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Research Article

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Evidence that Calcineurin Is Rate-limiting for Primary Human Lymphocyte Activation

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Abstract

Cyclosporine (CsA) is both a clinical immunosuppressive drug and a probe to dissect intracellular signaling pathways. *In vitro*, CsA inhibits lymphocyte gene activation by inhibiting the phosphatase activity of calcineurin (CN). In clinical use, CsA treatment inhibits 50–75% of CN activity in circulating leukocytes. We modeled this degree of CN inhibition in primary human leukocytes *in vitro* in order to study the effect of partial CN inhibition on the downstream signaling events that lead to gene activation. In CsA-treated leukocytes stimulated by calcium ionophore, the degree of reduction in CN activity was accompanied by a similar degree of inhibition of each event tested: dephosphorylation of nuclear factor of activated T cell proteins, nuclear DNA binding, activation of a transfected reporter gene construct, IFN- γ and IL-2 mRNA accumulation, and IFN- γ production. Furthermore, the degree of CN inhibition was reflected by a similar degree of reduction in lymphocyte proliferation and IFN- γ production in the allogeneic mixed lymphocyte cultures. These data support the conclusion that CN activity is rate-limiting for the activation of primary human T lymphocytes. Thus, the reduction of CN activity observed in CsA-treated patients is accompanied by a similar degree of reduction in lymphocyte gene activation, and accounts for the immunosuppression observed. (*J. Clin. Invest.* 1997; 100:1894–1901.) Key words: cyclosporine • calcineurin • lymphocytes • signal transduction • immunosuppression

Introduction

Cyclosporine A (CsA)¹ is a cornerstone of maintenance immunosuppression in organ transplantation, and is used widely in

bone marrow transplantation and autoimmune disease. *In vitro* CsA suppresses lymphocyte activation by binding to cyclophilins (CyP), ubiquitous intracellular proteins with isomerase activity (1–3). The CsA–CyP complex binds to the calcium-regulated enzyme calcineurin (CN), inhibiting its serine–threonine phosphatase activity (4–6). The immunosuppressive drug tacrolimus or FK-506 (FK) binds to an immunophilin, FK binding protein, creating a complex which similarly binds and inhibits CN (7, 8). Activity of CN is necessary to activate key transcription factors. By inhibiting CN activation, CsA or FK prevent the dephosphorylation (9, 10) and subsequent nuclear translocation of the nuclear factor of activated T cells cytoplasmic proteins (NFATc), a family of transcription factors (10–12). Other transcription factors are also partially CN-dependent in T cells, such as AP-1 (13–15) and some members of the NF- κ B/Rel family (16–19). These transcription factors govern the transcription of T lymphocyte activation genes, including many cytokine genes (IFN- γ , IL-2, GM-CSF, and others) and genes controlling membrane molecules such as the CD40 ligand (15, 20, 21). CsA or FK prevent the transcription of these genes (4, 22, 23). Various observations support the essential role of CN in T lymphocyte activation: for example, the transfection of constitutively active CN makes cells relatively resistant to the effects of CsA (4), and a knockout which eliminates one isoform of CNA, CNA α , reduces the immune responsiveness of the lymphocytes of the resulting mice (24).

While the qualitative role of CN inhibition in the effects of CsA on T lymphocyte activation is clear *in vitro*, the actual effect of CN inhibitors *in vivo* is less clear. The peripheral blood leukocytes (PBL) of CsA-treated transplant patients have reduced CN activity compared with healthy controls (25, 26). Even at peak CsA blood concentrations, complete inhibition of CN activity is uncommon (27, 28). Studies of CsA effect on CN activity and on gene expression in mice also revealed that CsA produced only partial inhibition except at very high concentrations (29). Thus, there is a discrepancy between the *in vivo* and the *in vitro* results: a given CsA concentration on a cell suspension in aqueous medium *in vitro* causes \sim 10-fold greater inhibition of CN activity compared with a similar blood concentration *in vivo*, or with the same concentration of cyclosporine in whole blood *in vitro* (25, 30). This discrepancy is due, at least in part, to the large number of binding sites for CsA *in vivo*, reducing the exposure of lymphocytes *in vivo* despite relatively higher CsA concentrations (31). Thus, modeling CsA effect by using the same concentrations *in vitro* (highly aqueous conditions) as those observed *in vivo* (with many CsA binding sites) may greatly overestimate its effect and recruit effects of the drug which are irrelevant to its *in vivo* actions. In spite of these considerations underscoring the importance of understanding CN inhibition and its immunologic consequences in quantitative terms, investigations of the quantitative relationships among CsA concentration, CN inhibition, and downstream effects have been limited (32).

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1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; CI, confidence interval; CsA, cyclosporine; CN, calcineurin; CyP, cyclophilin; EMSA, electrophoretic mobility shift assay; FK, FK-506; MLR, mixed lymphocyte reaction; NFAT, nuclear factor of activated T cells; PBL, peripheral blood leukocytes; PHS, pooled normal human serum.

