Differential Regulation of Human Antigen-Specific Th1 and Th2 Lymphocyte Responses by Isozyme Selective Cyclic Nucleotide Phosphodiesterase Inhibitors

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ABSTRACT

Our study explores the relative efficacy of phosphodiesterase (PDE) inhibitors on antigen-specific Th1 and Th2 clonal responses. Proliferative responses for both phenotypes were down-regulated by the PDE4 inhibitor, rolipram, but not the PDE3 inhibitor, siguazodan. The Th2 clones were more sensitive than the Th1 clones to PDE4 inhibition (P < .05 at 10 and 100 μM rolipram). The addition of 1 μM of the adenylyl cyclase activator, isoproterenol, significantly decreased both the EC50 and IC50 of rolipram in both phenotypes (P < .05). Gene expression for interleukin-4, interleukin-5, or interferon-γ, assessed by reverse transcription-polymerase chain reaction, was down-regulated by the PDE4 inhibitor, but not the PDE3 inhibitor, in each respective clone. Cytokine protein secretion paralleled the results of reverse transcription-polymerase chain reaction for IL-4 and interferon-γ (P < .01 for each). No differential efficacy on cytokine generation parameters between T helper phenotypes was apparent. Rolipram treatment significantly elevated intracellular cyclic AMP (adenosine 3',5'-cyclic monophosphate) in clonal T cells (P < .01 for Th1 or Th2 clones); these elevations were consistently greater in the Th2 clones (P < .05). Finally, Th1 cells showed reduced gene expression for the PDE4C isofom and a lack of gene expression for the PDE4D isofom by reverse transcription-polymerase chain reaction, compared to the Th2 cells. These data demonstrate the potent immunomodulatory efficacy of PDE4 inhibition on antigen-specific T cell clones. The enhanced sensitivity of Th2 to PDE4 inhibition may be due, in part, to the differential expression of PDE4 isofoms between Th1 and Th2 cells.

The existence of distinct Th1 and Th2 phenotypes, defined on the basis of specific cytokine generation, is well established in the murine system; a large body of data have accumulated to support a similar distinction in humans (Mosmann et al., 1986; Romagnani, 1991; Wierenga et al., 1991). While Th2 cells produce IL-4, IL-5 and IL-10, Th1 cells produce IFN-γ and tumor necrosis factor-β. Such phenotypic specificity plays an important role in both protective and pathologic immune responses (Paul and Seder, 1994). While Th1-mediated responses confer protection from intracellular parasites and increase the severity of certain autoimmune diseases, Th2-mediated responses govern both protection from certain extracellular pathogens and the inflammation characteristic of allergic disease. Despite these phenotypic and functional differences, specific regulatory differences between Th1 and Th2 cells have been difficult to establish at a molecular level. Among the candidates for such molecular regulators are CD30 (Del Prete et al., 1995a and b), specific chains of the IFNγ receptor (Pernis et al., 1995) and intracellular cAMP (Novak and Rothenberg, 1990; Gajewski et al., 1990). Pertaining to cAMP, studies performed in mice have provided evidence for both increased resting levels of intracellular cAMP in Th2-like cells, as well as an enhanced sensitivity of lymphokine production to down-regulation by cAMP-elevating agents in Th1 cells. To date, similar studies in antigen-specific human Th1 and Th2 cells have not been performed.

The steady-state, intracellular level of cAMP is controlled predominantly by PDE. This superfamily of enzymes is comprised of seven distinct families, characterized on the basis of substrate specificity, inhibitor sensitivity and sequence homology (Beavo et al., 1994). Two of these families, PDE3 and PDE4, are primary constituents of lymphocytes (Essayan and Lichtenstein, 1994). Previous studies from our laboratory have documented the ability of PDE4 inhibitors to down-regulate antigen-driven proliferation and cytokine gene expression in peripheral blood mononuclear cells (Essayan et

ABBREVIATIONS: PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; PDE, cyclic nucleotide phosphodiesterase; RT, reverse transcription; RW, ragweed; APC, antigen-presenting cells; IL, interleukin; INF, interferon; cAMP, adenosine 3',5'-cyclic monophosphate; EIA, enzyme immunoassay; Th, T helper; PKA, protein kinase A;
al., 1994, 1995). The potential for differential regulation of PDE4 isoforms in Th1 and Th2 cells has been suggested (Essayan et al., 1995; Engels et al., 1994); however, to date, studies to directly correlate intracellular cAMP, PDE4 isoform expression and functional parameters in antigen-specific Th1 and Th2 clones have not been performed.

