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Lysophospholipid-Mediated Inhibition of Na^+,K^+-Adenosine Triphosphatase Is a Possible Mechanism of Immunosuppressive Activity of Cyclosporin A

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SUMMARY

The relationship between the phospholipase-stimulating and immunosuppressive properties of cyclosporin A (CsA) has been investigated *in vitro*. At concentrations of 0.025 μM and upwards, CsA caused dose-related inhibition of both mitogen- and alloantigen-stimulated uptake of tritiated thymidine by human mononuclear leukocytes (MNL), which was associated with a time- and dose-related enhancement of the generation of lysophosphatidylcholine (LPC), arachidonic acid, and prostaglandin E_2 from mitogen-stimulated cells. Arachidonate alone, at concentrations of up to 20 μM, did not affect lymphocyte activation, whereas cyclooxygenase and 5′-lipoxygenase inhibitors failed to protect the cells against the antiproliferative effects of CsA. However, LPC caused dose-related inhibition of MNL proliferation. Moreover, coincubation of MNL with α-tocopherol, a lysophospholipid-complexing agent, or with lysophospholipase protected the cells against CsA, as well as against LPC. The Na^+,K^+-ATPase activity of mitogen-activated lymphocytes was also inhibited by CsA, whereas inclusion of α-tocopherol or lysophospholipase protected this enzyme. Excessive production of lysophospholipids and consequent inhibition of Na^+,K^+-ATPase during CsA treatment of mitogen- or antigen-activated lymphocytes is a possible biochemical mechanism of the immunosuppressive activity of this agent.

CsA is a fungus-derived, hydrophobic, cyclic unadecapeptide with potent immunosuppressive properties. This agent is used primarily in clinical organ transplantation (1–4) but is also effective in the treatment of some autoimmune diseases and dermatological disorders (5–8). Although CsA-mediated immunosuppression is associated with inhibition of transcription of a restricted number of T cell activation genes (9, 10), the precise primary biochemical mechanism by which this is achieved has not been established. There is some evidence, however, that the antiproliferative effects of CsA are due to interference with the structure and functions of the lymphocyte plasma membrane. Exposure of lymphocytes to CsA is accompanied by an immediate drug-induced membrane depolarization, increased lipid membrane microviscosity, release of K^+, and increased levels of intracellular Ca^{2+} (11–13). These structural perturbations in the cell membrane and alterations in membrane physiology occur within the first hour of exposure of lymphocytes to CsA and are followed by increased PL activity and generation of AA and its oxygenated metabolites from membrane phospholipids (14, 15). It has been suggested that CsA-mediated immunosuppression is due to PL activation in target cells, leading to enhanced release of antiproliferative PGs such as PGE_2 (15, 16).

In the present study we have investigated the relationship between the PL-activating properties and immunosuppressive effects of CsA. Our data demonstrate that 1) lysophospholipids produced as a consequence of drug-mediated activation of lymphocyte membrane PLs are possible mediators of CsA-induced immunosuppression by interfering with the activity of Na^+,K^+-ATPase and 2) the antiproliferative immunosuppressive effects of CsA are effectively prevented by the addition of the lysophospholipid-complexing agent AT (vitamin E) (17) to CsA-treated lymphocytes.

Materials and Methods

Chemicals and reagents. Pure CsA was obtained from Sandoz (Basel, Switzerland) and dissolved to a stock concentration of 1 mM (1.2 mg/ml) in dimethylsulfoxide. Subsequent dilutions were made in cell suspension medium and the drug was used at final concentrations ranging from 0.005 to 4 μM in assays of lymphocyte proliferation and

ABBREVIATIONS: CsA, cyclosporin A; AA, arachidonic acid; AT, DL-α-tocopherol; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; TdR, thymidine; LPC, lysophosphatidylcholine; MNL, mononuclear leukocytes; NDGA, nordihydroguaiaretic acid; PC, phosphatidylcholine; PG, prostaglandin; PKC, protein kinase C; PL, phospholipase; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum; LDH, lactate dehydrogenase; TLC, thin layer chromatography; PHA, phytohemagglutinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

PLA_2 activity. All other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO), with the exception of AT (F. Hoffmann-La Roche, Basel, Switzerland) and piroxicam (Pfizer Laboratories, Johannesburg, South Africa). Radiochemicals were obtained from DuPont NEN Research Products (Boston, MA) and from Amersham International (Aylesbury, England).

MNL and pure T lymphocyte suspensions. MNL were prepared, as described previously, by density centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) of blood taken from healthy adult human volunteers (18). The cells were then resuspended to 4×10^6/ml in HEPES (4.2 mM)-buffered RPMI 1640 medium supplemented with 1% glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Suspensions of purified T lymphocytes were prepared using a standard differential adherence method. Briefly, a 5-ml sterile disposable syringe was loosely filled with 0.15 g of sterile nylon wool (Biotest, Dreieich, Germany) and rinsed with 10 ml of prewarmed (37°) RPMI 1640 medium. MNL were resuspended in 1 ml of warm medium, added immediately to the nylon wool column, and allowed to equilibrate. After temporary sealing of both ends, the syringe was incubated for 30 min at 37° in an atmosphere of 5% CO_2. Thereafter, the nonadherent cells were removed and collected by flushing of the column with 10 ml of warm RPMI 1640 medium. The resultant nonadherent T lymphocyte-enriched cell population was concentrated by centrifugation and adjusted to 2×10^6/ml, and the purity was determined using flow cytometry to enumerate numbers of B and T lymphocytes and monocytes. Lucigenin-enhanced chemiluminescence, with phorbol-12-myristate-13-acetate (20 ng/ml) as the stimulus of superoxide generation, was also used to assess the extent of contamination by phagocytes (19).

AA production. MNL (1×10^7/ml) were coincubated with 5 μCi/ml [5,6,8,9,11,12,14,15-^{3}H(N)]AA (210 Ci/mmol; DuPont NEN) for 30 min at 37° in RPMI 1640 medium, to allow incorporation of the radiolabeled unsaturated fatty acid into membrane phospholipids. The cells (5×10^6) were then transferred to 10-ml tissue culture tubes and preincubated for 10 min at 37° (5×10^5 MNL/ml of RPMI), followed by addition of CsA (0.25, 0.5, or 1 μM). After an additional 30 min of incubation at 37°, PHA (5 μg/ml) and FCS (final concentration, 10%) were added to the tubes, bringing the final volume in each tube to 10 ml, containing 5×10^5 cells/ml. The tubes were then incubated for 2 or 4 hr at 37° in an atmosphere of 5% CO_2. In some experiments AT (50 μM) was added to the MNL during preincubation before the addition of CsA and/or PHA. Appropriate solvent controls for CsA and AT were included. After incubation the cells were washed twice in ice-cold phosphate-buffered saline and resuspended in 1 ml of this solution (5×10^6 cells). The lipids were then extracted by the addition of 5 ml of *n*-hexane/isopropanol/concentrated HCl (final concentration, 0.1 M) (300:200:4, v/v/v), as described previously (20). The upper organic phase was removed and dried under a stream of nitrogen. The lipids were dissolved in 100 μl of hexane/isopropanol (3:2, v/v) containing 15 μg of unlabeled AA to facilitate visual detection with iodine vapors. Aliquots of 10 μl were then spotted onto precoated silica gel 60 TLC plates (Merck, Darmstadt, Germany). The plates were developed in chloroform/acetone (96:4, v/v). After exposure to iodine vapors the AA spots were localized and the silica was removed and assayed for radioactivity.