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In these studies, we examined the effect of CN inhibition, to the degree measured in patients, on the downstream molecular events involved in cytokine gene activation and proliferation in freshly isolated human lymphocytes. We found that each degree of CN inhibition leads to a similar degree of inhibition of each of the downstream steps involved in both early cytokine gene activation and the longer term proliferation of antigen-stimulated cells. These data support the hypothesis that CN phosphatase activity is rate-limiting in human lymphocyte activation, and that the effect of CsA at concentrations which partially inhibit CN are parallel degree of inhibition of the dephosphorylation and translocation of NFATc, activation of cytokine transcription and production, and antigen-specific lymphocyte proliferation. These data support the view that the CN inhibitor CsA (and probably FK) is immunosuppressive because CN is rate-limiting in human T cell activation.

Methods

Cells and culture conditions. Samples of whole blood were obtained from healthy volunteers, and PBL were isolated by standard Ficoll-Hypaque gradient centrifugation. To reduce erythrocyte contamination, PBL were suspended in a solution containing 0.15 M NH₄Cl, 10 mM NaHCO₃, and 300 μM EDTA, agitated for 3–5 min, and then washed twice with PBS. In cases of significant platelet contamination, 2.5 U thrombin were added to the PBL suspension, and the resulting platelet aggregates were removed by gentle centrifugation. Enriched T lymphocyte populations were prepared using a negative selection column (Celect Human T; Biotex Laboratories, Edmonton, Canada). T cell purity is reported to be > 95%. Experiments were usually performed with PBL because the number of human T cells was limiting, and because T cell preparation may alter the T cells. In some experiments, human T cells were used, as indicated.

PBL were suspended at 5×10^6 cells/ml and maintained in a humidified (95% humidity) atmosphere at 37°C and 5% CO₂. Culture medium was RPMI 1640 (GIBCO BRL, Gaithersburg, MD), supplemented with 10% pooled normal human serum (PHS) (NABI, Miami, FL), 0.2 mM glutamine (GIBCO BRL), and an antibiotic cocktail (GIBCO BRL) consisting of 250 μg/liter amphotericin B, 100,000 U/liter penicillin G, and 100 mg/liter streptomycin sulfate.

Stimulation and inhibition. For short-term assays, PBL were stimulated with the calcium ionophore A23187 5 μM (ICN Biochemicals, Inc., Mississauga, Canada). Unstimulated control groups were treated with the A23187 solvent dimethyl sulfoxide at a final concentration of 0.05%.

CsA, kindly supplied by Sandoz Canada Ltd. (Dorval, Quebec), was dissolved initially in 96% ethanol and Tween 80 (Fisher Scientific Co., Fair Lawn, NJ), and a stock solution (20 mg/ml) was stored at 4°C. Stock concentration was confirmed by measuring CsA by HPLC. Immediately before adding to culture, it was diluted to a desired concentration using medium. In all experiments, the concentration of CsA solvent was kept constant in all groups (0.35×10^{-3} % ethanol and 0.14×10^{-3} % Tween 80).

Calcineurin activity. CN activity was measured as described (25). In brief, PBL lysates were prepared and assayed for the ability to dephosphorylate a 19-amino acid peptide (DLVPIPIGRFDRRV-(³²P)SVAAE). Background activity due to protein phosphatase 2C, measured as ³²P released in the presence of CsA 1,000 μg/l, was subtracted.

Western blotting. After treatment, whole cell lysates were prepared by suspending T lymphocytes in lysis buffer containing 5 mM Tris-HCl, pH 8.0, 25 mM NaCl, 5% sodium dodecyl sulfate, 30 mM sodium pyrophosphate, 5 mM EDTA, 2 mM PMSF, 250 μM leupeptin, 100 mg/l aprotinin, and 5 mM iodoacetamide. Cell suspensions were boiled for 5 min, passed through a 26-gauge needle, boiled for 10 min, then centrifuged for 40 min. Proteins in the supernatant were quantitated (according to method of Bio-Rad Labs., Hercules, CA),

separated by 6% SDS-PAGE, transferred to nitrocellulose, then probed for NFATC2 (formerly NFATp; NFAT nomenclature based on recommendations of Gene Data Bank on the World Wide Web at <http://gdwww.gdb.org/gdb-bin/lite/bin/genes>) using a rabbit polyclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY). This antibody was raised in rabbits against a 464-residue recombinant peptide, and reacts with mouse and human NFATC2 (10). Bands of interest were quantitated by densitometry.

Electrophoretic mobility shift assay (EMSA). After treatment of PBL, nuclear extracts were prepared by washing the PBL first with PBS, then with buffer A (10 mM Hepes, pH 7.8, 15 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 0.1 mM PMSF). The pellet was then suspended in buffer A plus NP-40 (0.2%) and left on ice for five min. The cells were then centrifuged at 3,000 rpm for 3 min, and the nuclear pellet was resuspended in 315 μl of buffer B (50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% vol/vol glycerol). The proteins were precipitated with 15 μl 3 M NH₄SO₄, incubated with agitation on ice for 30 min, then spun at 10,000 g for 20 min. An equal volume of 3 M NH₄SO₄ was added to the supernatant, and the mixture was incubated on ice for 20 min, followed by centrifugation at 13,000 rpm for 30 min. The pellet was suspended in buffer B, and protein concentrations were measured.

