

# Regulation of antigen-induced human T-lymphocyte responses by calcineurin antagonists

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**Background:** Cyclosporin A (CS) and tacrolimus (FK506, FK) are calcineurin antagonists used widely as T-cell immunosuppressants; however, their relative efficacy on antigen-stimulated T-cell subsets remains undefined.

**Objective:** We have examined the effects of CS and FK on antigen-driven proliferation and cytokine generation from human PBMCs and T-cell clones.

**Methods:** Proliferation was assessed by tritiated thymidine incorporation. Cytokine generation was assessed by reverse transcription-PCR and ELISA.

**Results:** Ragweed- and tetanus toxoid-driven proliferation of PBMCs was down-regulated equally by CS or FK. Gene expression for proinflammatory cytokines (IL-4, IL-5, IL-13, and IFN- $\gamma$ ) assessed by reverse transcription-PCR was down-regulated in a concentration-dependent manner by either drug. Antigen-induced proliferation of ragweed-specific Th0, Th1, or Th2 clones was inhibited by either CS or FK. Cytokine gene expression and protein secretion into culture supernatants (IL-4, IL-5, IL-13, and IFN- $\gamma$ ) were down-regulated in a concentration-dependent manner by either CS or FK in all relevant T-cell subsets. Interestingly, down-regulation of IL-5 protein generation from Th0 and Th2 clones was consistently less sensitive to either drug than was the effect on either IL-4 or IL-13 protein generation.

**Conclusion:** CS and FK promote equivalent down-regulation of Th0, Th1, and Th2 responses; however, IL-5 generation is relatively insensitive to the immunomodulatory effects of calcineurin antagonists. (*J Allergy Clin Immunol* 1999;104:828-35.)

**Key words:** Human, T lymphocyte, cyclosporine, tacrolimus, IL-4, IL-5, IL-13, IFN- $\gamma$

Cyclosporin A (CS) and tacrolimus (FK506, FK) are calcineurin antagonists used widely as T-cell immunosuppressants to prevent allograft rejection after solid organ transplantation. Investigational indications for

### Abbreviations used

|                    |                                         |
|--------------------|-----------------------------------------|
| APC:               | Antigen-presenting cell                 |
| CS:                | Cyclosporin A                           |
| FK:                | Tacrolimus (FK 506)                     |
| IC <sub>50</sub> : | Concentration of drug at 50% inhibition |
| NF-AT:             | Nuclear factor of activated T cells     |
| NF- $\kappa$ B:    | Nuclear factor- $\kappa$ B              |
| RT:                | Reverse transcription                   |
| RW:                | Ragweed extract                         |
| TT:                | Tetanus toxoid                          |

these agents include prevention of graft-versus-host disease after allogeneic bone marrow transplantation and treatment of severe, steroid-dependent asthma. CS and FK act through ligation of distinct intracellular binding protein targets (cyclophilin A for CS, FK binding protein for FK); these drug-binding protein complexes inhibit the function of calcineurin, which normally activates various transcription factors including nuclear factor of activated T cells (NF-AT) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) that regulate the expression of key cytokines and signaling proteins (Fig 1).<sup>1,2</sup>

T lymphocytes play a central role in the pathophysiologic mechanisms of allergic inflammation. CD4<sup>+</sup> T cells may be classified into several subsets on the basis of their patterns of cytokine generation. Human Th1 cells secrete IL-2, IFN- $\gamma$ , IL-13, and TNF- $\beta$ ; they mediate immune responses against intracellular pathogens and promote delayed-type hypersensitivity. Human Th2 cells secrete IL-4, IL-5, IL-10, and IL-13; they mediate immune responses against extracellular pathogens and regulate allergic inflammation by the promotion of IgE class-switching and eosinophil activation. Th0 cells secrete both Th1- and Th2-type cytokines.<sup>3</sup> The potential differential regulation of human antigen-stimulated T-cell subsets by calcineurin antagonists has not been described.

We herein present the first comprehensive study of the effects of calcineurin antagonists on proliferation and cytokine generation in antigen-stimulated human PBMCs and nontransformed antigen-specific human Th0, Th1, and Th2 clones. Our data argue against significant differential regulation of Th1 and Th2 responses by CS and FK but demonstrate a relative resistance of IL-5 to down-regulation by calcineurin antagonists.