LPC assay. A previously described high performance TLC method (21) was used to assay LPC levels in the chloroform/methanol (2:1, v/v) extracts of control and CsA-treated MNL. The labeling and incubation procedures were almost identical to those used for the measurement of [^{3}H]AA release, except that the cells were treated with 20 μCi/ml [9,10-^{3}H(N)]palmitate (47 Ci/mmol; DuPont NEN) to promote incorporation of radiolabeled palmitate into the C1 position of the glycerol backbone of membrane phospholipids and an extended incubation period of 18 hr was also included. After incubation the cells were washed and resuspended in 1 ml of phosphate-buffered saline and the lipids were extracted by the addition of 3 ml of chloroform/methanol (2:1, v/v). Water (0.3 ml) was added to cause phase separation. The phospholipid-containing lower phase was removed and 1 ml was evaporated to dryness under a stream of nitrogen. The dried residues were dissolved in 50 μl of chloroform/methanol, and aliquots of 10 μl containing the appropriate standards (10 μg LPC and PC) onto precoated silica gel 60 high performance TLC plates (Merck). The plates were developed (three times) in chloroform/methanol/isopropanol/0.25% KCl/ethyl acetate (30:9:25:6:18, v/v/v/v/v). After exposure to iodine vapors the LPC and PC spots were localized and the silica was removed and assayed for radioactivity.

Radioassay for PGE_2. MNL were suspended in HBSS and preincubated in an atmosphere of 5% CO_2 for 30 min at 37° before the addition of CsA (0.05–4 μM) and PHA (5 μg/ml) individually and in combination. The final volume in each tube was 1 ml, containing 5×10^5 MNL. After incubation for 30 or 120 min at 37° the reactions were terminated by the addition of 1 ml of ice-cold HBSS and the tubes were placed in an ice bath. The cells were removed by centrifugation in a refrigerated centrifuge and the PGE_2 in the cell-free supernatants was measured by radioimmunoassay (DuPont NEN). This is a competitive binding radioimmunoassay based on the differential precipitation (by 16% polyethylene glycol) of immune complexes formed between a standard amount of added ^{125}I-labeled PGE_2 and specific antiserum to PGE_2 after an 18-hr incubation period at 4°. The amount of radioactivity in the polyethylene glycol precipitate is inversely proportional to the amount of competing nonradiolabeled PGE_2 in the test (supernatant) samples. The concentration of PGE_2 in the supernatants was calculated by using a standard curve with added pure PGE_2 in the range of 0.25–25 pg/ml. The results are expressed as pg of PGE_2/5×10^5 MNL.

Measurements of the effects of CsA on the activity of purified PLA_2 and lysophospholipase. The effects of CsA (4 μM) on the activity of purified PLA_2 (from porcine pancreas; final concentration, 10 units/ml) or lysophospholipase (2-LPC acylhydrolase-PLB from *Vibrio* species; final concentration, 200 milliunits/ml) were assayed by radiometric TLC using L-[5,6,8,9,11,12,14,15-*arachidonyl*-^{3}H]PC (specific activity, 135 Ci/mmol; Amersham) and L-[1-*palmitoyl*-^{14}C]LPC (specific activity, 56 mCi/mmol; Amersham) as the respective substrates (22, 23).

Mitogen-activated MNL proliferation. Fifty microliters of MNL or purified T lymphocyte suspension (1×10^5 cells) were added to 100 μl of RPMI 1640 medium, followed by 20 μl of CsA (final concentration, 0.005–4 μM) or drug-free solvent control system. Appropriate volume adjustments were made when CsA was used in combination with the other agents described below. After an incubation period of 30 min at 37°, 20 μl of 50% autologous, heat-inactivated serum (5% final concentration) were added to each well, followed immediately by 20 μl of RPMI 1640 medium or the mitogen PHA (final concentration, 2.5 or 5 μg/ml), in control and stimulated systems, respectively. After 48 hr of culture at 37° in an atmosphere of 5% CO_2, 0.2 μCi of [^{3}H]TdR was added to each well, the plates were reincubated for 18 hr, and the cells were then assayed for [^{3}H]TdR uptake (9). In some experiments the potential of various lipid- and water-soluble antioxidant chemicals and enzymes, the LPC-hydrolyzing enzyme lysophospholipase, and inhibitors of cyclooxygenase, 5-lipoxygenase, and PKC to protect against CsA (0.1, 0.25, or 0.5 μM)-mediated inhibition of lymphocyte proliferation was investigated. These agents and the final concentrations at which they were used in the assays of lymphocyte proliferation are shown in Table 1. At the predetermined concentrations shown, none of the test agents alone either inhibited or enhanced the uptake of [^{3}H]TdR by PHA-activated lymphocytes. In other experiments the effects of the following on PHA-activated MNL proliferation were investigated: 1) pretreatment of MNL with AT (10 or 50 μM) for 60 min at 37°, followed by washing and exposure to CsA (0.1, 0.25, or 0.5 μM), 2) coincubation of MNL with AA (1–50 μM) or PC (1–50 μM) alone, and 3) coincubation of MNL with LPC (5–20 μM) in the presence or absence of AT (10 or 50 μM).

Mixed lymphocyte cultures. Fifty microliters of responding MNL were co-cultured with an equal number of mitomycin C (150 μM)-

TABLE 1

Antioxidants, enzymes, and enzyme inhibitors assayed for possible protective activity against CsA-mediated inhibition of mitogen-induced lymphocyte proliferation

Agent	Mode of action	Final concentration
AT	Lipid-soluble antioxidant	10 and 50 μM
AT acetate	Lipid-soluble antioxidant	10 and 50 μM
Retinol	Lipid-soluble antioxidant	10 μM
Butylated hydroxytoluene	Lipid-soluble antioxidant	25 μM
Butylated hydroxyanisole	Lipid-soluble antioxidant	15 μM
Cysteine	Water-soluble antioxidant	250 μM
Catalase	Antioxidant enzyme	500 units/ml
Indomethacin	PG synthetase inhibitor	15 μM
Piroxicam	PG synthetase inhibitor	15 μM
NDGA	5′-Lipoxygenase inhibitor	10 μM
Staurosporine	PKC inhibitor	25 and 50 nM
H-7	PKC inhibitor	25 μM
Lysophospholipase	LPC-hydrolyzing enzyme	25–200 milliunits/ml

pretreated MNL from an unrelated donor, in a final volume of 200 μl of RPMI 1640 medium containing 5% heat-inactivated AB serum with or without CsA (0.005–0.5 μM). After 6 days of incubation at 37° in an atmosphere of 5% CO_2, 0.2 μCi of [^{3}H]TdR was added to each well and the plates were reincubated for 24 hr, after which the cells were assayed for [^{3}H]TdR.