Complementary double-stranded oligonucleotides were synthesized using PCR-Mate[®] (Applied Biosystems, Inc., Foster City, CA). The sequence generated is the P2 sequence identified by Campbell et al. (33) as an NFAT binding site in the human IFN-γ promoter. The sequence was 5' GATCTAAAATTCCAGTCCTTGA 3'. The oligo probe was radiolabeled with ³²P using Klenow to fill in the ends. Competitors included Mutant P2, 5' GGGAGGTACAAAATCGACGC-CAGTCCTTGAATCCTGTGAA 3', IL-4 P site, 5' GATCTGTGACTGACAATCTGGTGAAGAAAATTCCAATGTA 3' (33a).

Labeled probe (~25,000 cpm) was added to the binding reaction, and 12 μg of nuclear extract was incubated with binding buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 12.5% glycerol, 50 mM KCl, and 0.1% Triton-X 100. Poly dIdC (2 μg) and 5 μg BSA were added to each sample. Samples were incubated at room temperature for 30 min before running on a 6% TBE gel for 90 min. The gel was dried and autoradiographed. For competition/supershift experiments, 100–200-fold excess of unlabeled competitor oligos were added to the extracts and incubated for 10 min at room temperature before the addition of labeled probe. Characterization of the binding protein(s) was carried out by determining the effect of incubating the protein-DNA complex with anti-NFAT antibodies. These were added to the binding buffer for 4 h at 4°C before running the gel. The antibodies included an anti-mouse anti-NFATC2 polyclonal antibody (see *Western blotting*, above) that was raised in rabbits against a 464-residue recombinant peptide, and is cross-reactive with human NFATC2 (10). The other antibody (a generous gift from Dr. G. Crabtree, Stanford University, CA) was the antihuman anti-NFATC1 mAb G1-D10 raised against a 134-residue peptide that lies just amino-terminal to the Rel homology domain.

Transient transfection and chloramphenicol acetyltransferase (CAT) assay. Transient transfection experiments were performed with PBL-blasts that were prepared by culturing freshly isolated PBL in medium (with 20% PHS) with 1% PHA (GIBCO BRL) for 72 h. Blasts were suspended at 20×10^6 cells/ml with 80 μg DNA, comprising a reporter gene construct containing three repeats of the CsA-sensitive P2 sites found in the human IFN-γ gene (33) upstream of the pCATPR plasmid (Promega Corp., Madison, WI). This plasmid has the SV40 promoter and the CAT gene. After 10 min at room temperature, the blast/DNA mix was electroporated (Bio-Rad Labs.) at 0.250 V and 960 μF in 250–400 μl aliquots and then left at room temperature for another 10 min, after which the aliquots were mixed, suspended at 10^6 cells/ml, and incubated at 37°C for 1 h before addition of CsA. 10 min after addition of CsA, cells were stimulated with A23187 5 μM for 48 h.

To obtain cell extracts, cells were washed twice in PBS, once in TEN (40 mM Tris-HCl, pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl),

and resuspended in 100 μ l 0.25 M Tris-HCl, pH 7.5. The cells were then lysed by three cycles of freeze-thaw and centrifuged at 10,000 g for 5 min. The supernatant was removed and heated to 65°C for 10 min. CAT activity was assayed by combining 75 μ l of the supernatant, 4 μ l 14 C-chloramphenicol (Amersham Corp., Arlington Heights, IL), 4 μ l 5 mg/ml *N*-butyryl CoA (Sigma Chemical Co.), and 42 μ l 2 M Tris-HCl, pH 7.5. After incubation at 37°C for 24 h, 300 μ l xylene was added, and the samples were vigorously mixed. After 3 min centrifugation, the top layer was removed, and 100 μ l Tris-HCl was added. The mixture was vortexed and recentrifuged, and the top layer was counted in a liquid scintillation counter.

RNA extraction and Northern blotting. After stimulation, cells were harvested, washed with PBS, and total cellular RNA was extracted as described (34, 35). RNA was electrophoresed on 1.5% agarose/2.2 M formaldehyde gel using 15 mg RNA per well and transferred on nitrocellulose membrane for hybridization with 32 P-labeled cDNA probes. Probing was performed using a standard procedure (36). Quantitation of 32 P-labeled Northern blots and EMSAs was performed using a Bio-Imager (BASS 1000; Fuji Photo Film Co., Tokyo, Japan).

Measurement of IFN- γ . IFN- γ in tissue culture supernatants was assessed using an IFN- γ ELISA kit (Biosource International, Camarillo, CA, or Endogen, Inc., Woburn, MA).

Mixed lymphocyte reaction (MLR). MLR was performed using 5×10^4 stimulator and 5×10^4 responder PBL in a total of 0.2 ml medium in sterile 96-well plates. Stimulator cells were irradiated with 2,000 rads before addition to culture. 16–24 h before harvesting, 2 mCi [3 H]thymidine was added to each well. Cells were harvested, washed, counted, and added to scintillation vials with 4 ml of scintillation fluid, and the cpm was counted.

Statistical analysis. Curve fitting and statistical analyses were performed as described, using Prism software (GraphPAD Software for Science, San Diego, CA).