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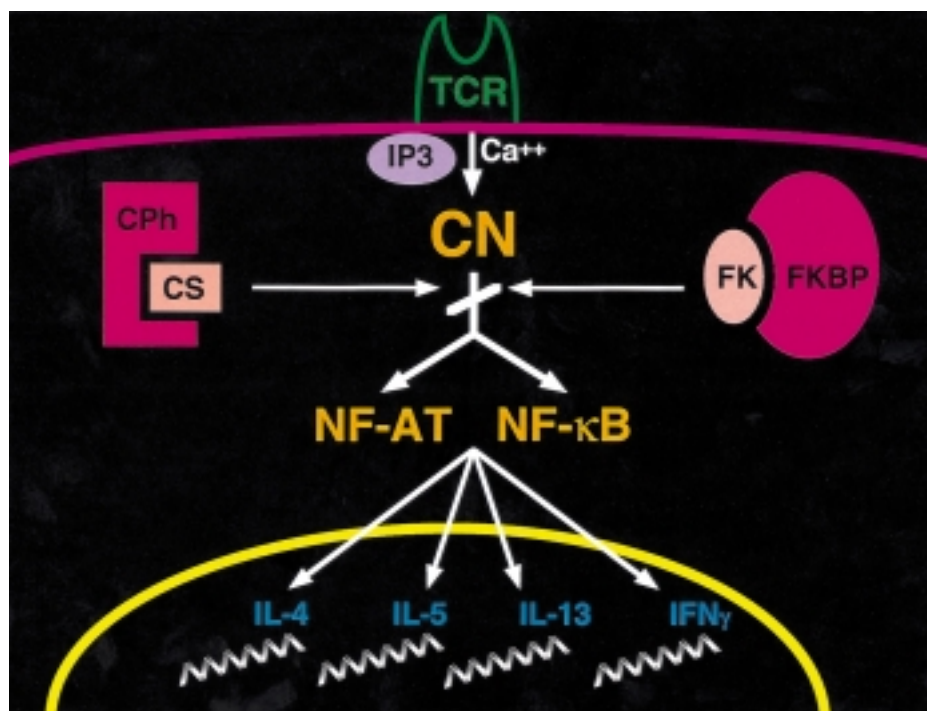


**FIG 1.** Modulation of signal transduction in T cells by CS and FK. CN, Calcineurin; CPh, cyclophilin A; FKBP, FK binding protein; IP3, inositol 1,4,5-triphosphate; TCR, T cell receptor.

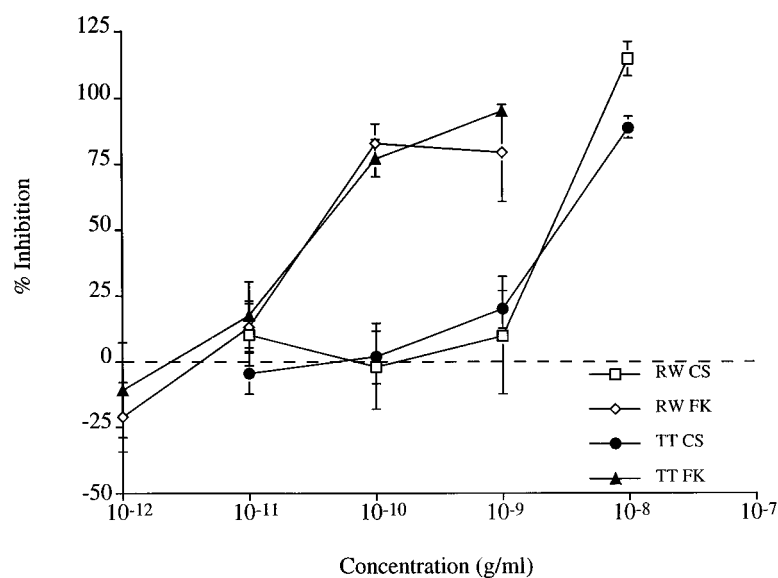


**FIG 2.** Modulation of RW- and TT-stimulated PBMC proliferation by CS and FK. Data are presented as percent inhibition  $\pm$  SEM relative to stimulated drug-free cultures, corrected for background with media alone.  $n = 4$  individual experiments with 4 different subjects.

## METHODS

### Selection of subjects

Subjects were allergic volunteers associated with the Johns Hopkins Asthma and Allergy Center who had previously been shown to have a positive epicutaneous skin test response to a 100,000 allergen

unit stock of short ragweed extract (RW, ALK Laboratories, Milford, Conn). All subjects had a history of upper or lower airway symptoms induced by ragweed. Subjects had received tetanus immunizations within the preceding 4 years and had not received any immunomodulatory medications for the month preceding the study; none of the subjects had undergone immunotherapy.