Assay of transmembrane fluxes of K^+ in MNL and purified T lymphocytes. ^{86}Rb was used as tracer for measuring K^+ uptake (24, 25). Briefly, MNL or purifed T lymphocytes (5×10^5/ml) were preincubated at 37° for 30 min in RPMI 1640 medium, followed by addition of CsA (0.1–1 μM). After 15 min PHA (5 μg/ml) and FCS (10%) were added and the cells were incubated for 2 or 4 hr at 37°. The final volume in each tube was 10 ml, containing a total of 5×10^6 cells. The cells were then pelleted by centrifugation, washed twice in isotonic Tris buffer (122 mM NaCl, 4 mM KCl, 1 mM $MgS0_4$, 1 mM KH_2PO_4, 20 mM Tris, 5 mM glucose, pH 7.4), and resuspended in Tris buffer supplemented with 10% FCS. The cells were preincubated for 15 min at 37°, followed by the addition of 2 μCi of ^{86}Rb (rubidium-86 chloride, 37 MBq; Amersham). The final volume in each tube was 1 ml, containing 5×10^6 cells. After 30 min, ice-cold Tris buffer was added to terminate the reaction, the cells were washed twice, the pellets were finally dissolved in 0.4 ml of 1% Triton X-100/0.1 M NaOH, and the radioactivity was assayed in a liquid scintillation counter. Na^+,K^+-ATPase-mediated K^+ uptake was taken as the difference in ^{86}Rb uptake in the presence and absence of 2 mM ouabain.

In another series of experiments MNL or purified T lymphocytes were incubated overnight (18 hr) with CsA in RPMI 1640 medium, washed, resuspended in FCS-supplemented Tris buffer, and incubated for 2 or 4 hr with PHA, and uptake of ^{86}Rb was measured as described above. PHA was not included during the overnight incubation because prolonged exposure to the mitogen caused extensive agglutination of the cells. AT (50 μM) or lysophospholipase (200 milliunits/ml) was added to some systems. These agents were added before addition of PHA to cells suspended in Tris buffer.

The activity of Na^+,K^+-ATPase in purified lymphocyte membranes was measured by a previously described spectrophotometric method (26). Briefly, after overnight incubation with CsA (0.1 or 1 μM) MNL were resuspended to 5×10^6/ml in FCS (10%)-supplemented Tris buffer and then concentrated by centrifugation, and the pellets were resuspended in 3 ml of 0.34 M sucrose containing 0.1 mM EDTA (2×10^7 cells/ml), followed by sonication. Cellular debris was removed by centrifugation at 1400 rpm for 10 min at 4°, and the membrane fractions in the supernatants were harvested by centrifugation at 25,000 rpm for 30 min. The membrane pellets were resuspended and dispersed in 2 ml of sucrose and were assayed for ouabain-inhibitable Na^+,K^+-ATPase activity exactly as described by Schwartz *et al.* (26); the data are expressed as nmol of NADH oxidized at 340 nm. This method was also used to investigate the effects of CsA (0.2–4 μM) with or without PHA (5 μg/ml), as well as reagent LPC (5–40 μM), on the activity of purified Na^+,K^+-ATPase (from dog kidney; Sigma). The enzyme was used at a final concentration of 100 milliunits/assay.

Measurement of the cytotoxic potential of CsA and LPC. The cytotoxic potential of CsA with or without PHA or of LPC was measured using a standard spectrophotometric LDH assay (27). Briefly, MNL were coincubated with 4 μM CsA, with or without PHA (5 μg/ml), or with 5–20 μM LPC in 1 ml of HBSS. After 120-min incubation at 37° in an atmosphere of 5% CO_2, the cells were removed by centrifugation and the supernatants were assayed for cytosolic LDH. Intracellular ATP levels were also measured in control and CsA (0.5–4 μM) (with or without PHA)-treated MNL, using a luciferin/luciferase chemiluminescence method (28). The cells (1×10^6/ml) were treated with CsA with or without PHA, in RPMI 1640 medium supplemented with 5% autologous serum, for 2, 4, or 18 hr, after which they were pelleted, lysed, and assayed for ATP.

Measurement of possible complexing of AT with CsA. The UV absorption spectra of mixtures of AT, AT acetate, butylated hydroxyanisole, or butylated hydroxytoluene and CsA, relative to identical concentrations of the individual agents, were measured using a Pye Unicam SP 1700 UV spectrophotometer, according to a previously described method (29). CsA was used at a final concentration of 50 μM, AT and AT acetate at 400 μM, and butylated hydroxyanisole and butylated hydroxytoluene at 1000 μM.

Expression and statistical analysis of results. The results are expressed as the mean value ± standard error for each series of experiments. Levels of statistical significance were calculated using the Student's *t* test (paired *t* statistic).

Results

Effects of CsA with or without PHA on the generation of [^{3}H]AA and [^{3}H]LPC by MNL. These data are shown in Table 2. In the presence of CsA or PHA alone the generation of [^{3}H]AA and [^{3}H]LPC was similar to control values. However, treatment of the cells with the combination of CsA and PHA caused a time- and concentration-dependent increase in the production of [^{3}H]AA and [^{3}H]LPC, which was maximal after 18 hr of incubation (Table 2). Inclusion of AT (50 μM) did not interfere with the CsA/PHA-mediated generation of [^{3}H]AA. The values (mean percentage of the corresponding control system) for MNL treated with PHA and 1 μM CsA for 4 hr in the presence and absence of AT (50 μM) were 220 ± 21% and 275 ± 36%, respectively (two experiments).

Effects of CsA with or without PHA on the generation of PGE_2 by MNL. The data shown in Fig. 1 are those for the 120-min incubation period. CsA alone caused a dose-related increase in the production of PGE_2 by MNL that was statistically significant at a concentration of 4 μM ($p < 0.025$). PHA alone caused a slight but statistically insignificant increase in the generation of PGE_2 by MNL. However, the combination of CsA and PHA caused a striking increase in the production of PGE_2 by MNL ($p < 0.01$ to $p < 0.005$ for 0.5, 1, 2, and 4 μM CsA).

Measurement of the effect of CsA on the activities of purified PLA_2 and lysophospholipase. CsA at a fixed concentration of 4 μM did not affect the activities of purified PLA_2

TABLE 2

Generation of radiolabeled AA and LPC by MNL treated with CsA and PHA individually and in combination

The results of four separate experiments are expressed as percentages of the corresponding untreated control systems (means ± standard errors). The absolute values for generation of [^{3}H]AA after 2 hr and 4 hr were 2,695 ± 325 and 2,375 ± 75 cpm, respectively. After 2, 4, and 18 hr the respective absolute values for generation of [^{3}H]LPC in control systems were 3,085 ± 631, 2,648 ± 301, and 2,115 ± 250 cpm. The extent of labeling of PC in [^{3}H]palmitate-treated MNL after 2, 4, and 18 hr was 124,065 ± 4,975, 181,995 ± 8,085, and 198,385 ± 19,670 cpm, respectively.

