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Differential efficacy of lymphocyte- and monocyte-selective pretreatment with a type 4 phosphodiesterase inhibitor on antigen-driven proliferation and cytokine gene expression

David M. Essayan, MD [[MEDLINE LOOKUP](#)]

Shau-Ku Huang, PhD [[MEDLINE LOOKUP](#)]

Anne Kagey-Sobotka, PhD [[MEDLINE LOOKUP](#)]

Lawrence M. Lichtenstein, MD, PhD [[MEDLINE LOOKUP](#)]

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Abstract

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Elevations of intracellular cyclic AMP, achieved with the use of phosphodiesterase (PDE) inhibitors, cause functional downregulation of most inflammatory cells. Rolipram, an inhibitor selective for the PDE4 isozyme, can markedly downregulate antigen-driven proliferation and cytokine gene expression of unfractionated human peripheral blood mononuclear cells. However, it is unclear

whether PDE4 inhibitors in a mixed-cell system exert their immunosuppressive effect on the lymphocyte or on the monocyte fraction. We have used an adherence-based protocol for separating peripheral blood mononuclear cells, isolated from atopic individuals, into lymphocyte and monocyte fractions and have selectively treated these populations with rolipram prior to reconstituting the cell cultures to their original lymphocyte/monocyte proportions. Cellular responses to both ragweed and tetanus toxoid were analyzed for both proliferation and gene expression of proinflammatory cytokines. A dose-dependent downregulation of ragweed- and tetanus toxoid-driven proliferative responses was achieved by pretreatment of lymphocytes from peripheral blood with rolipram. This downregulation was significantly greater than that achieved with pretreatment of monocytes. Pretreatment of both populations failed to show synergistic downregulation of proliferation. Lymphocyte pretreatment with rolipram also resulted in marked downregulation of gene expression for IL-4, IL-5, and interferon- γ compared to monocyte pretreatment in both ragweed- and tetanus toxoid-driven systems. Interestingly, monocyte pretreatment in these systems resulted in significant downregulation of IL-2 gene expression compared to lymphocyte pretreatment. Flow cytometric analysis failed to show alterations in any of a panel of surface activation and signal transducing molecules by rolipram treatment with or without antigen stimulation. We conclude that, in a mixed cell system, PDE4 inhibitors downregulate antigen-driven proliferation and gene expression of proinflammatory cytokines predominantly through their effects on lymphocytes rather than monocytes. (J Allergy Clin Immunol 1997;99:28-37.)

cAMP	Cyclic AMP
CM	Complete medium
DMSO	Dimethyl sulfoxide
IFN-γ	Interferon- γ
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
RT	Reverse transcription
RW	Ragweed extract
TT	Tetanus toxoid

(Click on a term to search this journal for other articles containing that term.)

Key Words: [Interleukin 4](#), [interleukin 5](#), [interferon- \$\gamma\$](#) , [T lymphocyte](#), [monocyte](#), [phosphodiesterase](#), [ragweed](#)

Cyclic nucleotide phosphodiesterases (PDEs) constitute the primary metabolic pathway for the key intracellular second messengers, cyclic AMP (cAMP) and cyclic GMP. Originally ascribed to the activity of a single enzyme, PDE activity is now attributed to a superfamily of isoenzymes comprising at least seven distinct families, differentiated biochemically on the basis of inhibitor

sensitivity, substrate specificity, allosteric modulation, and calcium/calmodulin dependence.^{1 2} At least three of these isozyme families consist of multiple distinct isoforms that may be subject to differential regulation. Highly selective tissue, cellular, and subcellular localization have made these isozymes targets for pharmacotherapy in diseases as diverse as asthma, cancer, and depression.³⁻⁵ In atopic patients, investigators have localized an elevated cAMP-specific PDE activity to monocytes; it has been suggested that this activity plays a primary role in the genesis of atopic disease and a dominant role relative to that of the lymphocytes in these patients.^{6 8}