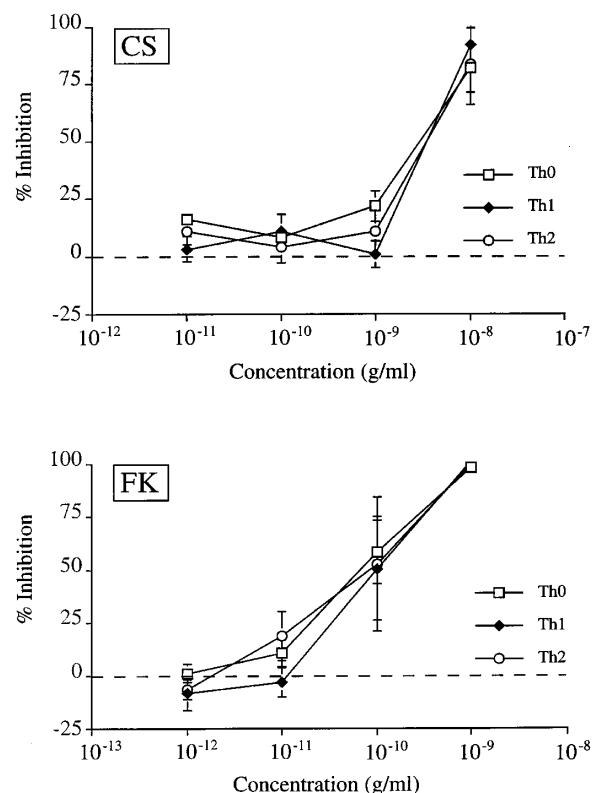


**FIG 3.** Modulation of antigen-stimulated Th0, Th1, and Th2 proliferation by CS and FK. RW-specific T-cell clones were stimulated in presence of various concentrations of each calcineurin antagonist. Data are presented as percent inhibition  $\pm$  SEM relative to stimulated drug-free cultures, corrected for background with media alone.  $n = 12$  individual experiments with 2 different clones of each phenotype.

### PBMC isolation and derivation of antigen-specific T-cell clones

PBMCs were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ) and resuspended in RPMI 1640 supplemented with 1% penicillin-streptomycin and 5% heat-inactivated human AB serum (Gibco/BRL, Gaithersburg, Md) as previously described.<sup>4,5</sup> Platelet contamination of these preparations was  $<1\%$ ; viability by trypan blue exclusion was uniformly  $\geq 98\%$ . These cells were either used immediately for PBMC studies or irradiated for use as antigen-presenting cells (APC) in conjunction with the T-cell clone studies.

Antigen-specific T-cell clones were derived and characterized as previously described.<sup>6</sup> Briefly, PBMCs from an asthmatic-rhinitic and a rhinitic subject, each with epicutaneous skin test reactivity to RW, were cultured in RPMI 1640 supplemented with 1% penicillin-streptomycin and 5% heat-inactivated human AB serum in the presence of short ragweed antigen  $10 \mu\text{g/mL}$ . This primary culture was followed by two successive biweekly restimulations of the antigen-specific T cells with a major short ragweed antigen, Amb a 1, in the presence of irradiated autologous PBMCs as APCs. The resulting antigen-specific T-cell line was cloned and subcloned with use of the limiting dilution technique. All T-cell clones were  $\text{CD}3^+\text{CD}4^+\text{CD}8^-$  by flow cytometry. Proliferative responses restricted to Amb a 1 confirmed antigen specificity. Cytokine profiles were determined by

both reverse transcription (RT)-PCR and ELISA for secreted protein; on the basis of these data, phenotypic assignment to Th0, Th1, or Th2 was made.<sup>7</sup>

### Proliferation assays

Proliferation assays were performed as previously described under conditions optimized for cell number, clone/APC ratio, antigen concentration, and kinetics.<sup>4,6,7</sup> Briefly, either  $2 \times 10^5$  PBMCs per well or  $2 \times 10^4$  clonal T cells with  $1.5 \times 10^5$  APCs per well were cultured in RPMI 1640 supplemented with 1% penicillin-streptomycin and 5% heat-inactivated human AB serum in 96-well flat-bottom plates in the absence or presence of antigen (RW  $25 \mu\text{g/mL}$  or tetanus toxoid [TT]  $0.1 \text{ IF/mL}$ ) and various concentrations of CS or FK (the kind gift of Drs Vincenzo Casolaro and Gianni Marone). Cells were preincubated with CS or FK for 2 hours before the addition of antigen. Negative (media alone) and positive (antigen alone) controls were performed with each culture. No exogenous cytokine was used in these assays. All conditions were performed in triplicate and incubated for 72 hours at  $37^\circ\text{C}$  with 5% carbon dioxide. Each culture was pulsed with  $1 \mu\text{Ci}$  of tritiated thymidine, incubated for an additional 20 hours, harvested onto glass fiber filters in a multi-channel cell harvester (Cambridge Technologies, Watertown, Mass), and counted for 1 minute in an automated beta counter (Beckman Instruments, Fullerton, Calif). Viability remained  $\geq 98\%$  in the presence of CS or FK, as determined by trypan blue exclusion.