In this report, we delineate the cAMP-dependent modulation of antigen-driven proliferation, cytokine gene expression and cytokine protein production in a set of Amb a 1- (a major allergen of short RW, Ambrosia artemisiifolia) specific human T cell clones that have been previously characterized as Th0, Th1 or Th2 (Essayan et al., 1996). Data are included on the modulation of intracellular cAMP by PDE inhibitors and by an adenylyl cyclase activator, as well as assessment of PDE4 isoform composition in the Th1 and Th2 clones.

Methods

Derivation of antigen-specific T lymphocyte clones. The derivation of the antigen-specific T cell clones used in these experiments has been described (Essayan et al., 1996; Huang et al., 1996). Briefly, PBMCs from an atopic asthmatic subject with epicutaneous presence of short RW antigen (10^6 Ci of [3H]-thymidine for an additional 20 hr, the PDE3 inhibitor (siquazodan), the PDE4 inhibitor (rolipram) or both for the entire culture period. Again, no exogenous cytokine was used. The cells were preincubated with drug for 90 min immediately before the addition of antigen and further culture for 12 hr. Cultures for PDE4 isoform gene expression contained 3 x 10^6 clonal T cells. To obtain data specific to the designated T cell phenotype, no APCs were used in these cultures; since the lack of APCs obviated the use of antigen, PHA (5 μg/ml) was used to activate the clonal T cells. Cells were incubated for 12 hr in the absence or presence of PHA in slanted 14 ml polypropylene tubes.

After the incubation step, the cultured cells were pelleted and washed; total cellular RNA was isolated using the RNXol method (Tel-Test, Inc., Friendswood, TX) according to the manufacturer’s instructions. Diethylpyrocarbonate-treated water without sodium dodecyl sulfate was used for the final resuspension. Strict RNase-free conditions were maintained throughout the experiments. The RNA was stored at -70°C. Normalization of RNA to approximately 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. A_{260/280} values > 1.7 were uniformly obtained. RT-PCR was performed with 5 mM magnesium and oligo dT priming, using standard reagents (Perkin-Elmer Cetus, Norwalk, CT) and cytokine- and PDE4-specific primer pairs designed in our laboratory and made at the DNA Core Facility of the Johns Hopkins University (table 1). All PCR products were visualized by ethidium bromide-stained gel electrophoresis and photographed.