The role of PDE inhibitors in the cAMP-mediated modulation of inflammatory responses has been the subject of intense research for more than two decades.^{9 10} Elevation in cAMP is generally felt to mediate downregulatory responses within the immune system. In T lymphocytes, elevation of intracellular cAMP inhibits the expression of both IL-2 and IL-2R.^{11 12} The effect on IL-2 expression may be induced by the use of nonselective as well as type 4 selective PDE inhibitors.^{13 14} In monocytes, PDE4 inhibitors have been shown to downregulate endotoxin-stimulated tumor necrosis factor- α production and calcium ionophore-induced leukotriene B₄ generation.^{15 17} In macrophages, zymosan-stimulated hydrogen peroxide production may be inhibited by treatment with a nonselective PDE inhibitor, and proliferation of macrophages in response to colony stimulating factor-1 may be inhibited by elevation of intracellular cAMP.^{18 19} Previous studies from our laboratory have examined the ability of PDE4 inhibitors to downregulate proliferative responses as well as gene expression for proinflammatory cytokines by using an antigen-driven, mixed cell system.^{20 21} However, the differential efficacy of PDE4 inhibitors on the monocyte and lymphocyte populations in an antigen-driven system remains unknown.

In this study we examine the relative contributions of PDE4 inhibition in these cell populations to antigen-driven proliferation and cytokine gene expression, by using a method for selective pretreatment of monocyte- and/or lymphocyte-enriched populations with the PDE4 inhibitor rolipram. We include data on alterations of cell-surface receptor populations in response to treatment with rolipram in the presence and absence of antigen.

Methods

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Materials

Disposable tissue culture ware, 96- and 24-well tissue culture plates, and 200 ml filter systems with 0.22 μ m nylon membranes were purchased from Corning Inc. (Corning, N.Y.). RPMI 1640 medium, penicillin G (5000 U/ml)/streptomycin (5000 μ g/ml), human AB serum, and Dulbecco's phosphate-buffered saline were purchased from Life Technologies, Inc. (Gaithersburg, Md.). Ficoll/Paque was obtained from Pharmacia Inc. (Piscataway, N.J.). Filter units (0.22 μ m) were obtained from Millipore Corp. (Bedford, Mass.). Isopropanol, agarose type I, ethidium bromide, bovine serum albumin, paraformaldehyde, human IgG, mouse IgG1, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Phycoerythrin-conjugated polyclonal goat anti-mouse IgG was purchased from Biosource International (Camarillo, Calif.). NuSieve GTG and SeaKem ME were obtained from FMC BioProducts (Rockland, Maine). Chloroform was purchased from Fisher

Scientific (Pittsburgh, Pa.). Ethyl alcohol was obtained from Aldrich Chemical Co., (Milwaukee, Wi.). All cultures were kept in a water-jacketed incubator (Forma Scientific, Marietta, Ohio) with 5% CO₂ at 37° C. Tritiated thymidine (6.70 Ci/mmol) was obtained from Dupont NEN (Boston, Mass.). Cultures for proliferation were harvested on a PHD cell harvester onto glass fiber filter strips 240-1, both from Cambridge Technologies Inc. (Watertown, Mass.), and counted on a Beckman LS 5000 TD beta counter (Beckman Instruments Inc., Fullerton, Calif.) with Budget-Solve from RPI Corp. (Mount Prospect, Ill.).

Ragweed (RW) was a defatted, lyophilized preparation obtained through the courtesy of Dr. David Marsh, Johns Hopkins Asthma and Allergy Center, Baltimore. Tetanus toxoid (TT) was the generous gift of Dr. Ram Sriram, Vanderbilt University, Nashville, Tennessee. Racemic rolipram was the generous gift of Drs. Mary Barnette and Ted Torphy, Smith Kline Beecham, King of Prussia, Pennsylvania. Unconjugated primary antibodies against CD3, CD11a, CD14, CD25, CD29, CD49d, CD54, CD58, CD69, CD71, CD80, CD86, and HLA-DR were the generous gift of Dr. Bruce S. Bochner, Johns Hopkins Asthma and Allergy Center, Baltimore.

