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SHORT COMMUNICATION

Regulation of Interleukin-13 by Type 4 Cyclic Nucleotide Phosphodiesterase (PDE) Inhibitors in Allergen-Specific Human T Lymphocyte Clones

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ABSTRACT. Interleukin-13 (IL-13) is a proinflammatory cytokine of T cell origin. Structural and functional studies suggest a key role for IL-13 in the genesis of chronic allergic inflammation; as such, its pharmacologic inhibition is of potential clinical utility. We studied the pharmacologic regulation of IL-13 expression by cyclic nucleotide phosphodiesterase (PDE) inhibitors in a panel of Amb a 1 (a major allergen of short ragweed, *Ambrosia artemisiifolia*)-specific T cell clones derived from a ragweed allergic, asthmatic subject. Proliferative responses of these cells were down-regulated by rolipram, a PDE4 inhibitor (% inhibition_{MAX} = 67%; IC_{50} = 20 μM). While the PDE3 inhibitor siguazodan provided no independent efficacy ($IC_{50} > 10^{-4}$ M), an increased efficacy of rolipram in the presence of 10^{-5} M siguazodan was noted at 10^{-6}, 10^{-5}, and 10^{-4} M rolipram ($P < 0.03$, 0.01, and 0.04, respectively). The EC_{50} values remained unchanged between assays using the PDE4 inhibitor with or without the PDE3 inhibitor. Both IL-13 gene expression and protein secretion into culture supernatants were down-regulated by the PDE4 inhibitor ($P \leq 0.005$). Once again, the use of a PDE3 inhibitor provided no independent efficacy ($P \geq 0.2$), and in this instance, increased efficacy of the PDE4 inhibitor with the PDE3 inhibitor was not apparent ($P \geq 0.3$). IL-13 production from clones with Th0, Th1, and Th2 phenotypes appeared equally sensitive to treatment with the PDE4 inhibitor. We conclude that the anti-inflammatory effects of PDE4 inhibitors may be mediated, in part, by down-regulation of IL-13. BIOCHEM PHARMACOL 53;7:1055–1060, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. human; T lymphocyte; interleukin-13; allergy; phosphodiesterase; cAMP

IL-13† is a T cell-derived cytokine that shares many structural and functional properties with IL-4. Specifically, IL-13 is capable of inducing IL-4-independent class switching to IgE in human B cells, as well as enhanced expression of CD23 and MHC class II [1, 2]. IL-13 has been shown to increase VCAM-1 expression in human umbilical vein endothelial cells and may act as a monocyte chemoattractant [3, 4]. While IL-13 has no direct effects on T cell proliferation, IL-13 does cross-react extensively at the IL-4 receptor, inducing both early and late phosphorylation events involving multiple proteins, including Raf-1 kinase [5–7]. This pattern of activity is consistent with the hypothesis that IL-13 exacerbates chronic allergic inflammation [8]. Also consistent with this hypothesis is our recent demonstration of increased IL-13 gene expression and protein production in allergen-challenged bronchoalveolar lavage samples from patients with asthma [9]. Thus, the ability to down-regulate IL-13 production from T cells would have strong potential clinical utility. To date, no published studies have examined the pharmacologic regulation of IL-13 from human T lymphocytes.

Elevations of intracellular cAMP have been shown to down-regulate a wide variety of immune cell functions, including IL-2 production, IL-2R expression, IgE production, and lymphokine-activated NK cell activity [10–13]. While levels of intracellular cAMP may be transiently elevated through the activation of adenylyl cyclase, sustained elevations may be difficult to achieve due to up-regulation of PDE activity [14]. The PDE isozyme family constitutes the primary metabolic pathway for cyclic nucleotide second messengers; selective inhibition of these isozymes results in elevated intracellular levels of specific cyclic nucleotides. The seven identified PDE isozymes may be differentiated on the basis of substrate specificity, inhibitor sensitivity, and sequence homology; while lymphocytes contain primarily PDE3–5, only PDE3 and PDE4 exhibit significant hydrolytic activity on cAMP [15]. We have shown previously the efficacy of PDE4 inhibitors in down-regulating antigen-

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† *Abbreviations:* IL, interleukin; PDE, cyclic nucleotide phosphodiesterase; cAMP, cyclic AMP; RT, reverse transcription; PCR, polymerase chain reaction; and PBMCs, peripheral blood mononuclear cells.