System	Generation of [^{3}H]AA after		Generation of [^{3}H]LPC after		
	2 hr	4 hr	2 hr	4 hr	18 hr
	% of control		% of control		
MNL + 1 μM CsA	107 ± 5	111 ± 12	103 ± 6	97 ± 5	122 ± 17
MNL + 5 μg/ml PHA	93 ± 10	98 ± 12	110 ± 8	103 ± 8	92 ± 12
MNL + PHA + 0.25 μM CsA	109 ± 4	109 ± 12	102 ± 1	126 ± 26	172 ± 11[a]
MNL + PHA + 0.5 μM CsA	134 ± 10[a]	151 ± 2[a]	120 ± 20	138 ± 6[a]	382 ± 28[a]
MNL + PHA + 1 μM CsA	156 ± 19[a]	216 ± 45[a]	187 ± 35[a]	205 ± 7[a]	865 ± 106[a]

[a] $p < 0.05$ to $p < 0.005$.

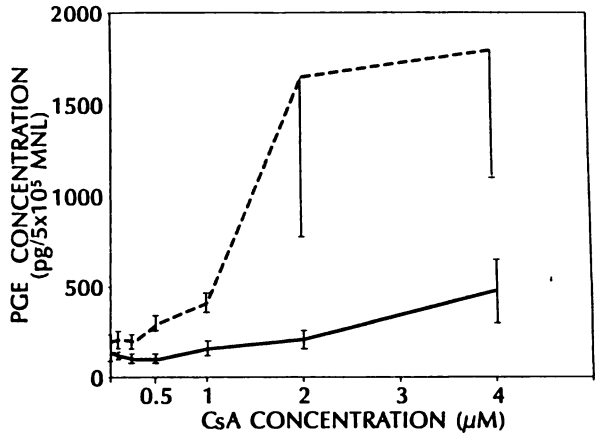

Fig. 1. Effects of CsA alone (——) and in combination with 5 μg/ml PHA (– – –) on the release of PGE_2 by MNL. Data from two separate experiments are expressed as means ± standard errors.

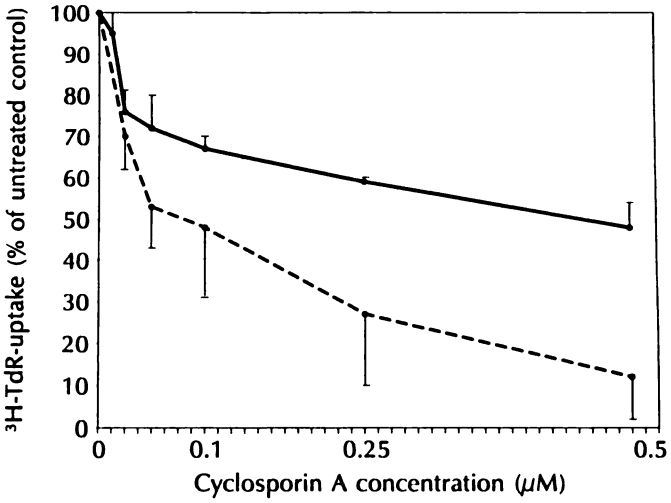

Fig. 2. Effects of CsA on the proliferative responses of MNL activated by 5 μg/ml PHA (——) and with mitomycin C-treated allogeneic lymphocytes (– – –). The results of five experiments are expressed as the mean ± standard error of percentages of the corresponding CsA-free control systems. The absolute values for the unstimulated and PHA-stimulated control systems were 872 ± 56 and 32,014 ± 1,008 cpm, respectively. The corresponding values for the mixed lymphocyte culture systems were 621 ± 35 and 13,081 ± 571 cpm.

or lysophospholipase. The amounts of AA released from radiolabeled PLC in the presence of PLA_2 were 19 ± 1 nmol and 21 ± 1 nmol for the control and CsA-treated systems, respectively (data from four different experiments). The amounts of LPC hydrolyzed by lysophospholipase were 103 ± 16 nmol and 88 ± 13 nmol for the control system and the system containing CsA, respectively (data from two different experiments).

Effects of CsA on PHA- and alloantigen-stimulated uptake of [^{3}H]TdR by MNL. These data are shown in Fig. 2. CsA caused dose-related inhibition of both mitogen- and alloantigen-stimulated lymphocyte proliferation, with statistical significance being achieved at threshold concentrations of 0.013 μM CsA ($p < 0.025$) and 0.025 μM CsA ($p < 0.05$), respectively. These results are in good agreement with previously published data (1, 2).

Measurement of the effects of cyclooxygenase, 5-lipoxygenase, and PKC inhibitors, as well as antioxidants, on CsA-mediated inhibition of PHA-stimulated MNL proliferation. The data for a fixed concentration of 0.5 μM CsA are shown in Table 3. Indomethacin, piroxicam, NDGA, H-7, staurosporine, cysteine, catalase, retinol, butylated hydroxytoluene, butylated hydroxyanisole, and AT acetate did not protect MNL against the antiproliferative effects of CsA. These agents also failed to protect the PHA-induced proliferative responses of MNL against lower concentrations of CsA (0.1 or 0.25 μM) (data not shown). However, both AT and lysophospholipase protected the MNL against the antiproliferative effects of CsA. The effects of AT on CsA-mediated

TABLE 3

Effects of water- and lipid-soluble antioxidants and inhibitors of cyclooxygenase, 5′-lipoxygenase, and PKC on CsA-mediated inhibition of PHA-activated MNL proliferation

Data from three experiments are expressed as percentages of the corresponding control systems (means ± standard errors). The average values for the resting and PHA-activated control systems were 1,072 ± 257 and 27,184 ± 3,751 cpm, respectively.

System	PHA-activated MNL transformation
	% of control
CsA (0.25 μM) only	48 ± 8
CsA + 50 μM AT acetate	31 ± 3
CsA + 10 μM retinol	44 ± 2
CsA + 25 μM butylated hydroxytoluene	38 ± 5
CsA + 15 μM butylated hydroxyanisole	28 ± 7
CsA + 250 μM cysteine	57 ± 11
CsA + 500 units/ml catalase	37 ± 8
CsA + 15 μM indomethacin	48 ± 9
CsA + 15 μM piroxicam	40 ± 5
CsA + 50 nM staurosporine	46 ± 2
CsA + 25 μM H-7	30 ± 11

inhibition of PHA-activated MNL proliferation are shown in Fig. 3. Coincubation of MNL with 10 μM AT partially protected the cells ($p < 0.005$) against the antiproliferative effects of 0.5 μM CsA, whereas complete protection was observed with lower concentrations (0.01 or 0.25 μM) ($p < 0.005$) of the immunosuppressive agent. At the higher concentration (50 μM), AT completely protected the MNL against the antiproliferative effects of all three concentrations of CsA. The level of protection remained the same whether AT was added 60 min before or 60 min after CsA. However, preincubation of the MNL with AT (50 μM) for 60 min, followed by washing (twice) and exposure to CsA (0.1–0.5 μM), did not protect the proliferative responses of the cells. These data, which are shown in Table 4, demonstrate a requirement for the continuous presence of AT to neutralize the antiproliferative effects of CsA. AT, at both concentrations tested, also protected the alloantigen-activated proliferative responses of MNL against CsA-mediated inhibition. These results are shown in Table 5.