Results

We showed previously that PBL freshly removed from the blood of CsA-treated patients have $\sim 50\%$ reduction of their CN activity at trough CsA blood levels (25). Moreover, CN inhibition rises and falls with the rise and fall of CsA blood levels, rising from 50% at trough CsA levels 12 h after dose to 75% for a brief time at peak CsA concentrations, then declining to baseline (reference 27, and Batiuk, T.D., F. Pazderka, and P.F. Halloran, manuscript submitted for publication). These clinical observations prompted us to explore the effects of 50–75% CN inhibition in PBL in vitro (Fig. 1). We established CsA concentrations in aqueous medium which partially inhibit CN to approximate the state of PBL in CsA-treated humans. For four individual experiments, the mean IC_{50} was 10 μ g/liter (95% CI, 9–14 μ g/liter); individual IC_{50} s ranged from 8 to 23 μ g/liter. The data fit a sigmoidal dose response curve with a goodness of fit of $r^2 = 0.9995$. Similar results were found with purified T cells (data not shown).

Having established the appropriate range of in vitro CsA to mimic the degree of partial CN inhibition obtained clinically, we investigated the consequences of partial CN inhibition on a series of steps involved in cytokine activation in T lymphocytes or whole PBL that occur subsequent to CN activation: NFAT activation and translocation, activation-induced DNA binding of nuclear proteins to a site in the IFN- γ promoter, activation of a CAT construct in transfected cells via a sequence in the IFN- γ promoter, IFN- γ mRNA induction and IFN- γ cytokine secretion during short term stimulation, and proliferation of PBL and IFN- γ production in cells responding during MLR.

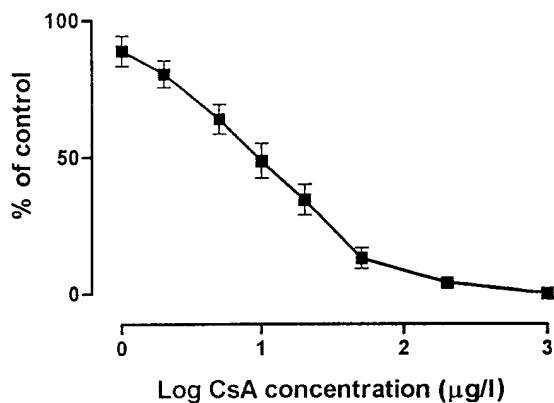


Figure 1. In vitro CsA inhibition of PBL CN activity. PBL were exposed to CsA at concentrations of 0 (solvent), 1, 2, 5, 10, 20, 50, 200, and 1,000 μ g/liter. PBL were lysed, and CN activity was measured. The results show the mean \pm SD of four individual experiments. The mean IC_{50} was 10 μ g/liter (95% CI, 9–14 μ g/liter); individual IC_{50} s ranged from 8 to 23 μ g/liter.

Activation of NFATc. CN activation leads to dephosphorylation of multiple serines in NFATc, accompanied by a reduction in the apparent molecular weight of NFATc (9, 10). To examine this step, T cells were exposed to CsA for 30 min, stimulated with A23187 5 μ M for 5 min, and immunoblotted with a polyclonal anti-NFATc2 antibody (Fig. 2). Extracts from unstimulated cells (lane 1) showed a sharp band at ~ 140 kD. In the absence of CsA, stimulation resulted in the disappearance of this band and the appearance of a new, less distinct band at ~ 120 kD. These bands are consistent in size with phosphorylated and dephosphorylated forms of NFATc2, respectively (9, 10). The bands were quantitated using densitometry. With increasing concentrations of CsA, the loss of the upper band was inhibited, and the ratio of the upper band (phosphorylated NFATc2) to the sum of both bands increased, indicating that dephosphorylation was inhibited by CsA in a concentration-dependent manner, with an IC_{50} of 20 μ g/liter (95% CI, 15–26 μ g/liter). Thus, the IC_{50} was in the same range as that for CsA inhibition of CN.

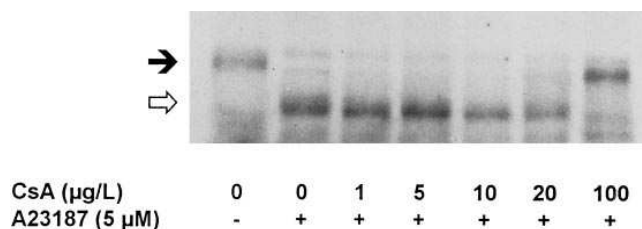


Figure 2. Anti-NFATc2 immunoblot of whole cell extracts from T lymphocytes stimulated for 5 min by A23187. T lymphocytes were purified from freshly isolated human PBL, and exposed to CsA for 30 min before stimulation with A23187 5 μ M (lanes 2–7) for 5 min. Whole cell extracts were prepared, and for each group, 200 μ g protein was separated by 6% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-NFATc2 polyclonal antibody. Upper band (black arrow) is at ~ 140 kD, and lower band (white arrow) is at ~ 120 kD.

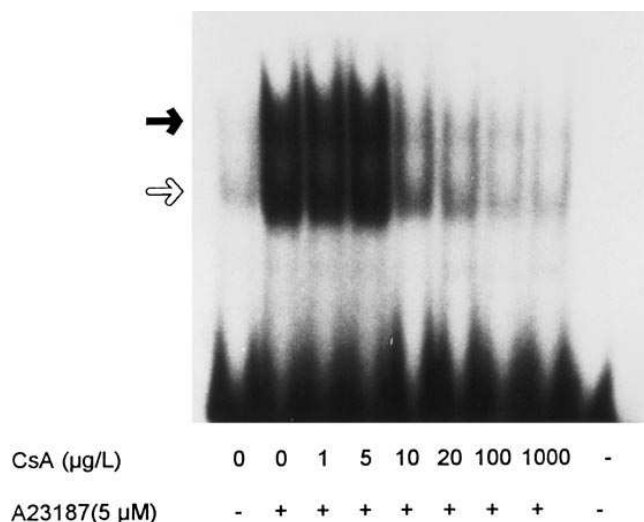


Figure 3. Effect of in vitro CsA on binding activity of nuclear proteins from stimulated PBL to DNA. PBL were treated with CsA, as indicated, for 30 min before stimulation with A23187 5 µM for 5 min. Nuclear proteins were isolated, quantitated, and for each group, equal amounts of protein were incubated with the ³²P-labeled DNA sequence corresponding to the P2 NFAT-binding site in the human IFN-γ promoter (33). The mixtures were then run through a 6% TBE gel, dried, and autoradiographed.