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driven proliferation and cytokine gene expression for IL-5 and IFN-γ in human PBMCs [16, 17]. Interestingly, despite their anti-inflammatory properties, PDE4 inhibitors appeared ineffective in down-regulating IL-4 gene expression. Therefore, we hypothesize that a portion of the anti-inflammatory efficacy of PDE4 inhibitors may be mediated by their inhibition of IL-13 production by human T lymphocytes. In the present study, we demonstrated that IL-13 gene expression and protein production are down-regulated by the PDE4 inhibitor rolipram, but not by the PDE3 inhibitor siguazodan, in a phenotypically diverse group of allergen-specific T cell clones.

MATERIALS AND METHODS

Derivation of Antigen-Specific T Lymphocyte Clones

Antigen-specific T cell clones were derived as previously described [18, 19]. Briefly, PBMCs from an asthmatic subject with epicutaneous skin test reactivity to short ragweed (*Ambrosia artemisiifolia*) and short ragweed-specific IgE = 1761 ng/mL were cultured in the presence of short ragweed antigen (10 μg/mL). This primary culture was followed by successive, biweekly stimulation of the antigen-specific T cells with a major short ragweed antigen, Amb a 1, in the presence of autologous, irradiated PBMCs as a source of antigen presenting cells (APCs). The resulting antigen-specific T cell line was cloned and subcloned using the limiting dilution technique. Eight clones were selected from a panel of 30 based only on their similar degree of proliferation to Amb a 1 antigen. Proliferative responses restricted to Amb a 1 confirmed antigen specificity. Cytokine profiles were determined by both RT-PCR and ELISA for cytokines secreted into culture supernatants. Based on these data, phenotypic assignment to Th0, Th1, or Th2 was made (Table 1).

Proliferation Assays

Proliferation assays were performed as previously described [16, 19]. Briefly, 2×10^4 clonal T cells/well were cultured with 1.5×10^5 APCs/well in the absence and presence of short ragweed antigen in 96-well plates. Culture conditions were designated by the absence or presence of various concentrations of the PDE3 inhibitor (siguazodan), the PDE4 inhibitor (rolipram), or both for the entire culture period. No exogenous cytokine was used. The cells were preincubated with drug for 2 hr prior to the addition of antigen, an interval previously determined as optimal [16]. All conditions were cultured in triplicate and incubated for 72 hr. The cultures were then pulsed with 1 μCi/well of [^{3}H]thymidine for an additional 20 hr, harvested on a multichannel harvester (PHD, Cambridge Technologies Inc., Watertown, MA), and counted in a beta counter (LS 5000 TD, Beckman Instruments Inc., Fullerton, CA).

Cytokine Gene Expression Assays

Cytokine gene expression was determined as previously described [17, 20]. Briefly, 2×10^5 clonal T cells/well were cultured with 3×10^6 APCs/well in the absence and presence of short ragweed antigen in 24-well plates. Culture conditions were designated by the absence or presence of 10^{-5} M of the PDE3 inhibitor (siguazodan), the PDE4 inhibitor (rolipram), or both for the entire culture period. Once again, no exogenous cytokine was used, and the cells were preincubated with drug for 2 hr prior to the addition of antigen. After a 12-hr culture interval, the cells were pelleted, washed, and subjected to RNA isolation by the RNAzol^B technique (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. Diethylpyrocarbonate-treated water without SDS was used for the final resuspension step. RNA was stored at −70°. Normalization of RNA to 100 ng/μL was achieved with a combination of spectrophotometry, ethidium bromide-stained gel electrophoresis, and RT-PCR for a constitutive marker gene, β actin, as previously described [17]. $A_{260/280}$ values > 1.7 were uniformly obtained. RT-PCR was performed on 200 ng RNA with 5 mM magnesium and oligo dT priming, using standard reagents (Perkin-Elmer Cetus, Norwalk, CT) and target-specific primer pairs designed in our laboratory and made at the DNA Core Facility of the Johns Hopkins University (β actin 5′-TGACGGGGTCACCCACACTGTGCCCATCTA; β actin 3′-CTAGAAGCATTGCGGTGGACGATGGAGGG; IL-13 5′-