Cytokine protein secretion assays. Cytokine protein secretion was assessed by enzyme linked immunosorbant assay (Biosource, Int., Camarillo, CA) according to the manufacturer’s instructions. Quantititation was achieved using the WHO standard provided by the company. Briefly, duplicate cultures to those used in the cytokine gene expression experiments were constructed and incubated for 12 hr. Supernatants from these cultures were harvested, and cellular debris was removed by centrifugation. Supernatants were stored at -20°C until assayed. Dilutions of samples, when necessary, were performed in culture medium. All standards and samples were tested in duplicate. Most samples were tested at two different dilutions and compared for internal consistency.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
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<tr>
<td>IL-4</td>
<td>5’-ATGGGCTCTCACCTCCCAACTGCT 3’-GTTTCTTCAAGCTCCTTTGTCG</td>
</tr>
<tr>
<td>IL-5</td>
<td>5’-GCTTCCTGATTTGAGTTGCTAGCT 3’-TGCCCCTGATTTTGTTTATTAG</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5’-ATGAAATATACAGTTATACCTGGTTT 3’-GATCGTCTGATCTGGAAACAGCAT</td>
</tr>
<tr>
<td>β Actin</td>
<td>5’-TGACGGGGTCACCCACACTGTGCCCATCTAGG 3’-CTAGAAGCATTGCGGGTGGACGATGGAGGG</td>
</tr>
<tr>
<td>PDE4A</td>
<td>5’-ACACGCTGAACACTGCTGCAAGTCTA 3’-CTAGAAGCATTGCGGGTGGACGATGGAGGG</td>
</tr>
<tr>
<td>PDE4B</td>
<td>5’-AGCTCATGACCCCAACTGTGCCCATCTA 3’-ATAACCATCTTCCTGAGTGTC</td>
</tr>
<tr>
<td>PDE4C</td>
<td>5’-TCGACAACCAAGAAGCTTGAAGCAGT 3’-GGATAAGGACCAGGAAAGAG</td>
</tr>
<tr>
<td>PDE4D</td>
<td>5’-CGGAGACGTGGATGTTGGTGC 3’-CGTCTCGAAACCATGTTGTC</td>
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TABLE 1
Cytokine-, β actin- and PDE4-specific RT-PCR primers
cAMP assays. Intracellular cAMP levels were assessed by EIA (Amersham Corporation, Arlington Heights, IL) according to the manufacturer's instructions. Quantitation was achieved using the nonacylated standard provided by the company. Again, to obtain data specific to the designated T cell phenotype, no APCs were used in these cultures. Briefly, 5 × 10^6 clonal T cells were incubated with rolipram (10^{-5} M) for 90 min, with or without a terminal 5 min incubation with isoproterenol (10^{-6} M). Untreated aliquots of the T cell clones and aliquots treated with siguazodan (10^{-6} M) were studied for comparison. On completion of the drug incubation step, the reactions were quenched with ethanol at -20°C. Particulate matter was removed by centrifugation and washed with 65% ethanol; the two ethanol-containing supernatants were combined, dried and re-suspended in the assay buffer provided in the EIA kit. The samples were stored at -70°C until assayed.

Statistical analysis. Mean and standard error values, as well as t test comparisons, were derived using StatView (BrainPower, Inc., Calabasas, CA) on a Macintosh PowerBook 145B computer. The t tests were paired, two-tailed. Proliferation data are depicted as percent inhibition for each condition, calculated based on inhibition relative to stimulated, drug-free mean counts, subtracted in every case for background counts with media alone. ELISA and EIA data are presented in standard units. IC_{50} values represent the concentration of drug at 50% inhibition; EC_{50} values represent the concentration of drug at 50% efficacy.

Results

Proliferation assays. Figure 1 depicts the percent inhibition of proliferation achieved with rolipram alone (PDE4 inhibitor), siguazodan alone (PDE3 inhibitor) and rolipram in the presence of 10^{-5} M siguazodan from multiple T cell clones assessed on several occasions, segregated by T cell phenotype; solubility of the compounds precluded the use of higher concentrations. Although a modest degree of independent efficacy was evident with the use of siguazodan in the Th1 clones, no independent efficacy was evident with this agent in the Th2 clones. Rolipram showed marked independent efficacy in down-regulating the antigen-driven proliferative response of both Th1 and Th2 clones; however, both IC_{50} and EC_{50} values were 4-fold greater in the Th1 clones than the Th2 clones (IC_{50} = 30 μM vs. 8 μM; EC_{50} = 10 μM vs. 2 μM; P < .05). Both Th1 and Th2 clones showed a modest but statistically significant additive efficacy of 10^{-5} M siguazodan with rolipram at 10^{-5} and 10^{-4} M (P < .05 for each). Th0 clones showed a similar pattern of drug efficacy, but their proliferative responses were midway in magnitude between those of the Th1 and Th2 clones (data not shown).

Figure 2 depicts the percent inhibition of proliferation achieved with rolipram alone, isoproterenol (an adenylyl cyclase activator) alone and rolipram in the presence of 10^{-6} M isoproterenol from multiple T cell clones assessed on several occasions, segregated by T cell phenotype. Although a minimal degree of independent efficacy was evident with the use of isoproterenol in the Th1 clone, no independent efficacy was evident with this agent in the Th2 clone up to a concentration of 10^{-5} M; toxicity (cellular pyknosis with the inability to exclude trypan blue) was evident at 10^{-4} M isoproterenol. However, the addition of 10^{-6} M isoproterenol with rolipram caused a significant increase in the potency of rolipram, evidenced by a left shift of the rolipram dose response curve and a decrease of both the EC_{50} and IC_{50} values (P < .05).