### Cytokine gene expression assay

Cytokine gene expression was determined as previously described under conditions optimized for cell number, clone/APC ratio, antigen concentration, and kinetics.<sup>5,6,8</sup> Briefly, either  $5 \times 10^6$  PBMCs or  $2 \times 10^5$  clonal T cells with  $3 \times 10^6$  APC were cultured in RPMI 1640 supplemented with 1% penicillin-streptomycin and 5% heat-inactivated human AB serum in the absence or presence of antigen (RW  $25 \mu\text{g/mL}$  or TT  $0.1 \text{ IF/mL}$ ) and various concentrations of CS or FK. Cells were preincubated with CS or FK for 2 hours before the addition of antigen. After a 12-hour incubation with antigen in slanted polypropylene tubes, the cells were pelleted, washed, and subjected to RNA isolation by the RNeasy<sup>®</sup> technique, according to the manufacturer's instructions (Tel-Test, Friendswood, Tex). Diethylpyrocarbonate-treated water without sodium dodecyl sulfate was used for the final resuspension step. RNA was stored at  $-80^\circ\text{C}$  until studied. Normalization of RNA to approximately  $100 \text{ ng}/\mu\text{L}$  was achieved with a combination of ethidium bromide-stained gel electrophoresis and RT-PCR for the constitutive marker gene,  $\beta$ -actin.  $A_{260/280}$  values  $>1.7$  were uniformly obtained. Semiquantitative RT-PCR was performed with  $5 \text{ mmol/L}$  magnesium and oligodeoxythymidine priming with use of standard reagents (Perkin Elmer Cetus, Norwalk, Conn) and cytokine-specific primer pairs designed in our laboratory and made at the DNA Core Facility of the Johns Hopkins University. Strict ribonuclease-free conditions were maintained throughout these experiments. All PCR products were visualized by ethidium bromide-stained gel electrophoresis and photographed.

### Cytokine protein secretion assays

Cytokine protein secretion was assessed by ELISA, with use of Cytoscreen Immunoassay kits (Biosource Int, Camarillo, Calif) according to the manufacturer's instructions. Quantitation was achieved with the World Health Organization standards provided by the company. Culture supernatants were collected after 12 hours, centrifuged free of debris, and stored at  $-20^\circ\text{C}$  until assayed. Dilutions of samples, when necessary, were performed in culture medium. All standards and samples were tested in duplicate. Most samples were analyzed at 2 different dilutions and compared for internal consistency.

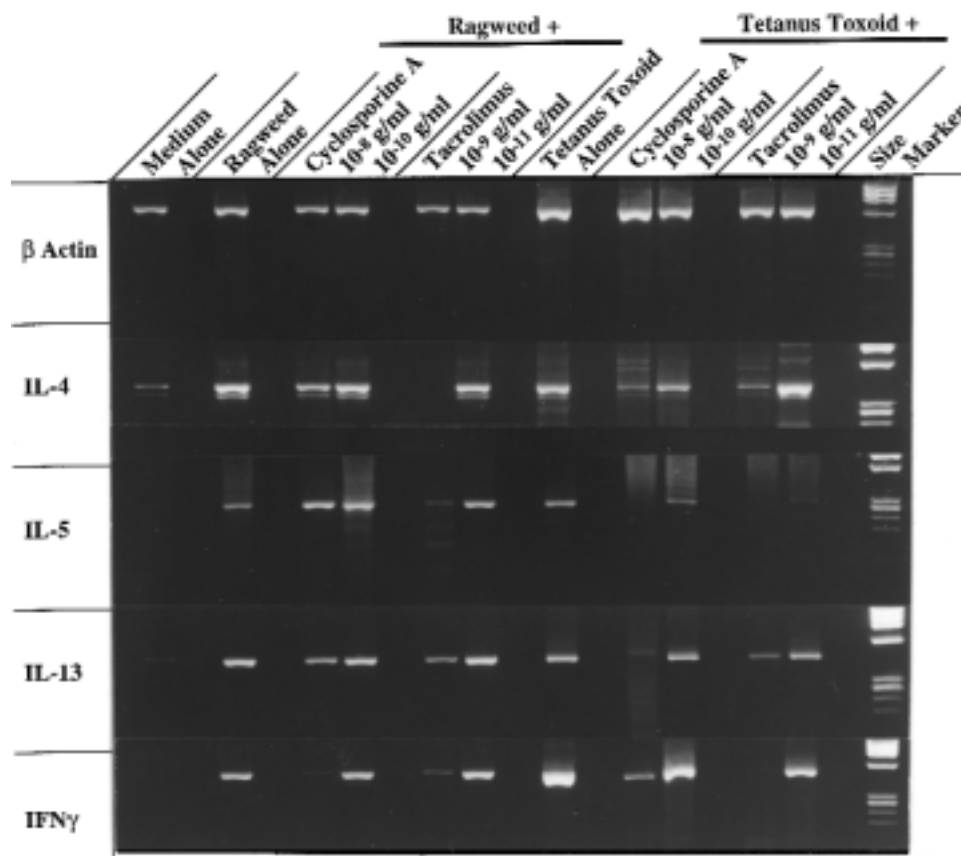


**FIG 4.** Modulation of cytokine gene expression by calcineurin antagonists in RW- and TT-stimulated PBMCs. Representative results of RT-PCR amplification products for  $\beta$ -actin, IL-4, IL-5, IL-13, and IFN- $\gamma$  are shown from single experiment for specific culture conditions indicated. Adequate normalization of RNA is demonstrated by equivalent  $\beta$ -actin gene expression at subsaturating cycle number (row 1). DNA size marker is shown on far right.  $n = 4$  individual experiments on 4 different subjects.