Studies involving gene expression utilized RNazol B, purchased from Tel-Test Inc. (Friendswood, Texas). Reverse transcription-polymerase chain reaction (RT-PCR) was performed on a thermal cycler (Model 9600) with the Gene-Amp kit, both from Perkin Elmer Cetus (Norwalk, Conn.). β actin- and cytokine-specific PCR primer pairs, designed in our laboratory and made at the DNA

Core Facility at Johns Hopkins University, were used as previously described.^{21 22} PCR products were visualized by ethidium bromide-stained gel electrophoresis on a Wide Mini Sub (Bio-Rad Laboratories, Hercules, Calif.) and photographed onto Polaroid 55 paper (Polaroid Corp., Cambridge, Mass.). 1X TBE was used as a running buffer. All reagents were carefully wrapped and autoclaved; RNase-free conditions were maintained at all times. Densitometry data were obtained with a densitometer (Molecular Dynamics, Sunnyvale, Calif.) running ImageQuant software according to manufacturer's instructions. Studies involving flow cytometry were performed on an EPICS Profile I flow cytometer (Coulter Inc., Hialeah, Fla.).

Selection of subjects

The subjects selected for participation were volunteers with allergy who were associated with the Johns Hopkins Asthma and Allergy Center. All subjects showed epicutaneous skin test sensitivity to a 100,000 AU stock of Short Ragweed Extract (ALK Laboratories Inc., Milford, Conn.) and had received TT immunizations within the preceding 2 years. All subjects reported upper and/or lower airway allergic symptoms that were attributable to ragweed. Subjects were not taking medications for the month preceding study; none of the subjects had undergone immunotherapy. All subjects were asked to abstain from the ingestion of methylxanthine-containing beverages on the morning of the study.

Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were prepared as previously described.²⁰ In brief, 50 ml of heparinized whole blood was diluted 1:1 with serum-free RPMI 1640 supplemented with 1% penicillin/streptomycin and overlaid in 25 ml partitions onto 10 ml Ficoll-Paque in 50 ml centrifuge tubes. These tubes were centrifuged at 800 × *g* at room temperature for 30 minutes. PBMCs were collected from the interface and washed twice in the serum-free medium. The cells were resuspended in RPMI 1640 with 5% human

AB serum (complete medium [CM]) in preparation for fractionation. Platelet contamination of these preparations was less than 1%; viability by trypan blue exclusion was uniformly greater than or equal to 99%.

For both proliferation and cytokine gene expression studies, adherent cell fractions were directly isolated on untreated culture plates by incubating the PBMCs for 2 hours, followed by gentle resuspension and removal of the nonadherent cell fractions to polypropylene tubes; this procedure had been previously optimized in our laboratory.²³ Nonadherent and/or adherent cell fractions from each culture well were then pretreated for 2 hours with rolipram or CM; adherent cells were pretreated in their respective culture plate wells, whereas nonadherent cells were pretreated in polypropylene tubes to minimize cellular adhesion and loss. The doses of rolipram were selected to enhance cellular penetration and prolong the duration of drug effect on the target population even after removal of the drug. After this preincubation, both cell fractions were washed free of drug, and individual culture conditions were reconstituted by overlaying individual nonadherent cell fractions onto their respective adherent cell fractions with antigen-containing CM. This protocol ensured both the specificity of the drug effect on a particular target cell population and the reconstitution of individual cultures to their original lymphocyte/monocyte ratio (usually approximately 2:1). Cell loss in the washing steps was assessed by counting the effluents in a hemocytometer; no significant cell loss was ever detected. The final cell density for proliferation assays was 2×10^5 cells/well in 96-well plates; cultures for cytokine gene expression were performed in 24-well plates at a cell density of 5×10^6 /well. The plates were incubated at 37° C with 5% CO₂.