Although this effect was evident for both Th1 and Th2 clones individually, no differential efficacy of isoproterenol with rolipram between the clones was evident (data not shown). These data support the conclusion that rolipram achieves its efficacy through the elevation of intracellular cAMP.

In both figures 1 and 2, the percent inhibition with the lowest doses of siguazodan and isoproterenol tended to be less than zero; this is an interesting observation for which we have no firm explanation. As the concentrations of various drugs used in this system drop below their ED_{50} values, we often see increases in the S.E. of the measurements as well as percentage inhibition that drops below zero (enhancement). This may be due simply to low level, nonspecific cellular activation from manipulation of the cells. This effect shows no dose dependence; it rarely reaches statistical significance (data not shown).

Cytokine gene expression. Figure 3 shows the β actin- and cytokine-specific RT-PCR amplification products from a representative study of Th1 and Th2 clones cultured with antigen and APCs in the absence or presence of 10^{-5} M rolipram and/or 10^{-6} M siguazodan. Doses of drugs were selected based on efficacy in preliminary dose-response studies (10^{-7}-10^{-4} M, data not shown); these concentrations gave consistent results although remaining within the range of
selectivity for the individual isozymes. Data from the Th2 clone are shown in the top three rows, whereas data from the Th1 clone are shown in the fourth and fifth rows. Resting clonal cells cultured with APCs in the absence of antigen did not express message for proinflammatory cytokines (data not shown). Adequate normalization of RNA was confirmed by the equality of RT-PCR amplification products at subsaturating cycle number for $\beta$-actin gene expression (first and fourth rows for Th2 and Th1 clones, respectively). Exposure to $10^{-2}$ M rolipram (second column) caused a clear down-regulation of gene expression for IL-4, IL-5 (Th2 clone, second and third rows) and IFN-$\gamma$ (Th1 clone, fifth row) when compared to the drug-free condition (first column). Siguazodan showed no independent efficacy in down-regulating gene expression of proinflammatory cytokines (third column). Finally, in contrast to the proliferative response data, no additional efficacy of siguazodan with rolipram was evident (fourth column). These data confirm and extend our previous findings in PBMCs (Essayan et al., 1995).

**Cytokine protein secretion.** Figure 4 depict the amounts of IL-4 and IFN-$\gamma$ secreted from Th0, Th1 and Th2 clones cultured with antigen and APCs in the absence or presence of $10^{-5}$ M rolipram and/or $10^{-5}$ M siguazodan. In both figures, the same individual T cell clones are depicted by the same symbols; Th0 clones are in gray although the Th1 clones are in black and the Th2 clones are in white. The accuracy of the individual values for a clone was confirmed by both duplicate culture experiments as well as replicate ELISA assays at different dilutions of the culture supernatants (data not shown). A clear distinction between Th1 and Th2 clones was evident in the cytokine secretion patterns. Culture with $10^{-2}$ M rolipram, with or without $10^{-5}$ M siguazodan, produced a significant down-regulation of both IL-4 secretion (2048 + 6481 vs. 588 ± 127 or 853 ± 207 pg/ml; P < .01) and IFN-$\gamma$ secretion (1938 ± 608 vs. 198 ± 68 or 238 ± 91 pg/ml; P < .01). Siguazodan as a single agent was ineffective in down-regulating IL-4 or IFN-$\gamma$ production (2350 ± 591 and 2084 ± 665 pg/ml, respectively); no significant additional efficacy of siguazodan with rolipram was evident. These results are analogous to the results of cytokine gene expression discussed above.
cAMP assays. Figure 5 depicts the levels of intracellular cAMP in Th1 and Th2 clones cultured in the absence of antigen and APCs, using $10^{-5}$ M rolipram and/or $10^{-6}$ M isoproterenol to modulate cAMP levels; results with $10^{-6}$ M siguazodan are shown for comparison. The resting levels of cAMP were essentially identical in the Th1 and Th2 clones (985 ± 57 and 975 ± 54 fmol/10⁶ cells, respectively). Exposure to rolipram or isoproterenol individually caused significant increases in intracellular cAMP in both Th phenotypes ($P < .01$); these values are in close agreement with those obtained from purified peripheral CD4⁺ T cells (Giembycz et al., 1996). Although additive efficacy was evident between isoproterenol and rolipram in the Th1 clone, no such effect was evident in the Th2 clone ($P > .01$ and NS, respectively). Moreover, the Th2 cells were consistently more sensitive to rolipram or isoproterenol as single agents than the Th1 cells, suggesting a mechanistic difference in cAMP regulation between the two lymphocyte subtypes ($P < .05$ for rolipram or isoproterenol). Siguazodan caused significant and equivalent elevations of intracellular cAMP in both Th1 and Th2 clones.