The anti-NFATC2 antibody was raised in rabbits against murine epitopes. When similar experiments were performed in murine spleen cells, the IC₅₀s ranged between 7 µg/liter for a crude splenocyte preparation and 18 µg/liter for purified splenic T cells (our unpublished data). Western blots of PBL preparations not enriched for T lymphocytes were too insensitive to detect these changes.

DNA binding activity. We then examined the effect of CsA on the DNA binding characteristics of nuclear proteins from stimulated PBL. Nuclear proteins from control and stimulated PBL were incubated with a ³²P-labeled DNA probe with a sequence corresponding to the NFAT-binding P2 site from the human IFN-γ gene promoter (33). This mixture was then electrophoresed through a 6% TBE gel, dried, and autoradiographed (Fig. 3). Nuclear extracts from unstimulated cells showed no bands (lane 1), whereas stimulation led to two bands (lane 2, arrows), both of which were reduced with increasing concentrations of CsA (lanes 3–8). Band quantitation by Bio-Imager showed an IC₅₀ of 8 µg/liter (95% CI, 5–12 µg/liter) for each band.

Characterization of the proteins and binding specificity involved in these bands was undertaken using the proteins from the stimulated group (Fig. 3, lane 2). Incubation of the nuclear extracts with antibodies to NFATC1 or NFATC2 specifically eliminated both bands, with NFATC2 treatment leading to the appearance of a new, supershifted band (Fig. 4). Also, preincubation of the proteins with nonradioactive DNA probes corresponding to the IFN-γ P2 site or the homologous P2 site from the human IL-4 promoter abolished the bands, whereas a mutant P2 probe had no effect. Thus the DNA-binding activity in stimulated PBL that was inhibited by CsA was recognized by two different anti-NFAT antibodies, and specifically bound NFAT-binding sites. The presence of two bands may represent

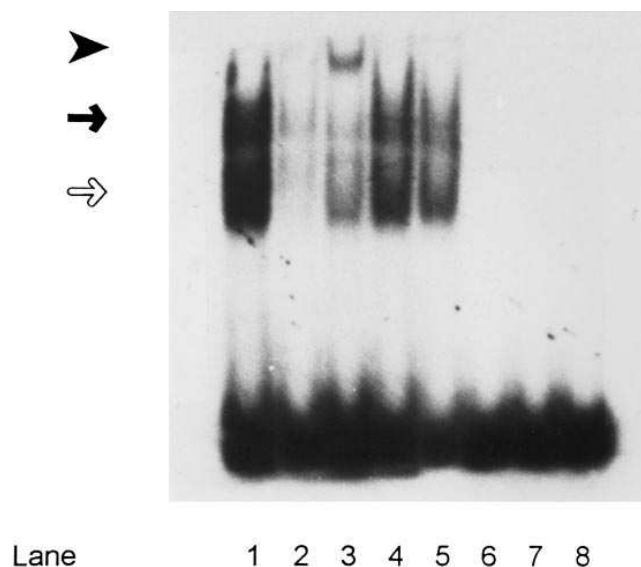


Figure 4. Characterization of DNA-binding proteins from nuclei of stimulated PBL. Nuclear extracts of stimulated PBL (Fig. 3, lane 2) were incubated for 3 h at room temperature with nothing (lane 1), anti-NFATC2 polyclonal antibody (lane 2), anti-NFATC1mAb (lane 3), a nonspecific polyclonal antimurine control antibody (lane 4), a mutant nonradioactive P2 DNA sequence (lane 5), nonradioactive P2 DNA (lane 6), or a nonradioactive NFAT-binding sequence from the IL-4 promoter (lane 7). The mixtures were then incubated with the ³²P-labeled DNA probe described in Fig. 3. The mixtures were then run through a 6% TBE gel, dried, and autoradiographed. Lane 8 represents the DNA probe in the absence of nuclear proteins.

the binding of NFAT alone (lower band, white arrow) or NFAT in conjunction with other proteins such as AP-1 (upper band, black arrow), as has been shown in other studies of NFAT (10, 11).