## Statistical analysis

Mean and SE values, as well as  $t$  test comparisons, were derived with use of StatView (BrainPower, Calabasas, Calif) on a Macintosh Classic computer. Probability values are paired 1-tailed values. Percent inhibition for each condition was calculated on the basis of inhibition relative to stimulated, drug-free mean counts, corrected in every case for background counts with media alone.  $IC_{50}$  values represent the concentration of drug at 50% inhibition.

## RESULTS

### PBMC proliferation in the presence of calcineurin antagonists

Fig 2 shows the proliferative responses of human PBMCs stimulated with RW or TT, each in the presence of varying concentrations of CS or FK. Both calcineurin antagonists induced significant concentration-dependent inhibition of RW- or TT-driven proliferation. Although no significant differences between the antigens could be discerned for either drug, the  $IC_{50}$  of FK was approximately 100-fold lower than the  $IC_{50}$  for CS for either antigen ( $4 \times 10^{-11}$  vs  $2 \times 10^{-9}$  g/mL, respectively,  $P <$

.001). Of note, the molecular weight of CS is 1203, and the molecular weight of FK is 822. Thus the correction factor for  $IC_{50}$  between grams per milliliter and molar concentrations is less than 1.5, and the difference remains significant.

### T-cell clonal proliferation in the presence of calcineurin antagonists

Fig 3 shows the proliferative responses of human Amb a 1-specific Th0, Th1, and Th2 clones in the presence of either CS (top) or FK (bottom). Both calcineurin antagonists induced significant concentration-dependent inhibition of T-cell clonal proliferation. No significant differences between Th0, Th1, or Th2 clonal proliferative responses could be discerned for either drug (CS  $IC_{50} = 3 \times 10^{-9}$  g/mL; FK  $IC_{50} = 6.6 \times 10^{-11}$ ,  $9.3 \times 10^{-11}$ ,  $7.1 \times 10^{-11}$  g/mL, respectively for Th0, Th1, and Th2,  $P > .2$ ); however, the difference between  $IC_{50}$  values for the 2 drugs remained significant. Interestingly, unlike corticosteroids and phosphodiesterase inhibitors, there was little difference in the  $IC_{50}$  values for calcineurin antagonists between PBMC and T-cell clones.

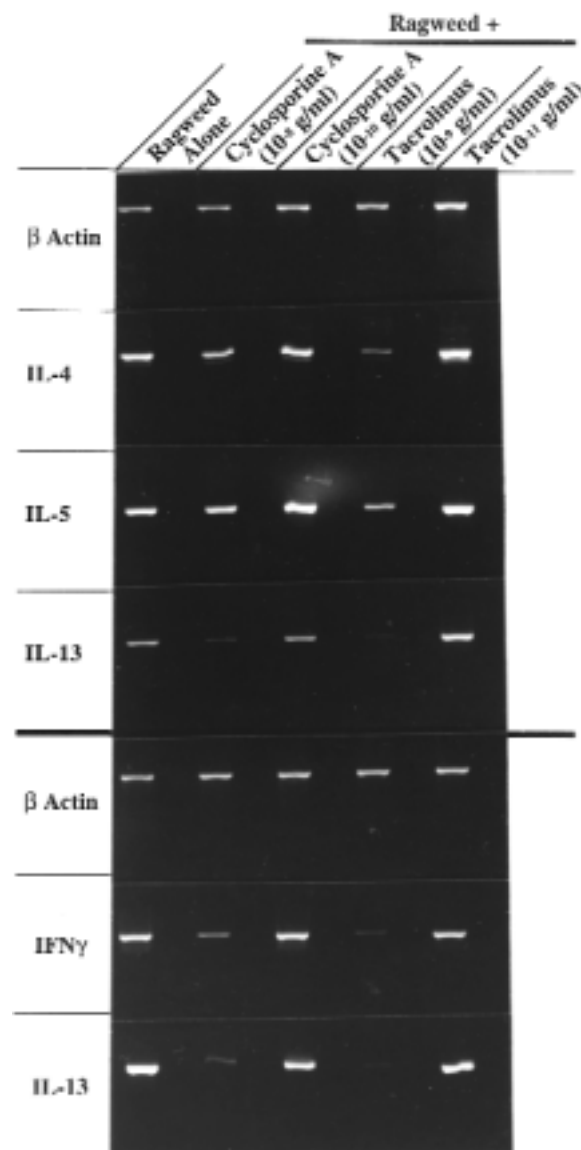


**FIG 5.** Modulation of cytokine gene expression by calcineurin antagonists in Th2 (top) and Th1 (bottom) clones. Representative results of RT-PCR amplification products for  $\beta$ -actin and phenotype-specific cytokines are shown from single experiment for specific culture conditions indicated. Adequate normalization of RNA is demonstrated by equivalent  $\beta$ -actin gene expression at subsaturating cycle number (rows 1 and 4).  $n = 8$  individual experiments with 2 different clones of each phenotype.