PDE gene expression. Figure 6 shows the β actin- and PDE4-specific RT-PCR amplification products from a representative study of Th1 and Th2 clones cultured without APCs in the absence or presence of 5 µg/ml PHA (for cellular activation in the absence of APCs). Adequate normalization of RNA was confirmed by the equality of RT-PCR amplification products at subsaturating cycle number for β actin gene expression (first row). Cellular activation by PHA neither enhanced nor diminished gene expression for any of the PDE4 isoforms in either T cell subset. Although identical levels of gene expression for PDE4A and PDE4B were seen in the Th1 and Th2 clones, a consistently lower level of gene expression for PDE4C and a lack of gene expression for PDE4D was evident in the Th1 cells when compared by RT-PCR to the Th2 cells.

Discussion

We have reported the differential expression of PDE4 isoforms in Th1 and Th2 cells, and provided evidence for the differential efficacy of a PDE4 inhibitor between these T cell phenotypes. These data suggest that Th1 and Th2 clones maintain equivalent steady-state intracellular concentrations of cAMP, but that the Th2 clones exhibit a more marked elevation in cAMP in response to treatment with a PDE4 inhibitor. The functional significance of this finding is that an augmented sensitivity of antigen-driven proliferative responses to treatment with a PDE4 inhibitor exists; the relative excess of PDE4C and specific presence of PDE4D may confer this enhanced sensitivity. Thus, the differential regulation of intracellular cAMP, via the specific use of PDE4 isoforms, may represent an important molecular difference between Th1 and Th2 phenotypes.

A comparison of these data with those of Novak and Rothenberg (1990) provides additional insights into the dif-
to inhibition by 8-Br-cAMP (an analogue of cAMP), compared to its effect on IL-4 production by Th2 clones. However, the converse was observed with IL-2-driven proliferation of Th1 and Th2 clones: The proliferative response of the Th2 clones was 10-fold more sensitive than that of the Th1 clones to inhibition by 8-Br-cAMP (8-bromoadenosine 3',5'-cyclic monophosphate). These observations are consistent with our data in human, antigen-driven T cell clones. Regarding proliferative responses, our Th2 clones were 4-fold more sensitive to PDE4 inhibition than were our Th1 clones. Although there was a suggestion in the ELISA studies that rolipram-induced greater suppression of IFN-γ (in the Th1 clones) than of IL-4 (in the Th2 clones), study design and low sample numbers preclude adequate comparative statistical analysis.