TABLE 1. Characterization of Amb a 1-specific T cell clones

Clone	Phenotype	IL-4 (pg/mL)	INF-γ (pg/mL)	IL-13 (pg/mL)	$Proliferation_{MAX}$/ Control (cpm)
2D2	Th2	3,880	64	13,860	69,730/610
3	Th2	3,500	331	23,000	111,300/610
1B5	Th0	3,300	3,180	12,500	35,500/640
2B7	Th0	1,800	2,500	5,400	55,040/640
13	Th0	1,750	962	11,600	86,080/590
11	Th0	1,200	486	5,200	25,730/710
12	Th1	750	3,200	10,300	61,310/580
2B8	Th1	200	4,780	4,500	64,770/730

Representative data from eight T cell clones, selected on the basis of their similar degrees of proliferation to Amb a 1 antigen. All clones are $CD4^+$.

GGAAGCTTCTCCTCAATCCTCTCCTGTT; IL-13 3′-GCGGATCCGTTGAACCGTCCCTCGCGAAA). All PCR products were visualized by ethidium bromide-stained gel electrophoresis, photographed, and quantitated on a Molecular Dynamics densitometer (Sunnyvale, CA) running ImageQuant software according to the manufacturer's instructions.

Cytokine Protein Secretion Assays

Cytokine protein secretion was assessed by ELISA (Biosource, Int., Camarillo, CA) according to the manufacturer's instructions, using the WHO standards provided by the company. Briefly, duplicate cultures to those used in the cytokine gene expression experiments were incubated for 24 hr; supernatants from these cultures were harvested and stored at −20° until assayed. Dilutions of samples, when necessary, were performed in culture medium. All standards and samples were run in duplicate; most samples were run in two different dilutions and compared for internal consistency.

Statistical Analysis

Mean and standard error values, as well as *t*-test comparisons, were derived using StatView (BrainPower, Inc., Calabasas, CA) on a Macintosh computer. The *t*-tests were paired, two-tailed tests. Percent inhibition for each condition was calculated based on inhibition relative to stimulated, drug-free mean counts, subtracted in every case for background counts with medium alone. The IC_{50} values represent the concentration of drug at 50% inhibition; the EC_{50} values represent the concentration of drug at 50% efficacy.

RESULTS

Proliferation Assays

Figure 1 depicts the mean values for percent inhibition for each of the eight allergen-specific T cell clones at four separate concentrations of three different drug conditions: the PDE4 inhibitor, rolipram, alone; the PDE3 inhibitor, siguazodan, alone; and rolipram in the presence of 10^{-5} M siguazodan (a pharmacologic but nontoxic concentration). While the PDE4 inhibitor was highly efficacious in down-regulating the allergen-driven proliferative response (% $inhibition_{MAX}$ = 67%; IC_{50} = 2×10^{-5} M), the PDE3 inhibitor was ineffective ($IC_{50} > 10^{-4}$ M). Despite the lack of independent efficacy of the PDE3 inhibitor on any of the clones tested, a 10^{-5} M concentration of siguazodan used in conjunction with the PDE4 inhibitor was additive in down-regulating the proliferative response at 10^{-6}, 10^{-5}, and 10^{-4} M rolipram ($P < 0.03$, 0.01, and 0.04, respectively). The EC_{50} values were not different between the two drug treatment conditions. These results closely parallel those from the antigen-driven PBMC model previously reported [16]. The low number of T cell clones precluded adequate subgroup analysis.