#### Cytokine gene expression in PBMC and T-cell clones in the presence of calcineurin antagonists

Fig 4 shows the effects of high and low concentrations of calcineurin antagonists on gene expression for IL-4, IL-5, IFN- $\gamma$ , and IL-13 in PBMCs stimulated with either RW or TT.  $\beta$ -Actin- and cytokine-specific RT-PCR amplification products from 1 representative subject are shown; similar results were obtained in 3 additional experiments on 3 additional subjects. Adequate normal-

ization of RNA for semiquantitative analysis was confirmed by the equality of PCR amplification products at subsaturating cycle numbers for  $\beta$ -actin gene expression (top row). Unstimulated PBMCs expressed minimal message for proinflammatory cytokines (column 1); control assays in the absence of reverse transcriptase were uniformly negative (data not shown). Expression of all 4 cytokines was strongly down-regulated or eliminated in the presence of high concentrations of either drug compared with the positive controls; low concentrations for either drug caused little or no inhibition of cytokine gene expression. This effect was similar for both RW and TT.

Fig 5 shows the influence of high and low concentrations of calcineurin antagonists on gene expression for IL-4, IL-5, IFN- $\gamma$ , and IL-13 in Th2 (top) and Th1 (bottom) clones.  $\beta$ -actin- and cytokine-specific RT-PCR amplification products from 1 representative experiment are shown; similar results were obtained in 3 additional experiments. Adequate normalization of RNA for semiquantitative analysis was again confirmed by the equality of RT-PCR amplification products at subsaturating cycle number for  $\beta$ -actin gene expression (rows 1 and 5). Neither unstimulated T-cell clones nor stimulated irradiated APCs expressed message for proinflammatory cytokines; control assays in the absence of RT were uniformly negative (data not shown). Similar to the data from PBMCs, Th1 and Th2 clones each showed strong down-regulation of gene expression for their respective cytokines when cultured with high concentrations of CS or FK; little effect was observed with low concentrations of either drug. Specifically, IL-13 gene expression from both Th1 and Th2 clones was down-regulated by high concentrations of CS or FK, and differential efficacy between Th1 and Th2 clones was not evident.

#### Cytokine secretion from T-cell clones in the presence of calcineurin antagonists

Figs 6 and 7 show the influence of high, intermediate, and low concentrations of calcineurin antagonists on cytokine protein secretion from Th0, Th1, and Th2 clones. Fig 6 depicts data using CS, whereas Fig 7 depicts data using FK. A low concentration of either calcineurin antagonist had little effect on IL-4, IL-5, IL-13, or IFN- $\gamma$  secretion, whereas a high but physiologic concentration of either antagonist resulted in marked inhibition of IL-4, IL-13, or IFN- $\gamma$  secretion. Interestingly, only partial inhibition of IL-5 secretion was achieved compared with either IL-4 or IL-13 secretion from the same clones (55% and 38%, respectively, for Th0 and Th2 clones cultured with CS and 70% and 86%, respectively, for Th0 and Th2 clones cultured with FK). The difference between IL-4 and IL-5 secretion from Th0 and Th2 clones was significant with both drugs ( $P < .05$ ); the difference between IL-13 and IL-5 secretion was significant with both phenotypes using CS and with Th2 clones using FK ( $P < .05$ ). With use of an intermediate concentration of either drug ( $10^{-9}$  and  $10^{-10}$  g/mL, respectively, for CS and FK), partial inhibition of IL-5 compared with IL-4 from both Th0 and Th2 clones was again seen with