A number of interesting conclusions may be drawn when comparing these current data to our previous studies of PDE4 inhibition in antigen-driven human PBMCs (Essayan et al., 1994, 1995). The current clonal data confirm our earlier finding of a differential response to PDE4 inhibitors between PBMCs stimulated with a Th1-promoting antigen (tetanus toxoid, TT) and a Th2-promoting antigen (RW extract, RW); a relative resistance to PDE4 inhibition was seen in the tetanus toxoid-driven PBMCs compared to the RW-driven PBMCs (Essayan et al., 1994). In that study, the PDE3 inhibitor was ineffective in down-regulating antigen-driven proliferative responses. A subsequent study of cytokine gene expression in RW- and TT-driven PBMCs treated with PDE inhibitors revealed significant down-regulation of IL-5 and IFN-γ by rolipram (Essayan et al., 1995); a significant contribution of Th0 cells to the antigen-driven response of PBMCs was shown, consistent with the frequency of the various T cell subsets in cloning experiments from our laboratory (Essayan et al., 1996). Valid analysis of cAMP levels in that study was not possible due to the mixed cell design. Evidence for differential expression of PDE4 isoforms in a variety of immune cell lines, including T cells, B cells and basophils, was also presented (Essayan et al., 1995). Specifically, although the HLA-DR2.2-restricted, Amb a 5-specific Th2 cell line, AP.1, expressed mRNA for both PDE4A and PDE4B, the Th1-like Jurkat cell line did not express PDE4A; this last finding has recently been confirmed by others (Engels et al., 1994). However, the current data suggest that the differences in PDE4 gene expression in nontransformed, antigen-specific T cell clones actually occur only in the PDE4C and PDE4D isoforms. Thus, the potential for selective efficacy of PDE4 inhibitors on Th2 cells may positively affect the development of inhibitors selective for the PDE4C and PDE4D isoforms. Finally, the involvement of PDE4 isoforms in the genotype of Jurkat cells, viewed in the context of studies showing modulation of tumor cell growth and differentiation by PDE4 inhibitors, raises the possibility of primary or secondary aberrations of PDE4 isoforms in carcinogenesis (Drees et al., 1993).

The lack of efficacy of siguazodan in these and our previous experiments raises questions about the expression of PDE3 isoforms in T cells and the bioactivity of this particular PDE3 inhibitor. Interestingly, both T cell and PBMC lysates demonstrate a PDE3 peak by diethylaminoethyl-Sepharose column separation that is inhibited by siguazodan (Essayan et al., 1994; Robicsek et al., 1991). However, we have been unable to detect the human cardiac PDE3 by RT-PCR in human T cells (data not shown); other human PDE3 isoforms have been observed in two replicate experiments.

Fig. 6. Resting and stimulated PDE4 isoform gene expression in Th1 and Th2 clones. Representative results of RT-PCR products for the four known isoforms of the PDE4 are shown for a Th1 clone and a Th2 clone, with and without stimulation by PHA, all in the presence of APCs. Normalization by equivalent β-actin gene expression at subsaturating cycle number is depicted for each condition in the first row. The DNA size marker is shown in the fifth column. Four individual clones were used in two replicate experiments.

Gajewski et al. (1990) have also studied cAMP-mediated effects in antigen-specific murine T cell clones. In Th1 clones, they observed a 30-fold greater sensitivity of IL-2 production

Differential regulation of T cell phenotypes by intracellular cAMP. These investigators, working in the murine system, found higher baseline levels of cAMP in the Th2 clones, D10.G4.1 and CDC-25, than in the Th1 clone, A.E7. However, in these experiments cAMP levels were assessed on day 7 after restimulation, and these data did not differ from those derived at day 4 after restimulation, when cellular activation should still be near maximal. This lack of difference between days 4 and 7 suggests that the cell populations studied by Novak and Rothenberg (1990) were not composed of resting cells. Studies from our laboratory indicate that D10.G4.1 cells may not return fully to a resting state for 10 to 14 days after antigen stimulation (data not shown). Thus the cAMP levels reported in the study of Novak and Rothenberg (1990) are likely to represent stimulated values; under their conditions, higher cAMP levels in the Th2 clones might be expected. However, our data suggest that resting human Th1 and Th2 cells show no differences in baseline intracellular cAMP levels.