Rolipram was dissolved in DMSO at a working concentration of 0.1 mol/L, diluted fresh with CM for each experiment, filtered through a 0.22 µm filter, and added to the various cultures to achieve final concentrations as designated. At no time did the final concentration of DMSO exceed 1:1000. Cultures were preincubated with drug for 2 hours prior to the addition of antigen; this 2-hour interval was previously found to be optimal⁹; preliminary studies failed to show increased efficacy of rolipram with prolonged preincubation. The final concentrations of antigens were 10 µg/ml for RW and 25 µg/ml for TT; these concentrations result in comparable levels of proliferation but are not saturating.

Proliferation assays

Assays were performed in triplicate for each condition as previously described.²⁰ Culture intervals were optimized for each stimulus in order to yield maximal stimulation indices. These intervals were 72 hours for RW and 96 hours for TT. Tritiated thymidine (1 µCi/well) was added 24 hours prior to harvest. The cell harvester and beta counter were used according to manufacturer's instructions. Each sample was counted for 1 minute.

Cultures for cytokine gene expression

Rolipram at 10^{-4} mol/L was used for all gene expression studies. Cultures for gene expression were harvested at 12 to 14 hours with vigorous resuspension, followed by a second resuspension with cold Dulbecco's phosphate-buffered saline in order to ensure that no cells remained adherent to the flask. These cells were then washed in Dulbecco's phosphate-buffered saline, pelleted, and stored at -70° C until RNA isolation was performed.

Isolation of RNA

Total cellular RNA was purified from the stored cell pellets by using the RNeasy RNeasy Lysis Buffer method according to the manufacturer's instructions. Diethylpyrocarbonate-treated water was used in the final resuspension of RNA. The concentration of RNA was determined by spectrophotometry and standardized to 100 ng/μl for each culture condition. The equivalence of this standardization was confirmed in two ways: (1) by direct visualization for integrity with RNA gel electrophoresis in ethidium bromide-stained 1.5% SeaKem ME and (2) by the equivalence of β actin PCR products for each culture condition, assayed at subsaturating cycle number. RNA was stored at -70° C until assayed. Typically, 5 × 10⁶ cells were processed for each condition, yielding approximately 5 μg of total cellular RNA. A_{260/280} values of greater than 1.7 were uniformly obtained.

Assay for gene expression

To assess each culture condition for each gene, 200 ng of RNA was used. Transcripts analyzed included the following: (1) β actin, (2) IL-4, (3) IL-5, (4) interferon-γ (IFN-γ), and (5) IL-2. RT was performed using the GeneAmp kit according to the manufacturer's instructions, with 5 mmol/L magnesium and oligo dT priming. The complementary DNA products were immediately amplified with the designated primer pairs (0.5 μmol/L/primer) and a subsaturating cycle number (35 cycles). The cycling profile was as follows: Denaturation at -95° C for 2 minutes; PCR at -95° C for 15 seconds, at 60° C for 15 seconds, and at 72° C for 30 seconds; final extension at -72° C for 10 minutes. The RT-PCR products were stored at -20° C until gel electrophoresis was performed in ethidium bromide-containing 2% NuSieve/1% Sigma agarose.

Flow cytometric analysis

Aliquots of PBMCs that were used for flow cytometric analysis were cultured for 12 hours under each of the following conditions: (1) medium alone, (2) medium with 10⁻⁵ mol/L rolipram, (3) Medium with 10 μg/ml ragweed, and (4) medium with both ragweed and roli-pram. Cells were harvested, resuspended in Dulbecco's phosphate buffered saline supplemented with 0.2% bovine serum albumin, and labeled with antibody as previously described.²⁴ In brief, 1 × 10⁶ cells were incubated in the dark with a saturating concentration of the primary antibody, washed twice, and then incubated with a saturating concentration of the conjugated secondary antibody for an additional 30 minutes prior to washing and fixation in 1% paraformaldehyde in Dulbecco's phosphate buffered saline. Cells were stored at 4° C until flow cytometric analysis.