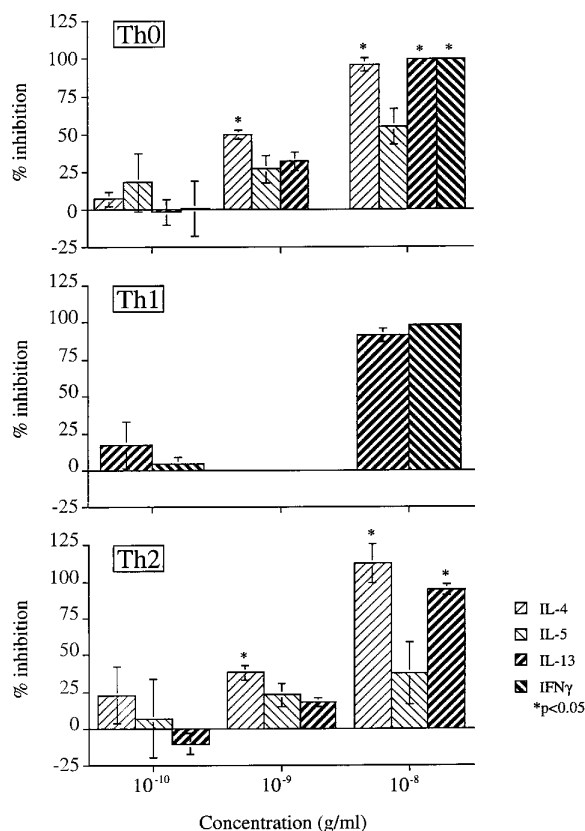


**FIG 6.** Modulation of cytokine secretion by CS in Th0, Th1, and Th2 clones. Secretion of phenotype-specific cytokines is shown for specific culture conditions indicated. Data are presented as percent inhibition  $\pm$  SEM relative to stimulated drug-free control cultures. Asterisk,  $P < .05$  compared with IL-5 at that concentration of CS.  $n = 4$  individual experiments with 2 different clones of each phenotype.

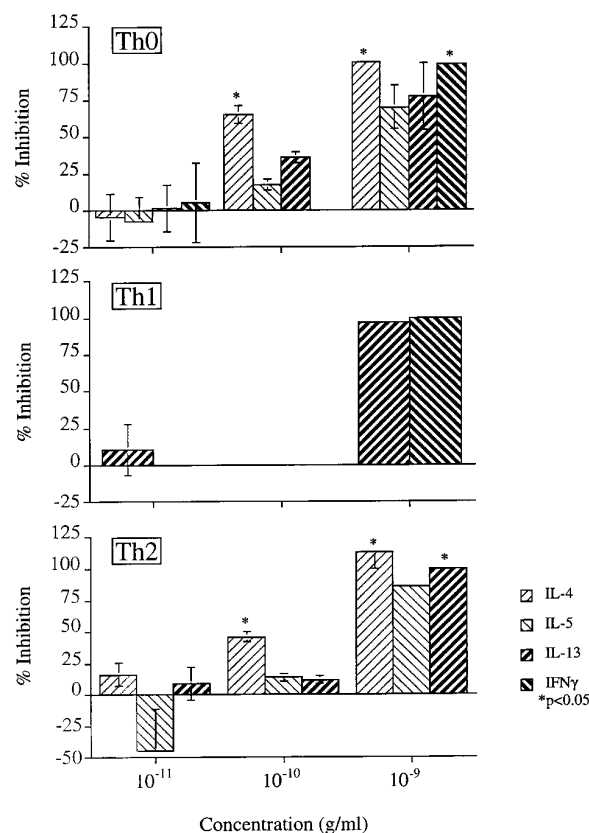


**FIG 7.** Modulation of cytokine secretion by FK in Th0, Th1, and Th2 clones. Secretion of phenotype-specific cytokines is shown for specific culture conditions indicated. Data are presented as percent inhibition  $\pm$  SEM relative to stimulated drug-free control cultures. Asterisk,  $P < .05$  compared with IL-5 at that concentration of FK.  $n = 4$  individual experiments with 2 different clones of each phenotype.

both CS and FK ( $P < .05$ ). Finally, concentrations of either drug 10-fold above the designated high concentrations ( $10^{-7}$  and  $10^{-8}$  g/mL, respectively, for CS and FK) resulted in cell death within a day.

## DISCUSSION

Cyclosporine and tacrolimus (FK 506) are calcineurin antagonists. These agents act, in part, through the inhibition of nuclear transcription factors such as NF-AT and NF- $\kappa$ B. Functionally, CS and FK inhibit the transcription of numerous cytokines and immunomodulators involved in T-cell activation and proliferation. A number of reports have suggested a relative resistance of Th2 cytokines to the effects of calcineurin antagonists.<sup>9,10</sup> Within the category of Th2 cytokines relative resistance of specific interleukins (ie, IL-4 vs IL-5) to the actions of calcineurin antagonists may have implications for the treatment of specific aspects of allergic and immunologic disease states (IgE vs eosinophilia). The current study is the first comprehensive analysis of calcineurin antagonists in human antigen-stimulated PBMCs and nontransformed, antigen-specific Th0, Th1, and Th2 clones.