Cytokine Gene Expression

Figure 2 (top panel) depicts a representative set of RT-PCR amplification products for an IL-13-specific primer pair, performed at subsaturating cycle number in a Th0 (2B7), Th1 (2B8), and Th2 (2D2) antigen-specific T cell clone under the following conditions: ragweed antigen (10 μg/mL) without drug (positive control); antigen with rolipram 10^{-5} M; antigen with 10^{-5} M siguazodan; antigen with both rolipram and siguazodan (both concentrations being pharmacologic but nontoxic). The IL-13 band appeared as the lower of the two bands for each culture condition. Gene expression for β actin (a constitutive marker gene) for each culture condition, appearing as the upper of the two bands, is shown for comparison. A 12-hr stimulation interval was used, obviating any effects of cellular proliferation. Prelimi-

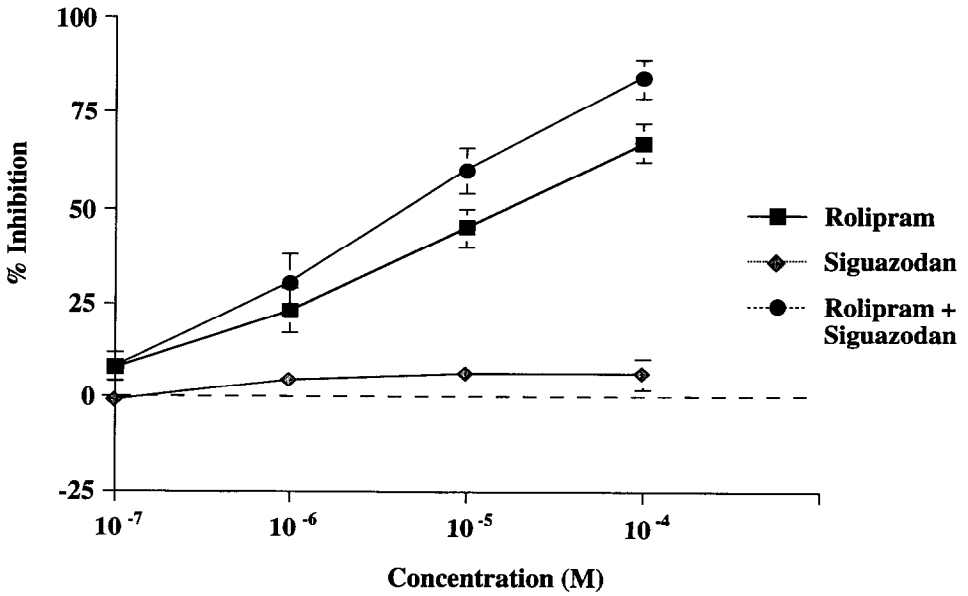

FIG. 1. Modulation of the clonal T cell proliferative response by PDE inhibitors. Group data are presented for stimulation of eight individual Amb a 1-specific T cell clones in the presence of various concentrations of PDE inhibitors. Data are presented as percent inhibition ± SEM relative to stimulated, drug-free cultures, subtracted for background with medium alone (58,000 ± 7,000 and 1,100 ± 200 cpm, respectively). Each clone was used in at least two separate duplicate experiments. N = 19 individual experiments.