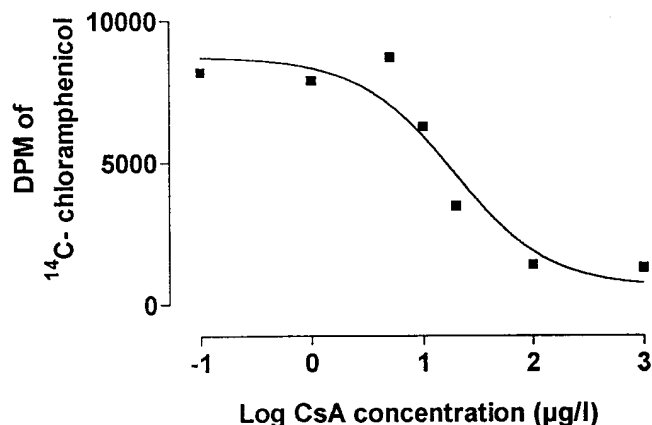


Figure 5. Effect of in vitro CsA on the induction of CAT activity in transfected PBL. PBL were exposed to 1% PHA for 3 d, washed and suspended in medium at 20 × 10⁶/ml, and incubated with 80 µl/ml of DNA encoding a plasmid containing three P2 sites upstream of an SV-40 promoter-driven CAT gene. Electroporation was performed on aliquots of cells. The transfectants were pooled, then split for CsA treatment (30 min) followed by exposure to A23187 (5 µM). After 48 h, cells were harvested, and CAT activity was measured.

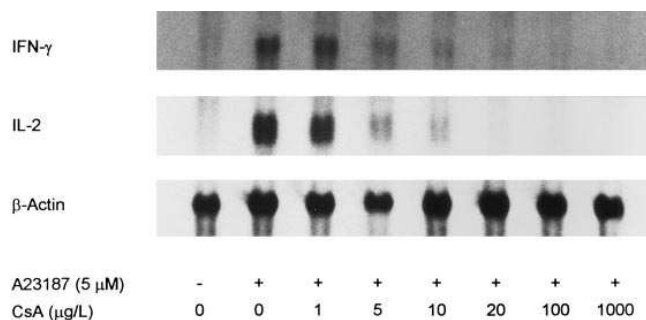


Figure 6. Effect of in vitro CsA on IFN- γ - and IL-2 mRNA levels in stimulated PBL. After exposure to CsA (or solvent control), PBL were unstimulated or stimulated with A23187 5 μ M for 1 h, and RNA was extracted with cesium chloride, run through an agarose gel, transferred to nitrocellulose, and probed with 32 P-labeled cDNAs for human IFN- γ , IL-2, and β -actin. The blots were then autoradiographed.

The effect of CN inhibition on a transfected reporter gene with NFAT sites. To investigate the effect of CN inhibition on functional activity of the transcription factors activating defined NFAT promoter sites, we transfected human PBL-blasts with a plasmid encoding three P2 sites upstream of a CAT reporter gene (Fig. 5). The construct without the P2 sites was not activated by A23187 exposure, whereas the construct with the P2 sites showed a 15-fold increase in CAT activity (data not shown). CsA pretreatment led to a stepwise reduction in CAT activity, with an IC₅₀ of 16 μ g/liter (95% CI, 2–146 μ g/liter).

Effect of partial CN inhibition on induction of IFN- γ - and IL-2 mRNA levels. We then studied the effect of in vitro CsA on steady state IFN- γ - and IL-2 mRNA levels in PBL stimulated by 5 μ M A23187. Northern blots of PBL stimulated for 1 h showed induction of both IFN- γ - and IL-2 mRNA levels that were reduced with increasing CsA concentrations (Fig. 6). Quantitation by Bio-Imager and correction for β -actin levels revealed IC₅₀s of 5 μ g/liter (95% CI, 4–7 μ g/liter) for both IFN- γ and IL-2. Similar Northern blots and identical IC₅₀s were seen when PBL were stimulated for 4 h (data not shown), indicating that CsA inhibition of mRNA induction did not simply shift the time course of induction during the first 4 h.

Effect of partial CN inhibition on IFN- γ secretion. We used an ELISA kit to measure IFN- γ protein secreted by PBL stimulated by 5 μ M A23187 for 18 h (Fig. 7). In two experiments, stimulation resulted in a 10–100-fold induction in IFN- γ in the supernatants. CsA inhibited accumulation of IFN- γ over the same concentration range described above, with IC₅₀s of 7 μ g/liter (95% CI, 5–9 μ g/liter) in both experiments.

Effect of partial CN inhibition on proliferation and IFN- γ production in the allogeneic MLR. We investigated the effect of CsA on the responses of lymphocytes stimulated by alloantigens. Responder PBL were mixed with irradiated stimulator PBL in a 6-d MLR in the absence or presence of CsA. For CN and IFN- γ measurement, cells and supernatants, respectively, were harvested on day 6. Proliferation and IFN- γ secretion were both induced during the MLR and, as shown in Fig. 8, all parameters were inhibited by treatment by CsA. The IC₅₀s were as follows: thymidine incorporation, 11 μ g/liter (95% CI, 0–7500 μ g/liter); CN activity, 8 μ g/liter (95% CI, 1–60 μ g/liter); and IFN- γ accumulation, 11 μ g/liter (95% CI, 0–1073 μ g/liter).