Previous studies have demonstrated the ability of calcineurin antagonists to down-regulate IFN- $\gamma$ . Batiuk et al<sup>11,12</sup> have demonstrated CS-induced inhibition of IFN- $\gamma$  in OKT3-stimulated human PBMCs; of note, these investigators reported excellent PBMC viability after exposure to CS in concentrations up to 0.1 mg/L ( $10^{-7}$  g/mL). Briscoe et al<sup>13</sup> have reported CS-induced inhibition of mitogen-stimulated IFN- $\gamma$  generation from T cells in the presence of endothelial cell costimulatory signals. However, cellular activation in these studies was achieved with mitogens; the relevance of these systems to APC and antigen-driven Th1 and Th2 phenotypic responses is unclear. Iacono et al<sup>14</sup> have described CS-induced down-regulation of both serum and bronchoalveolar lavage IFN- $\gamma$  levels in lung transplant recipients undergoing acute rejection. Although these data represent pharmacologic regulation of antigen-stimulated cytokine generation, only IFN- $\gamma$  data were described and phenotypic analysis was not sought.

Previous reports of the effects of calcineurin antagonists on IL-4 and IL-13 generation have provided conflicting results. Calcineurin antagonists have been shown to down-regulate IL-4 and IL-13 generation in atopic dis-

ease through an NF-AT-mediated pathway.<sup>15-17</sup> Moreover, Rafiq et al<sup>18</sup> reported CS-induced inhibition of IL-4 in anti-CD3- or mitogen-stimulated T cells costimulated through engagement of CD28; of interest, concomitant down-regulation of IL-10 and up-regulation of IFN- $\gamma$  production was demonstrated by these investigators. However, although IL-4 generation from human basophils was sensitive to treatment with FK, IL-13 generation was resistant; at least 2 reports have demonstrated up-regulation of IL-13 generation by T cells activated through CD3/CD28 in the presence of CS and FK.<sup>19-21</sup> Differences in cell populations and methods of cellular activation used in these experiments complicate their interpretation and likely account for the contradictory results. We and others have shown that mitogenic stimulation of phenotypically specific CD4<sup>+</sup> T cell clones (human or murine) will result in nonphenotypically selective (Th0-type) cytokine generation; subsequent restimulation with antigen and APCs will restore phenotypic specificity.<sup>6,22</sup> Thus mitogen stimulation appears to obviate phenotypic specificity. Moreover, mitogen stimulation is clearly less physiologic than antigen stimulation. Finally, Pazderka et al<sup>23</sup> have demonstrated that sensitivity to calcineurin antagonists may vary with the quality and quantity of the activation signal applied to the responder cells. The data presented in this study have been derived exclusively from antigen-specific T-cell responses and represent a refined approach to the analysis of pharmacologic regulation by calcineurin antagonists.

Previous reports of the effects of calcineurin antagonists on IL-5 generation have also provided conflicting results. Rolfe et al<sup>24</sup> have demonstrated transcriptional regulation of IL-5 by CS and FK. Mori et al<sup>25</sup> have demonstrated concentration-dependent inhibition by CS and FK of IL-5 production from PBMCs of atopic subjects stimulated with PMA and calcium ionophore; although their study suggested similar regulation of IL-5 and other proinflammatory cytokines, quantitative analysis was not provided. However, Valentine and Sewell<sup>26</sup> have reported the resistance of IL-2-induced IL-5 secretion to CS. Moreover, the potential for differential regulation of IL-5 among other human cytokines has been suggested by a number of investigators. Umland et al<sup>27</sup> have described different kinetics for IL-5 messenger RNA compared with that of IL-4 and IL-13. In their study of alloantigen-induced CD30<sup>+</sup> T-cell activation, Martinez et al<sup>28</sup> demonstrated relative IL-5 resistance to CS treatment; similar results were obtained by Eum et al<sup>29</sup> in a murine pulmonary allergen challenge model. The relative resistance of IL-5 generation suggests the potential existence of unique, calcineurin-sensitive elements in the regulation of IL-5 gene expression; a unique transcription factor, NF-IL-5, has recently been reported that may regulate IL-5 gene transcription independently of IL-2 or IL-4.<sup>30</sup> Our data suggest a quantitative, rather than qualitative, difference in IL-5 regulation by calcineurin antagonists and provide the first such data in a purely antigen-driven system.

Two final observations deserve note. First, IC<sub>50</sub> values

for the proliferative responses of PBMC and clonal T-cell populations were equivalent for each drug used; these data differ from those with corticosteroids or phosphodiesterase inhibitors, where the clonal T cells are routinely more sensitive to the drugs used.<sup>4,31,32</sup> Second, although there is evidence that calcineurin antagonists can effect T-cell responses through interference with antigen presentation from APCs, the similarity of PBMC and clonal T-cell data from this study and the use of irradiated autologous PBMCs as APCs militate against significant confounding of these data.<sup>7</sup>

In conclusion, proliferation and cytokine generation from antigen-stimulated PBMCs and nontransformed human Th0, Th1, and Th2 clones were strongly inhibited by either CS or FK. Down-regulation of IL-5 was relatively insensitive to the effects of calcineurin antagonists. Finally, our data argue against differential regulation of T-helper phenotypes by these agents.

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