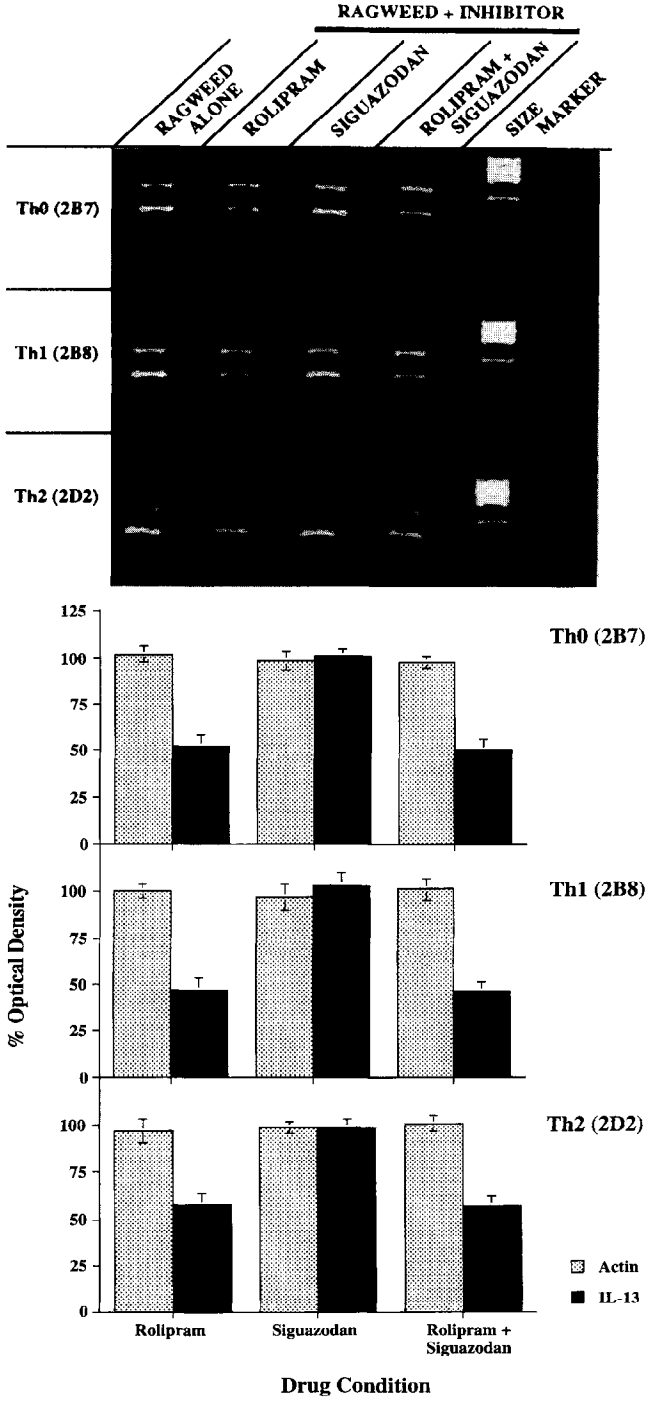

FIG. 2. (Top panel) Modulation of IL-13 gene expression by PDE inhibitors in three T cell clones. A representative set of RT-PCR products for IL-13 are shown (lower band) for three different antigen-stimulated T cell clones under four different culture conditions. Normalization by β actin gene expression at subsaturating cycle number is depicted for each condition (upper band). Fragment sizes: β actin = 636 base pairs, IL-13 = 441 base pairs. The ϕX174 *HaeIII* fragment size marker is shown on the far right for comparison. (Bottom panel) Densitometry data for the modulation of IL-13 gene expression by PDE inhibitors. Data are depicted for each culture condition of each clone for both β actin and IL-13. The numbers represent percent optical density ± SEM of PCR amplification product bands normalized to stimulated, drug-free controls, subtracted for background. The variability of β actin gene expression was less than 2%. N = 3 individual experiments.

nary studies have confirmed that clonal T cells cultured in the presence or absence of these drugs without antigen do not generate mRNA for proinflammatory cytokines (data not shown). Gene expression for β actin showed less than 2% variability between culture conditions within a single clone. Gene expression for IL-13 was down-regulated 42–53% (depending on the individual clone, $P \leq 0.005$) with the use of rolipram, with or without siguazodan. No independent efficacy was apparent with the use of siguazodan ($P \geq 0.2$). Unlike the proliferation studies, no additive effect of siguazodan with rolipram was seen ($P \geq 0.3$). This efficacy profile of the PDE inhibitors was apparent in all three T helper phenotypes, although the relative amount of IL-13 transcripts varied somewhat between the T cell clones. A quantitative representation of these data, using band densitometry, is shown in the bottom panel of Fig. 2.

Cytokine Protein Secretion

Figure 3 depicts the amounts of IL-13 secreted by each of the eight antigen-specific T cell clones stimulated with ragweed antigen for 24 hr under the same culture conditions as used for the cytokine gene expression studies. Th0 clones are depicted in gray, the Th1 clones are shown in black, and the Th2 clones are shown in white. Although Th2 clones generally secreted higher levels of IL-13 than did Th1 clones, small sample numbers precluded adequate statistical analysis. A significant decrease in the amount of secreted IL-13 was apparent in both the rolipram- and the rolipram with siguazodan-treated culture conditions, relative to drug-free control (10,800 ± 2,200 vs 5,700 ± 1,500 and 4,800 ± 1,300 pg/mL; $P < 0.005$ and 0.002, respectively). Siguazodan was ineffective in down-regulating IL-13 protein secretion from these T cell clones (11,000 ± 2,500 pg/mL; $P > 0.4$). Although lower cytokine protein production from the rolipram plus siguazodan-treated culture condition was apparent relative to the rolipram alone-treated cultures, this did not reach statistical significance ($P = 0.3$).