The protective effects of lysophospholipase (200 milliunits/ml) on CsA (0.1 or 0.25 μM)-mediated inhibition of PHA-activated MNL proliferation are shown in Table 6. Lysophospholipase was added at the outset, 30 min after CsA had been

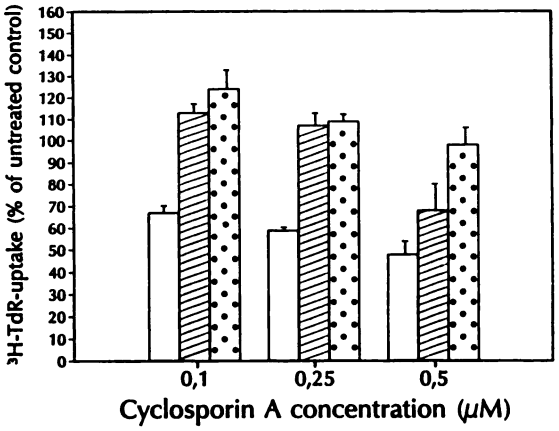

Fig. 3. Effects of CsA (0.1, 0.25, or 0.5 μM) on PHA-activated MNL transformation in the absence (□) or in the presence of 10 μM AT (▨) or 50 μM AT (⊡). The results of three to seven experiments are presented as the mean ± standard error of percentages of the corresponding CsA-free control systems. The absolute values for unstimulated and PHA-stimulated CsA-free control MNL were 967 ± 56 and 42,528 ± 2,673 cpm, respectively.

TABLE 4

Effects of pretreatment of MNL with AT, followed by washing and exposure to CsA, on the CsA-mediated inhibition of PHA-activated MNL proliferation

Data from three determinations are presented as means ± standard errors. In systems d and e the MNL were pretreated with AT for 60 min at 37°, followed by washing (twice) and exposure to PHA with or without CsA, whereas in systems f and g the AT was present throughout.

System	Uptake of [³H]TdR by MNL
	cpm
a. MNL only	742 ± 36
b. MNL + 5 μg/ml PHA	48,877 ± 2,340
c. MNL + PHA + 0.5 μM CsA	21,405 ± 1,712
d. MNL + PHA + pretreatment with 50 μM AT	45,613 ± 1,562
e. MNL + PHA + pretreatment with 50 μM AT + CsA	24,525 ± 2,943
f. MNL + PHA + 50 μM AT	45,944 ± 3,216
g. MNL + PHA + 50 μM AT + CsA	45,456 ± 7,273

TABLE 5

Effects of AT on CsA-mediated inhibition of alloantigen-activated MNL proliferation

The results of three experiments are expressed as means ± standard errors. The background value for unstimulated MNL was 835 ± 108 cpm.

System	Uptake of [³H]TdR by alloantigen-activated MNL
	cpm
MNL only	31,058 ± 2,071
MNL + 50 μM AT	33,152 ± 2,135
MNL + 0.1 μM CsA	14,908 ± 1,978
MNL + 0.1 μM CsA + 50 μM AT	29,505 ± 3,154
MNL + 0.25 μM CsA	13,666 ± 1,538
MNL + 0.25 μM CsA + 50 μM AT	27,021 ± 2,875

TABLE 6

Measurement of the effects of added lysophospholipase on CsA-mediated inhibition of PHA-activated MNL proliferation

Data from six experimenta are presented as means ± standard errors.

System	PHA-activated MNL transformation
	cpm
a. MNL only	640 ± 80
b. MNL + 200 milliunits/ml lysophospholipase	580 ± 46
c. MNL + PHA	29,438 ± 3,438
d. MNL + PHA + lysophospholipase	28,766 ± 3,526
e. MNL + PHA + 0.1 μM CsA	14,077 ± 1,449
f. MNL + PHA + 0.1 μM CsA + lysophospholipase	25,273 ± 2,771[a]
g. MNL + PHA + 0.25 μM CsA	4,077 ± 1,178
h. MNL + PHA + 0.25 μM CsA + lysophospholipase	12,165 ± 1,733[b]

[a] $p < 0.005$ for comparison of systems e and f.
[b] $p < 0.025$ for comparison of systems g and h.

added to the MNL, followed 30 min later by serum and PHA. Inclusion of the enzyme almost completely abolished ($p < 0.005$) the inhibitory effects of 0.1 μM CsA on MNL proliferation, whereas partial protection was observed with 0.25 μM CsA. In a single experiment the mean percentage of inhibition of PHA-activated MNL proliferation was 31% for 0.1 μM CsA alone and 44%, 27%, 7%, and 0% in the presence of 25, 50, 100, and 200 milliunits/ml lysophospholipase, respectively. Heat-inactivated (80° for 15 min) enzyme did not protect the MNL against the inhibitory effects of CsA (data not shown).

Effects of CsA with or without AT on the proliferative responses of purified T lymphocytes. The mean percentages of monocytes, B lymphocytes, and T lymphocytes in the unfractionated MNL preparations were 2 ± 1%, 17 ± 2%, and 77 ± 9%, respectively. The corresponding values after removal of adherent cells were 0%, 2 ± 1%, and 95 ± 6%. The phorbol-12-myristate-13-acetate-stimulated lucigenin-enhanced chemiluminescence responses of unfractionated and fractionated MNL were 814 ± 251 and 56 ± 15 $mV \cdot sec^{-1}$, respectively, with corresponding unstimulated values of 40 ± 1 and 16 ± 1 $mV \cdot sec^{-1}$.

The effects of CsA (0.25 or 0.5 μM), in the presence or absence of 10 or 50 μM AT, on the PHA-activated proliferative responses of purified T lymphocyte suspensions are shown in Table 7. As with unfractionated MNL, CsA caused a dose-related inhibition of the PHA-stimulated proliferative responses of purified T lymphocytes, which were more sensitive than MNL to the antiproliferative effects of this agent. How-

TABLE 7

Effects of CsA with and without AT on PHA-activated proliferative responses of purified T lymphocytes

Data from two experiments are presented as means ± standard errors. The background value for unstimulated MNL was 3,781 ± 193 cpm.

System	Uptake of [^{3}H]TdR by PHA (5 μg/ml)-activated T lymphocytes
	cpm
Cells only	39,412 ± 3,175
Cells + 50 μM AT	38,233 ± 2,578
Cells + 0.25 μM CsA	12,218 ± 4,871
Cells + 0.25 μM CsA + 50 μM AT	36,259 ± 3,718
Cells + 0.5 μM CsA	5,912 ± 2,078
Cells + 0.5 μM CsA + 50 μM AT	34,682 ± 2,478

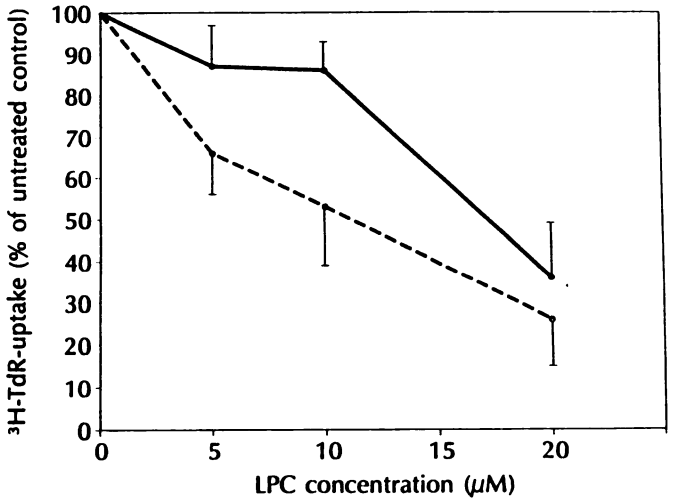

Fig. 4. Effects of LPC alone (——) and in the presence of 50 μM AT (– – –) on the PHA-stimulated uptake of [^{3}H]TdR by MNL. The results of five experiments are presented as the mean ± standard error of percentages of the corresponding LPC-free control systems. The absolute values for the unstimulated and PHA-stimulated control systems were 1,482 ± 171 and 40,667 ± 3,721 cpm, respectively.

ever, inclusion of AT protected the T lymphocytes against the inhibitory effects of CsA.