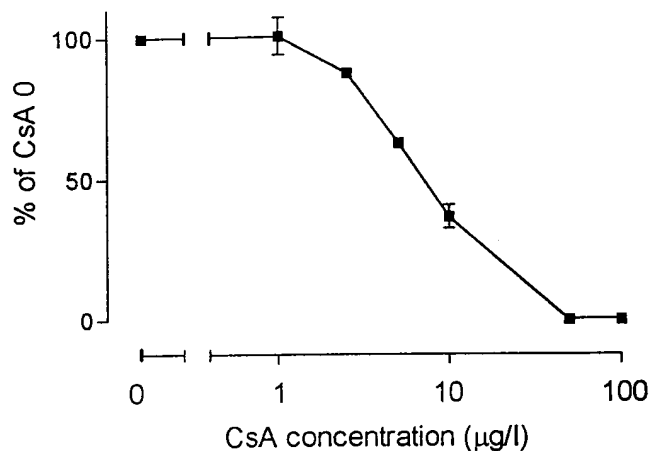


Figure 7. Effect of in vitro CsA on IFN- γ secretion by stimulated PBL. PBL were incubated with CsA for 30 min at 37°C followed by incubation with A23187 5 μ M for 18 h, at which time IFN- γ in the supernatants was measured by ELISA. The results represent the mean \pm SD of two experiments normalized against the stimulated CsA 0 group.

The latter values for CN activity and FN- γ accumulation are the same as those obtained in short-term experiments, indicating that there was no significant change in CsA effects over the 6-d experiment compared with previous short-term experiments.

Discussion

Because clinical measurements indicate that CsA treatment inhibits PBL CN activity, we studied the relationship between the degree of CN inhibition achieved clinically and the subsequent CsA-sensitive steps involved in cytokine gene activation and proliferation in human blood lymphocytes. The degree to which CsA inhibited each step studied—NFAT dephosphory-

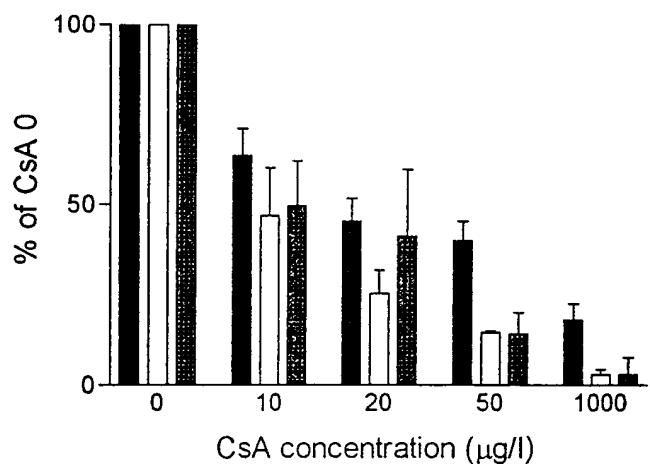


Figure 8. Effect of CsA on proliferation of PBL (black bars), CN (white bars), and IFN- γ accumulation (stippled bars) after a 6 d MLR. Responder PBL were stimulated by irradiated stimulator PBL. 6 d later, proliferation was measured by measuring [3 H]thymidine uptake. CN activity and IFN- γ were measured as previously described. Results represent standardized means \pm SD of five experiments for proliferation and IFN- γ , and three experiments for CN activity.

lation and translocation, appearance of NFAT-like DNA-binding activity in the nucleus, activation of a transfected reporter gene with NFAT sites in its promoter, IL-2 and IFN- γ mRNA accumulation, IFN- γ cytokine secretion, and cell proliferation in the MLR—was quantitatively similar to the degree to which CsA inhibited CN activity (Table I). The IC₅₀s for CsA inhibition of CN and of the downstream processes were similar, and the shapes of the concentration–inhibition curves each fit a sigmoidal dose-response kinetic model. Thus, a variety of molecular techniques each yielded a similar quantitative result for the pattern of cell response after exposure to CsA. These data agree with the previous demonstration that disruption of the CNA α gene, which leads to loss of 66% of total CN activity, is accompanied by T cell activation that is reduced but not eliminated (24). These data support the hypothesis that CN is not only essential but is rate-limiting for many activities of PBL or T cells.

The use of freshly isolated human cells may be important in establishing quantitative relationships between molecular events and may help avoid the potentially misleading results that can occur with the use of cell lines and additional CsA-insensitive stimuli such as phorbol esters. In T cell lines, intracellular signaling often differs from that observed in primary, freshly isolated, nonmalignant T cells. In particular, some T cell lines require a second chemical signal in order to become activated (37). In contrast, fresh PBL are activated by calcium ionophores alone, or by anti-T cell receptor antibodies (reference 37, and data not shown), and the addition of agents such as PMA is unnecessary. PMA recruits other pathways and may change the sensitivity of the activated cells to CsA. Furthermore, some T cell lines fail to express lymphocyte-specific cytokines such as IFN- γ (37), perhaps due to selection against the antiproliferative effects of IFN- γ . For these studies, we chose to focus on the T/natural killer cell-specific cytokine IFN- γ because this allowed us to use heterogeneous PBL preparations that have undergone minimal manipulation, and because purification of T cells often produced limiting cell numbers. Moreover, the MLR experiments indicated a similar relationship between CN inhibition and lymphocyte activation when the cells were stimulated through normal antigen recognition molecules, over much longer time intervals.