DISCUSSION

The cytokine network plays a central role in immune cell signaling and immune responsiveness in a variety of disease states, including atopic asthma [9, 21–23]. Dysregulation of a number of cytokines has been implicated in the pathogenesis of atopic disease. Among these, IL-13 is of particular interest due to its ability to induce IL-4-independent class switching to IgE and enhanced expression of both CD23 and MHC class II [1, 2]. Moreover, the production of IL-13 from human Th1 cells raises the potential for IgE generation in the absence of a true Th2 antigen response. From these properties, one may hypothesize a role for IL-13 in the maintenance of chronic allergic inflammation in the absence of continuous allergen exposure. This study represents the first published report concerning the pharmacologic regulation of IL-13.

Cyclic nucleotide PDE isozymes regulate the steady-state

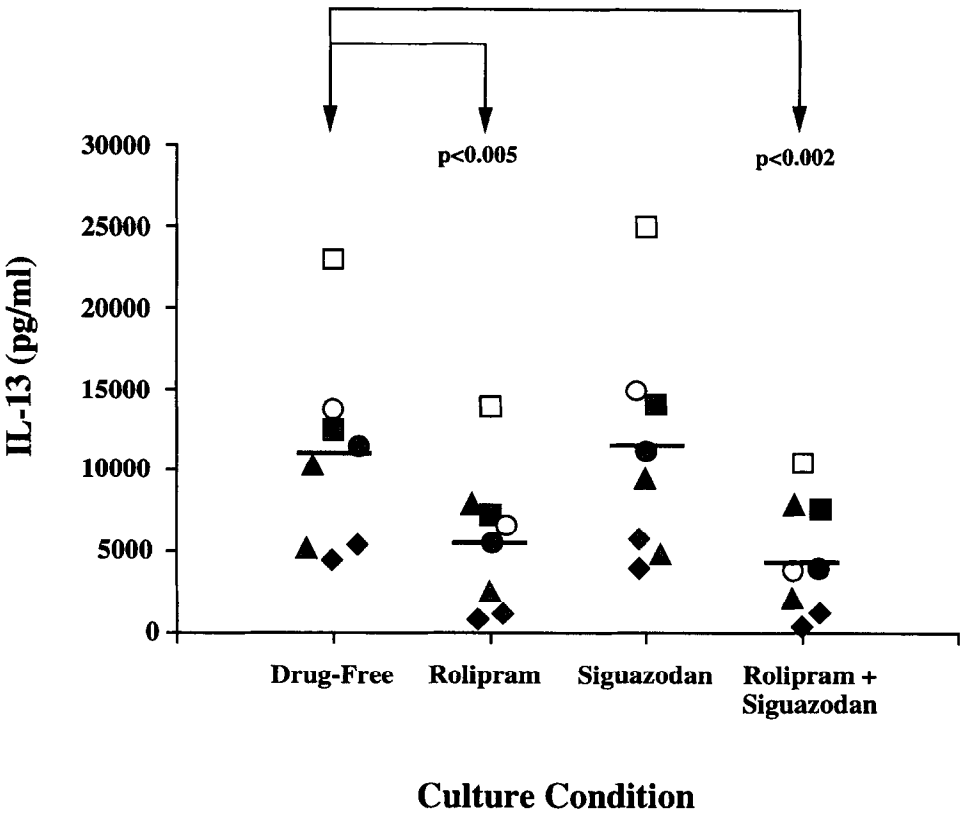

FIG. 3. Modulation of IL-13 secretion by PDE inhibitors in a panel of eight T cell clones. IL-13 protein, measured by ELISA, is depicted for each of eight T cell clones, as indicated by each symbol, under the same four culture conditions as in Fig. 2. Key: gray symbols = Th0; black symbols = Th1; and white symbols = Th2. Mean values in pg/mL are shown by the horizontal bars.