Effects of LPC, PC, and AA on PHA-stimulated MNL transformation. The effects of LPC (5–20 μM) on PHA-induced lymphocyte proliferation are shown in Fig. 4. This agent caused a dose-related inhibition of MNL proliferation that was statistically significant at a threshold concentration of 5 μM ($p < 0.05$). Treatment of MNL with 50 μM AT before exposure to LPC protected the proliferative responses of cells exposed to 5 μM ($p < 0.005$) or 10 μM ($p < 0.05$) LPC. However, AT added to MNL 30 min after LPC was unable to reverse the inhibitory effect of the lysophospholipid (data not shown). On the other hand, AA at concentrations of up to 20 μM did not detectably alter the uptake of [^{3}H]TdR by PHA-activated MNL. The respective values for PHA-treated control MNL and cells coincubated with 20 μM AA were 48,064 ± 1,531 and 44,413 ± 1,832 cpm (means ± standard errors of three experiments). The corresponding value for unstimulated control MNL was 412 ± 87 cpm. Likewise, PC at concentrations up to 20 μM did not affect PHA-activated MNL transformation. The respective values for unstimulated MNL and PHA-activated MNL without and with 20 μM PC were 1,222 ± 177, 56,808 ± 1,579, and 53,942 ± 3,551 cpm.

Effects of CsA on lymphocyte Na$^+$,K$^+$-ATPase activity. The data for uptake of ^{86}Rb by MNL after 4- and 18-hr exposure to CsA are shown in Table 8. Coincubation of MNL with CsA only, at concentrations of 0.1–1 μM, for 2, 4, or 18 hr in the absence of PHA did not affect the activity of cellular ATPase, and only data for the highest concentration (1 μM) of CsA are shown. As reported by others, addition of PHA increased the uptake of ^{86}Rb by MNL (24, 30, 31). The Na$^+$,K$^+$-ATPase activity of PHA-treated MNL was inhibited by CsA in a time- and dose-dependent manner (Table 8). After 2 hr of incubation only the highest concentration of CsA (1 μM) inhibited the uptake of ^{86}Rb by PHA-treated MNL (88 ± 3% of the control value; $p < 0.01$), whereas after 4 or 18 hr of exposure to this agent inhibition was observed with the other concentrations tested (Table 8). Similar results were observed using purified T lymphocytes, with 86 ± 3% ($p < 0.05$) and 63 ± 2% ($p < 0.005$) of the control values being observed after 4 and 18 hr of incubation of these cells with both 1 μM CsA and PHA (data from three separate experiments).

The effects of AT (50 μM) and lysophospholipase (200 milliunits/ml) on the uptake of ^{86}Rb by CsA-treated MNL are shown in Table 9. Inclusion of AT protected the Na$^+$,K$^+$-ATPase activity of PHA-activated MNL against both concentrations of CsA (0.1 and 1 μM), whereas partial protection was observed with lysophospholipase.

The activity of Na$^+$,K$^+$-ATPase in membrane fractions from PHA-activated MNL in the absence of CsA and in the presence of 0.1 μM and 1 μM concentrations of this agent were 1.2 ± 0.2,

TABLE 8

Effects of CsA on lymphocyte Na$^+$,K$^+$-ATPase activity

The results of 11 experiments (4 hr) and seven experiments (18 hr) are expressed as percentages of the corresponding control systems (means ± standard errors). The absolute values for the control systems (PHA-activated MNL in the absence of CsA) after 4 and 18 hr of incubation at 37° were 7,350 ± 1,004 and 5,916 ± 1,222 cpm, respectively. The absolute values for the corresponding PHA-free control systems containing MNL only or MNL plus 1 μM CsA were 4,050 ± 748 and 4,471 ± 906 cpm, respectively, for the 18-hr incubation period.

System	ATPase activity after	
	4-hr incubation	18-hr incubation
	% of control	
MNL + PHA + 0.1 μM CsA	96 ± 3	78 ± 3[a]
MNL + PHA + 0.25 μM CsA	97 ± 2	74 ± 4[a]
MNL + PHA + 0.5 μM CsA	82 ± 7[a]	70 ± 3[a]
MNL + PHA + 1 μM CsA	82 ± 4[a]	59 ± 3[a]

[a] $p < 0.01$ to $p < 0.005$.

TABLE 9

Effects of AT and lysophospholipase on CsA-mediated inhibition of MNL ATPase activity

The results of four separate experiments using MNL exposed to CsA for 18 hr are expressed as percentages of the corresponding untreated control systems (means ± standard errors). The absolute value for the uptake of ^{86}Rb by control PHA-activated MNL in the absence of CsA was 6,009 ± 1,453 cpm.

System	Na$^+$,K$^+$-ATPase activity
	% of control
MNL + PHA + 0.1 μM CsA	79 ± 4
MNL + PHA + 1 μM CsA	54 ± 3
MNL + PHA + 50 μM AT	92 ± 7
MNL + PHA + AT + 0.1 μM CsA	95 ± 8
MNL + PHA + AT + 1 μM CsA	94 ± 6
MNL + PHA + lysophospholipase	94 ± 3
MNL + PHA + lysophospholipase + 0.1 μM CsA	91 ± 9
MNL + PHA + lysophospholipase + 1 μM CsA	68 ± 8

0.9 ± 0.3, and 0.7 ± 0.1 nmol of NAD/min/4 × 10^7 MNL, respectively, corresponding to 25% (not significant) and 36% ($p < 0.01$) inhibition (data from two experiments).

CsA did not affect the activity of purified ATPase (activities of 6.4 ± 0.2 and 6.2 ± 0.2 nmol of NAD/min in the presence and absence of 4 μM CsA, respectively). However, LPC caused a dose-dependent inhibition of this enzyme; the mean percentages of inhibition of Na^+,K^+-ATPase activity observed with 5, 10, 20, and 40 μM LPC were 2 ± 1%, 7 ± 2%, 20 ± 6%, and 51 ± 13%, respectively (data from three separate experiments).

