactivity. Results of *in vitro* testing, however, have their limitation in explaining *in vivo* events involving CsA and metabolites. Clinically relevant immunosuppressive activity of CsA or metabolites does not depend only on its structural property. Other factors, such as distributions of metabolites and CsA in the body (M1, M17, and M8, for example, are almost entirely found in red blood cells) and the individual sensitivity of lymphocytes infiltrating the target organ, may play an important role in overall suppression. Extrapolation of the data obtained *in vitro* to *in vivo* situations will not be attempted herein. The three previous reports examining the effects of defined mixtures of CsA and metabolite or combinations of metabolites for synergism will now be discussed. The results of Zeevi *et al.* [29] showing synergism between CsA and M17 in the primed lymphocyte test of heart biopsy lymphocytes are consistent with our findings using similar concentrations of CsA and metabolite M17 (Table 1). It remains to be established if, as in our results, upon expansion of the number of combinations tested, antagonism between CsA and M17 can be demonstrated in the primed lymphocyte system. With regard to the results of Copeland *et al.* [31] showing no synergism but rather an additive effect of M17 and CsA combinations on the inhibition of mixed lymphocyte reaction, we used the concentration–response curves for M17 and CsA shown in the Copeland paper along with the values of inhibition obtained with the different concentrations to determine equi-effective concentrations in equation 1. Such treatment resulted in the following: the combinations tested and reported by Copeland to be additive are without exception strongly synergistic (sum of the fractions in equation 1 ranged from 0.10 to 0.40) which would be consistent with our findings of synergism for similar concentrations of M17 and CsA in the PHA assay. The criterium used by Copeland *et al.* [31], comparing the effect of the M17 and CsA used in combination with the sum of their effects when used alone, is based on assumptions that are in error [32]. Because of the nature of drug–receptor interactions, concentration–response curves for biologically active agents are rarely if ever linear.

Sewing *et al.* [30] described the inhibition of proliferation of rat mesangial cells and Con A-induced peripheral lymphocyte transformation by metabolites H320 (M13), H350 (M203-218), and H355. In both assays, the combination of the metabolites has a far higher inhibitory effect than the addition of their single points, especially at low concentrations. It was shown that even at ineffective concentrations of the single compounds, metabolites can potentiate (synergize) each other and gain biologically significant activity. In the study of CsA and metabolite combinations on Con A responses of lymphocytes, the concentrations tested ranged from approximately 1 μmol/L (1,203 ng/mL) to greater than 100 μmol/L (120,340 ng/mL) for M13; 13.8 μmol/L (17,000 ng/mL) to greater than 1,380 μmol/L (1,700,700 ng/mL) for M203-218; and 15.6 μmol/L (19,257 ng/mL) to greater than 1,560 μmol/L (1,925,700 ng/mL) for H355. These concentrations are several orders of magnitude higher than the concentrations used in our study and by other investigators and are far in excess of the levels found in the patient. Again, comparing the effects of CsA metabolites H320 (M13), H355, and H350 (M203-218) used in combination with the sum of their effects used alone is based on assumptions that are wrong. The effects of combinations of metabolites H350 (203-218) and H355 on proliferation of rat mesangial cells showing that the combination of the two metabolites has a far higher effect than the addition of their single effects is also difficult to interpret. The concentrations of H350 (M203-218) ranged from 0.015 mmol/L (18.5 μg/mL of culture) to over 1 mmol/L (1,200 μg/mL), and from 0.375 mmol/L (464 μg/mL) to over 25 mmol/L (30,860 μg/mL) for H355. Combinations of these concentrations would bring the amount of total metabolite present in the assay from approximately 0.5 mg/mL to over 32 mg/mL of culture. The significance of results obtained from these studies using milligram amounts of metabolite per milliliter of culture is questionable. We use 1 μg CsA (or metabolite) per mL of culture as the highest concentration tested since it represents the upper limit of the therapeutic level of CsA found in patients.

In summary, we have demonstrated that certain combinations of CsA and its immunosuppressive metabolite M17 have a synergistic effect on the inhibition of PHA-induced lymphocyte transformation *in vitro*, whereas other combinations result in an antagonistic effect. Likewise, certain combinations of CsA and its nonimmunosuppressive metabolite M8 have an antagonistic effect (reduction in the immunosuppressive activity of CsA), while others result in a synergistic effect (enhancement of the inhibition of PHA response). These diverse changes occur at therapeutic concentrations of CsA

and concentrations of metabolite found *in vivo* and, therefore, add to the complexity of determining the immunopharmacology of CsA therapy. Low levels of CsA in combination with metabolite do have a significant inhibitory effect, at least *in vitro*, on lymphocyte proliferation when concentrations of CsA and its metabolite alone would not be adequately immunosuppressive, but slight changes in levels of CsA and/or metabolite can result in a less than favorable interaction, i.e. antagonism or reduction in the immunosuppressive action of CsA and/or its metabolite. Metabolites lacking immunosuppressive activity can also affect the action of the parent drug, either favorably or unfavorably, depending on their levels, and these interactions have to be considered when looking at the overall effect of all metabolites and CsA on immunosuppression.

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