concentrations of cyclic nucleotide second messengers, used in a variety of signal transduction events [15]. The specificity of the PDE4 isoform for cAMP implicates elevations of intracellular cAMP in the down-regulation of IL-13. While PDE inhibitors are known to exert potent anti-inflammatory effects both *in vitro* and *in vivo*, PDE inhibitors have shown little effect in modulating antigen-driven IL-4 gene expression in a mixed cellular system [15, 17]. Thus, the efficacy of these agents on IL-13 gene expression and protein secretion may help to provide a resolution to this discrepancy. Our use of antigen-driven T cell clones, rather than mitogen-driven T cells, provides a more clinically relevant model for analysis. Parallel studies using PBMCs from allergic subjects have provided similar data, although measurements of cytokine protein secretion could not be performed due to a low number of antigen-specific responder cells in peripheral blood.

While numerous properties of IL-13 may be considered proinflammatory, IL-13 has been shown to inhibit the production of various proinflammatory mediators from activated monocytes [1]. Thus, the down-regulation of IL-13 by PDE4 inhibitors could enhance allergic inflammation. However, allergic inflammation is characterized by the generation of IgE; the two cytokines critical for class switching to IgE are IL-4 and IL-13. By down-regulating IL-13 in situations where IL-4 production remains high, PDE4 inhibitors may exhibit anti-inflammatory efficacy. The presence of increased IL-13 gene expression and protein production in allergen-challenged bronchoalveolar lavage samples from patients with asthma would support this hypothesis [9]. Furthermore, data from our laboratory employing selective treatment of cell populations with PDE4 inhibitors suggest that the target cells for this effect are the responder lymphocytes and not the antigen-presenting cells [24]. Thus, the hypothesis that PDE4 inhibitors could negate anti-inflammatory effects of IL-13 that are relevant in this system is probably not tenable.

We have taken a number of precautions in our methodology to ensure the reliability of our data. With respect to proliferation, the time interval for incubation has been optimized to maximize the signal/noise ratio. The phenotypic profiles of the T cell clones used in these experiments have been reanalyzed over the course of 6 months; their profiles have remained constant, precluding the effects of cellular differentiation events on these data. Replicate experiments using the same clone over a 3-month period have yielded highly reproducible results. With regard to the RT-PCR assay, we have continued to use a multi-step normalization process, as previously described, to ensure that valid quantitative comparisons may be made between culture conditions on a "per cell" basis. The 12-hr culture interval precludes the effect of cellular proliferation on comparative cytokine gene expression. The use of subsaturating cycle numbers in the PCR assay assures the validity of our comparative data. The close correlation of gene expression with cytokine protein secretion supports this contention. Finally, the viability of cultured cells in the presence of PDE inhibitors at concentrations up to 10^{-4} M was >99%; after

10 days of culture with PDE inhibitors, T cell clones may be restimulated with antigen without any change in phenotype or response characteristics, arguing against the possibility of drug-induced T cell evolution.

The precise mechanism by which inhibition of PDE4 down-regulates IL-13 is unknown. The specificity of PDE4 inhibitors suggests that their effects are mediated through elevations of intracellular cAMP; the ability of cAMP agonists to modulate T cell function is well described [15]. Through direct measurements of intracellular cAMP, we have correlated PDE4 inhibitor concentrations to functional alterations in T cell clones and cAMP concentrations (data not shown). Subsequent studies from our laboratory will address downstream signal transduction pathways related to elevated intracellular cAMP.

In conclusion, inhibition of the PDE4 isozyme in allergen-specific Th0, Th1, and Th2 clones resulted in significant down-regulation of IL-13 gene expression and protein secretion. Although an additive effect of PDE3 inhibition with PDE4 inhibition was apparent in the down-regulation of the proliferative response, no such increased efficacy was apparent on the generation of IL-13. The precise molecular mechanism for this effect remains to be elucidated.

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