In addition to the quantitative effects CN inhibition has on

T cell effector activities in vivo (e.g., cytokine production), there may also be qualitative effects. These data that CN inhibition in vitro is rate-limiting for IFN- γ secretion and lymphocyte proliferation after antigen-specific stimulation in an MLR are consistent with our previous mouse results showing that the degree of in vivo CN inhibition correlated with the degree of inhibition of IFN- γ production and MHC induction in CsA-fed mice rejecting an allogeneic tumor (29). However, activation of some leukocyte activities such as TGF- β and IL-10 is CsA-resistant. Thus, cellular and humoral immune responses might be deviated by imbalances between the CsA-sensitive and CsA-resistant activities, producing qualitative shifts in the immune response. Whether this could shift the response toward mechanisms promoting anergy is unknown, but possible. T cell response can deviate toward different, mutually exclusive patterns of cytokine and cell surface molecule expression (38, 39), possibly many different patterns (39, 40), which are themselves influenced by the cytokines being produced in the environment. Therefore, it is possible that due to the relative resistance of some activation genes, partial CN inhibition in a population of CsA-treated cells may alter the ratio between CsA-sensitive and CsA-resistant cytokines and thus shift the character of the response as well as reducing its magnitude.

Thus, an array of clinical, murine in vivo, and molecular data support the hypothesis that CN activity is not just essential but is rate-limiting for the activation of a number of lymphocyte genes. The demonstration of an enzyme activity quantitatively rate-limiting for lymphocyte activation must be distinguished from the evidence that it is essential. Genetic immunodeficiency syndromes in humans have provided definitive evidence that the γ_c receptor chain (41) and the tyrosine kinases JAK3 and ZAP-70 (42–45) are essential for lymphocyte response and function. The ability to breed mice lacking specific genes has allowed the study of other signal transduction molecules believed to be important in lymphocyte activation, including Ras, protein kinase C, and CD28. Knocking out each gene in these pathways has been associated with effects on lymphocyte behavior (46–48). The CD28 knockout mice, despite significant defects in lymphocyte function in vitro (49, 50), were still able to reject allogeneic skin grafts (51). However, the observations based on natural and induced mutations do not indicate whether these molecules function qualitatively as on-off switches, or whether there is a quantitative effect. It is possible that one or more other steps within the CN-dependent activation pathway may also be rate-limiting, and that by limiting amounts of, for example, ZAP-70 or NFAT, a parallel reduction in lymphocyte activation might be observed. Mice lacking the CNA α gene showed evidence for both immunosuppression and immunoreactivity (24). Despite lacking the CNA α , these animals retained 34% of normal CN activity because of other CNA isoforms. Thus, these animals have a similar degree of CN inhibition as that seen in transplant patients, and their immune systems behave in a similar fashion. Finally, Shibasaki et al. have demonstrated a vigorous kinase activity that resulted in the rephosphorylation, and therefore the inactivation and nuclear export, of NFATC3 (originally called NFAT-4) (52), suggesting that NFAT activation requires continuous CN activity. Under these circumstances, increments in CN activity would be expected to lead to increments in NFAT activation, promoter binding and activation, etc.

Coupled with the previous data that CN is only partially inhibited in CsA-treated patients, these results help explain the

Table I. Summary of IC₅₀s of CsA Effect on the Calcium-dependent Events Involved in Cytokine Gene Activation and Lymphocyte Proliferation

	Mean IC ₅₀ μg/liter (95% CI)	Sigmoidal dose-response curve fit <i>r</i> ²
CN activity	10 (8–14)	0.9995
NFATC2 activation (Western blot)	20 (15–26)	0.9964
DNA binding (EMSA)	8 (5–12)	0.9958
Promoter activation (transfection)	19 (4–92)	0.9186
mRNA induction – IFN- γ	5 (4–7)	0.9974
– IL-2	5 (4–7)	0.9861
IFN- γ secretion	7 (5–9)	0.9995
Proliferation (6-d MLR)	11 (0–7500)	0.9977
CN activity (6-d MLR)	8 (1–60)	0.9993
IFN- γ secretion (6-d MLR)	11 (0–1073)	0.9975

clinical characteristics of CsA, particularly the basis for its safety and efficacy. CsA produces incomplete CN inhibition in PBL of CsA-treated patients (25, 26). Its safety, as shown by the low incidence of opportunistic infections (53), is due to the fact that some CN activity and thus lymphocyte responsiveness is preserved, permitting specific immune responses against infectious agents and preventing immunodeficiency. Thus CsA-treated patients can reject allografts (54), express abundant T cell cytokines (55), and be immunized against exogenous antigens (56). The effectiveness of CsA, evident by the reduction in allograft rejection rates (57) and the successful treatment of some autoimmune disorders (58), is due to the reduction in CN activity and its subsequent quantitative (and qualitative?) effect on immune responses. Further study of the CNA α gene knockout mice, with their 66% reduction in CN activity, will be helpful in defining the impact of partial CN inhibition on in vivo immune responses. In addition, more detailed in vivo animal studies investigating the entire dosage range of CsA will provide additional data regarding the consequences of CN inhibition on the function of an intact immune system. We propose that the immunosuppressive effect of CsA, and presumably also FK506, is due to its inhibition of CN activity, which is rate-limiting for lymphocyte activation. Recently, studies of the effect of the immunosuppressive drug mycophenolate mofetil have suggested that it too only produces partial inhibition of its target enzyme inosine monophosphate dehydrogenase (59). Thus, partial inhibition of rate-limiting enzymes may be a general theme in immunosuppressive drugs used for maintenance therapy, where the immune response must be blunted for control of disease activity but retained for long-term host defense.

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