Effects of CsA and LPC on MNL LDH and ATP levels. LPC caused a dose-dependent release of LDH from MNL, demonstrating the cytotoxic potential of this agent. Using a 2-hr incubation period, the release of LDH from control MNL and cells treated with 5, 10, and 20 μM LPC was 5 ± 1%, 24 ± 2%, 47 ± 1%, and 81 ± 2% of total cellular enzyme, respectively (data from two experiments). The corresponding values for cellular ATP levels were 9.9 ± 1.4, 4.7 ± 0.2, 3.2 ± 0.2, and 1.9 ± 0.1 nmol of ATP/10^7 MNL. However, CsA at concentrations of up to 4 μM in the presence or absence of PHA did not affect LDH release or cellular ATP levels at any of the incubation times used (2, 4, and 18 hr). After 18 hr of incubation at 37° the intracellular ATP levels of 1) control MNL, 2) MNL plus 4 μM CsA, 3) MNL plus PHA, and 4) MNL plus 4 μM CsA plus PHA were 8 ± 2, 10 ± 2, 8 ± 3, and 11 ± 2 nmol of ATP/10^7 cells, respectively.

Spectrophotometric analysis of mixtures of AT, AT acetate, butylated hydroxyanisole, and butylated hydroxytoluene with CsA. These results are shown in Fig. 5. Mixing of CsA with all four lipid-soluble antioxidants caused major reductions in the intensity of the major CsA absorption peak at 215 nm, as well as peak shifts. These observations demonstrate that CsA interacts with the four lipid-soluble antioxidants.

Discussion

CsA inhibits the mitogen- and alloantigen-activated proliferative responses of T lymphocytes *in vitro* (1–3) by interfering with lymphokine production by helper T cells (32, 33) and generation of cytotoxic T lymphocytes (1). CsA blocks the expression of the gene for interleukin-2 production at the level of mRNA transcription (7–9) and interrupts ongoing interleukin-2 mRNA synthesis (34). This may not, however, represent a primary mechanism of CsA-mediated immunosuppression, because addition of recombinant interleukin-2 to lymphocytes does not prevent or reverse the inhibitory effects of CsA on the proliferative responses of these cells (13). It has been proposed that binding of CsA to the cytosolic protein peptidyl-prolyl *cis-trans*-isomerase (immunophilin) is required to promote immunosuppression (35, 36). Until recently the mechanism by which these immunophilin-CsA complexes induce T lymphocyte dysfunction had not been established. The target of these complexes has now been identified as the Ca^{2+}/calmodulin-dependent phosphatase 2B, known as calcineurin (37, 38). Calcineurin activity is strongly inhibited by cyclophilin-CsA complexes *in vitro*, whereas the individual components do not affect enzyme activity (37). Until the exact involvement of calcineurin in T lymphocyte activation is established, the mechanistic relevance of these interesting observations remains uncertain. There is also evidence, however, that suggests that the lymphocyte plasma membrane may be the primary target of CsA (11–16).

In the present study we have investigated the effects of CsA on the activity of lymphocyte PL, as well as the potential involvement of PL breakdown products in the mediation of CsA-induced immunosuppression. In all the assays of lymphocyte function identical cell concentrations (5 × 10^5/ml) were used, to maintain a constant drug to cell ratio, because it was previously reported that sensitivity to CsA is dependent on cell density, probably as a consequence of the lipophilic properties of this agent (39). Coincubation of lymphocytes for 2, 4, or 18 hr with CsA at therapeutically relevant concentrations of 0.25, 0.5, and 1 μM, or with a fixed concentration of the mitogen PHA (5 μg/ml), minimally affected the activity of PLA_2, as measured by the release of [^{3}H]AA and [^{3}H]LPC. However, when CsA and PHA were added in combination, increased release of [^{3}H]LPC and [^{3}H]AA was observed, which was dependent on the duration of incubation and the concentration of CsA. CsA and PHA had similar effects on the generation of PGE_2 by lymphocytes. PHA caused slight but insignificant release of PGE_2 from these cells, whereas CsA at the highest

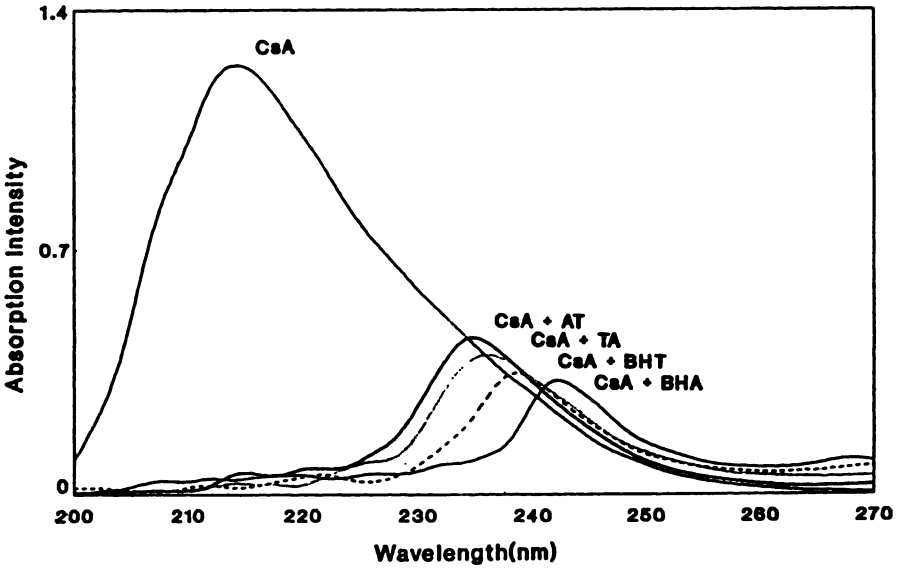

Fig. 5. UV spectra of CsA alone and in combination with AT, AT acetate (*TA*), butylated hydroxyanisole (*BHA*), or butylated hydroxytoluene (*BHT*).

concentration used (4 μM) significantly increased PGE_2 release. However, the two agents in combination caused marked, dose-related stimulation of the generation of PGE_2 by lymphocytes. With this extremely sensitive radioimmunoassay, the stimulatory effects of CsA combined with PHA on PGE_2 release were evident at lower concentrations (0.25 or 0.5 μM) of the immunosuppressive agent. PHA and CsA (4 μM) alone and in combination did not detectably affect the viability of the lymphocytes during the 2-hr incubation period. Other authors have reported that CsA alone increases the release of AA and AA metabolites from rat macrophages *in vitro* (15). Those authors used higher concentrations of CsA (2–50 μM) than those used in the present study. Our data indicate that CsA alone does not directly activate PLA_2, as demonstrated here using purified enzyme, but may affect membrane structure (11) such that the integral phospholipids are more susceptible to cleavage. This is compatible with the previously described effects of CsA on membrane potential and lipid order (11–13). Nevertheless, a direct effect of CsA on PLA_2 in the plasma membrane of intact cells cannot be excluded (40). The requirement for PHA may be related to the ability of this agent to increase intracellular calcium concentrations (41), which could then lead to activation of PLA_2. Moreover, the most profound antiproliferative effects of CsA are reported to occur after calcium ionophore activation of lymphocytes (42). Although effects on other cell types have been described (15, 43–45), the relative selectivity of CsA for T lymphocytes may be related to the incomplete effects of CsA alone on phospholipid hydrolysis in these cells. The apparent selectivity may therefore be due to the additional requirement for a Ca^{2+}-mobilizing lymphocyte activator, such as mitogen or antigen. In addition, proliferating cells may be more susceptible to the inhibitory effects of phospholipid hydrolysis products.