The current studies demonstrate a PDE3 peak by diethylaminoethyl-Sepharose column separation that is inhibited by siguazodan (Essayan et al., 1994; Robicsek et al., 1991). However, we have been unable to detect the human cardiac PDE3 by RT-PCR in human T cells (data not shown); other human PDE3 isoforms...
have not been cloned. Thus, although PDE3 clearly is expressed in human T cells, further molecular analysis cannot be performed at this time. Functionally, treatment of human Th1 or Th2 clones with siguazadon $10^{-6}$ M resulted in a significant elevation of intracellular cAMP, implicating either compartmentalization of PDEs or cGMP-dependence as key modulators of the efficacy of PDE3 inhibitors during T cell activation. Finally, we have used three additional PDE4 inhibitors, each of which is known to bind its intracellular target and to inhibit cAMP hydrolysis in cell lysates. Each of these inhibitors has shown a lack of efficacy in modulating proliferation and cytokine generation from PBMCs (Essayan et al., 1994b). Thus, although PDE3 inhibitors have their predicted biochemical effect in human T cells, their lack of efficacy in modulating proliferation and cytokine generation is a consistent finding.

We have taken a number of precautions in our methodology to ensure the reliability of our results. First, the time interval for incubation of our proliferation assays has been optimized to maximize the signal/noise ratio. Second, the phenotypic profiles of the T cell clones used in these experiments have remained constant through repeated analyses over the course of 6 mo, precluding the effects of cellular differentiation events on these data. Third, replicate experiments using the same clone over a 3-mo period have yielded nearly identical results. Fourth, we have continued to use a complex, multi-step normalization process with our RT-PCR assay, as previously described, to ensure valid quantitative comparisons between culture conditions; the 12-hr culture interval precludes the effect of cellular proliferation on comparative cytokine gene expression. Fifth, the possibility of cellular senescence as an explanation of our findings is negated by $>99\%$ cell viability by trypan blue exclusion at the beginning and end of the culture period. Moreover, cells cultured in the absence or presence of PDE4 inhibitors restimulated with antigen, after drug removal, exhibit responses independent of prior drug treatment. Finally, the meticulous use of subsaturating cycle numbers in the PCR assays assures the validity of the comparative data. The close correlation of gene expression with cytokine protein secretion supports this conclusion.

Although the differential regulation of two PDE4 isoforms between Th1 and Th2 clones is not surprising, a significant difference in the sensitivity of these two cell phenotypes to PDE4 inhibition might not have been predicted. Because rolipram is believed to have no isoform selectivity, a difference due to the specific inhibitor is unlikely. However, a number of other mechanisms could account for this finding. One hypothesis is that PDE4C and PDE4D quantitatively constitute the predominant isoforms in Th2 cells; unfortunately, precise quantitation of PDE4 isoforms in lymphocytes has not been performed. A second hypothesis might invoke functional compartmentalization of PDE4 isoforms, with the C and D isoforms controlling proliferation and possibly cytokine generation. Because the treatment of intact, resting Th2 cells with rolipram induced a more marked elevation of cAMP than did treatment of intact, resting Th1 cells, our data appear to lend more support to the first hypothesis. Clarification of these issues must await improved techniques for PDE4 isoform isolation and quantitation.

Our data also support the differential regulation of intracellular cAMP in T cell subsets. However, the precise mechanisms by which elevations of intracellular cAMP downregulate immune responses remain unclear. Increases in intracellular cAMP may selectively inhibit Ras/Raf-1 binding through hyperphosphorylation of Raf-1 on serine 43; however, the potential role of PKA isoforms in this model has not been clarified (Wu et al., 1993; Cook and McCormick, 1993). Type I PKA has been shown to interact with the T cell receptor-CD3 complex, but specific consequences of this interaction are unknown (Skalhegg et al., 1994). Finally, elevations of intracellular cAMP, acting through PKA, have been shown to disrupt multiple promoter-enhancer interactions; however, the involvement of specific isoforms of PKA in this model has also not been proven (Chen and Rothenberg, 1994). An improved understanding of the precise pathways involved in cAMP-mediated signal transduction will help to define the potential for differential regulation of downstream effectors between T cell subsets and identify potential new targets for pharmacologic regulation of immune responses.

In conclusion, we provide the first documentation in human Th1 and Th2 clones of the differential regulation of cAMP, associated with differential expression of the PDE4C and PDE4D isoforms. The increased sensitivity of Th2 cells, compared to Th1 cells, to PDE4 inhibition suggests the potential to target treatment to specific T cell phenotypes.

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