We also confirmed the well recognized inhibitory effects of CsA on the proliferation of lymphocytes stimulated by the mitogen PHA and alloantigens (1–3). Using both unfractionated MNL suspensions and purified T lymphocytes, inhibition of lymphocyte proliferation was observed at a threshold concentration of 0.05 μM CsA. To identify the products of PL activation with antiproliferative activity, we investigated the effects of the major degradation products of PLA_2, i.e., LPC and AA, on PHA-activated lymphocyte proliferation. LPC, at a threshold concentration of 5 μM, caused dose-related inhibition of T lymphocyte activation, whereas AA at concentrations of up to 20 μM had no effect. Likewise, PC (20 μM), the major phospholipid substrate for PLA_2, did not affect MNL proliferation. Although implicating LPC, which possesses detergent and membrane-destabilizing properties (17), these data did not exclude other metabolites and reactive oxidants generated during the metabolism of AA by cyclooxygenase and lipoxygenase enzymes. However, inclusion of a water-soluble antioxidant (cysteine) or enzyme (catalase), lipid-soluble antioxidants (retinol, butylated hydroxytoluene, or butylated hydroxyanisole), PG synthetase inhibitors (indomethacin or piroxicam), or an inhibitor of 5-lipoxygenase (NDGA) all failed to protect the cells against the antiproliferative effects of CsA. These observations demonstrate that oxidants originating from contaminating cells such as neutrophils and monocytes, reactive oxidants generated during AA metabolism, PGs, or 5-lipoxygenase products are not primarily involved in CsA-mediated inhibition of lymphocyte proliferation. Although AT acetate did not protect MNL against the antiproliferative effects of CsA, AT, originally included as a lipid-soluble antioxidant, proved to be a striking exception, indicating a critical requirement for the hydroxyl group on the chromanol nucleus of AT. This agent (AT) almost completely blocked the inhibitory effects of CsA on the proliferative responses of both unfractionated MNL and purified T lymphocytes. The protective effects of AT were eliminated by washing of the cells, demonstrating a requirement for the continuous presence of this agent. Although spectrophotometric analysis of mixtures of AT with CsA revealed interactions between these two agents, it is improbable that the protective effects of AT are due to interaction of this agent with CsA, because 1) complex formation was also detected with mixtures of CsA and AT acetate, butylated hydroxyanisole, or butylated hydroxytoluene, none of which interfered with the immunosuppressive activity of CsA; 2) the protective effects of AT were still evident when this agent was added to the cells 1 hr after CsA; and 3) AT did not inhibit the effects of CsA/PHA on the activity of PLA_2. It also seemed unlikely that these protective effects of AT were related to the antioxidant properties of the molecule, because other lipid-soluble antioxidants were ineffective. Notwithstanding its well documented antioxidant properties, AT possesses other biological activities that could account for the observed protection against the antiproliferative effects of CsA. AT has been reported to inhibit both the activation and translocation of cytosolic PKC (46, 47) and to interfere with the activity of 5-lipoxygenase (48) and PLA_2 in some experimental systems (22), but not in others (49). The failure of staurosporine, H-7, and NDGA to protect the mitogen-activated proliferative responses of lymphocytes appears to discount PKC or 5-lipoxygenase inhibition, respectively, as potential mechanisms of prevention of CsA-induced immunosuppression. Likewise, we were unable to demonstrate any inhibitory effects of AT on lymphocyte PLA_2. AT also complexes with and neutralizes lysophospholipids through two types of interaction, namely formation of a hydrogen bond between the AT chromanol nucleus hydroxyl group and the carboxyl group of the lysophospholipid and interaction of the acyl chains of the lysophospholipids with the chromanol nucleus methyl groups of AT (17). In the present study we observed that pretreatment of lymphocytes with AT effectively protected these cells against the antiproliferative activity of LPC. The most compelling evidence in favor of the involvement of LPC in CsA-mediated immunosuppression was derived from experiments with the LPC-hydrolyzing enzyme lysophospholipase. The antiproliferative effects of CsA were partly neutralized by coincubation of mitogen-activated MNL with this enzyme.

These data clearly implicate LPC as a potential mediator of CsA-induced immunosuppression. Upon exposure to CsA, mitogen- or antigen-activated lymphocytes generate excessive amounts of LPC. Although cellular lysophospholipases may confer partial protection, the lymphocytes are apparently ill equipped to neutralize excess LPC. LPC-mediated damage to the plasma membrane and consequent cytotoxicity seemed a likely mechanism of antiproliferative activity, and this was supported by observations of membrane damage and loss of viability of cells treated with reagent LPC. However, several lines of evidence indicated that this was not the case. Firstly, damage resulting from the addition of exogenous reagent LPC to MNL was not reversed by AT and, secondly, treatment of MNL with CsA at concentrations of up to 4 μM, with or without

PHA, did not affect cell viability. Clearly, the mechanism of antiproliferative activity mediated by gradual intramembrane accumulation of endogenously generated LPC is different from that observed with exogenous LPC, which is primarily cytolytic. The membrane-associated enzyme Na^+,K^+-ATPase was identified as the likely target of endogenous LPC. The activity of this enzyme is up-regulated during activation of T lymphocytes, and it is indispensable for the differentation and proliferation of these cells (24, 30, 31). Only when CsA (0.1–1 μM) was used in combination with PHA was inhibition of enzyme activity observed. Nevertheless, the magnitude of CsA-mediated inhibition of Na^+,K^+-ATPase activity (approximately 40% with 1 μM CsA combined with PHA) may not account for all of the immunosuppressive activity of CsA, indicating the existence of interactive mechanisms (37, 38). However, it is noteworthy that the relative inhibitory activity increases considerably in magnitude if related to the increment in the activity of Na^+,K^+-ATPase during exposure of lymphocytes to PHA. Importantly, both AT and lysophospholipase protected the enzyme against CsA-mediated inactivation, implicating LPC as the probable inhibitor. The inhibitory effects of LPC on Na^+,K^+-ATPase activity have been described in a variety of different cell types (25, 50), and it has been proposed that LPC may interact with boundary phospholipids in the inner membrane, preventing essential interactions with Na^+,K^+-ATPase (25).

Nephrotoxicity is the most serious side effect associated with administration of CsA. Interestingly, lysophospholipids have previously been reported to cause cardiac (22), lens (51), and pulmonary damage (52) and, although the biochemical mechanism of CsA-related renal dysfunction has not been established, it may be due to increased accumulation of toxic lysophospholipids in renal tubular cells. If this is the case, then AT status may be a determinant of both efficiency of immunosuppression and susceptibility to development of nephrotoxicity. Moreover, administration of AT may be useful in controlling CsA-mediated nephrotoxicity, possibly at the expense of immunosuppression. Interestingly, administration of CsA to rats is associated with decreased activity of renal cortical Na^+,K^+-ATPase and nephrotoxicity (53).

Finally, the data presented here are compatible with a membrane site of action of CsA leading to enhancement of phospholipid hydrolysis in mitogen- or antigen-activated T lymphocytes and increased release of the antiproliferative agent LPC as being a potential mechanism, but not necessarily the only mechanism (37, 38), of CsA-mediated immunosuppression. LPC is a possible mediator of both the therapeutic activity and renal side effects of CsA.

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