Statistical analysis

Mean and standard error values for each triplicate set, as well as *t* test comparisons, were derived with StatView (BrainPower, Inc., Calabasas, Calif.) on a Macintosh PowerBook 145B computer. Probability values are paired, one-tailed. Percentage of inhibition for each condition was calculated on the basis of inhibition relative to stimulated, drug-free mean counts, corrected in each case for background counts with medium alone.

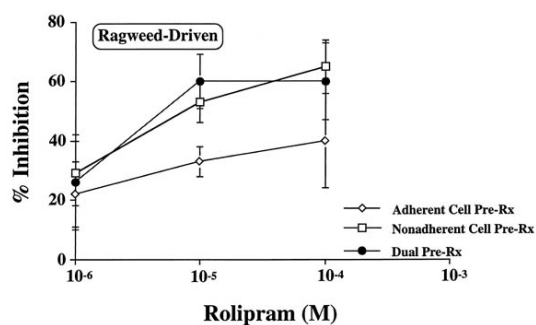
Cell separation

The purity of the adherent and nonadherent cell fractions was assessed by both flow cytometry and direct visualization following staining with Dif-Quick. The purity of lymphocytes in the nonadherent fraction was greater than 97% by both methods. Estimates of monocyte purity in the adherent cell fraction by flow cytometry was confounded by extensive cell disruption resulting from scraping of the cells for removal from plastic. Direct visualization following staining revealed a monocyte purity of approximately 99%. Viability by trypan blue exclusion at this step remained greater than 99%. Finally, responder cells previously cultured in the presence of rolipram were capable of antigen-specific restimulation with a profile similar to that of rolipram-naïve responder cells (data not shown).

Proliferative response

Fig. 1 depicts the percent inhibition of proliferation to RW at three different doses of rolipram for the culture conditions of adherent cell pretreatment alone, nonadherent cell pretreatment alone, and both adherent and nonadherent cell pretreatment (hereafter called dual pretreatment).

Fig. 1. Proliferative responses to ragweed. Results are shown for adherent, nonadherent, and dual pretreatment with rolipram at three concentrations. Data are presented as percentage inhibition $\pm$ SEM relative to stimulated, drug-free cultures (mean counts per minute, 10624 ± 1860), corrected for background with medium alone (mean counts per minute, 604 ± 140). $n = 4$.



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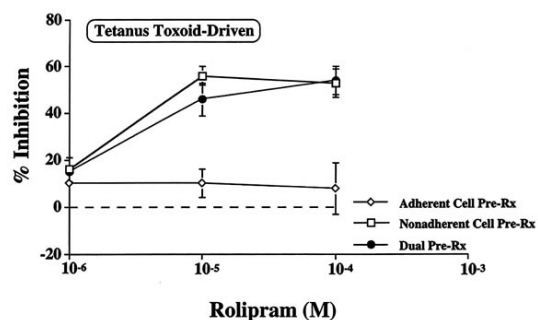
Percent inhibition was calculated based on the maximal proliferative response of drug-free cultures. Of note, the mean counts per minute of cultures undergoing the separation protocol did not differ from the mean counts per minute of unfractionated PBMCs in antigen-free, rolipram-free cultures (negative control), of antigen-stimulated, rolipram-free cultures (positive control), or of antigen-stimulated cultures in the presence of rolipram (data not shown). As depicted in Fig. 1 ■, selective pretreatment of the nonadherent cell fraction resulted in a dose-dependent inhibition of proliferation that was significantly greater than that achieved with selective pretreatment of the adherent cell fraction; this effect was evident at 10^{-5} and 10^{-4} mol/L ($p < 0.05$). This effect was lost at 10^{-6} mol/L,

consistent with a declining drug effect and a known IC_{50} for rolipram of 10^{-5} mol/L. The dose-response curves for the nonadherent cell pretreatment and dual pretreatment groups were essentially superimposable.

Several additional control experiments, not depicted graphically, deserve mention. First, proliferation and cytokine gene expression were unaltered by the presence of DMSO in concentrations of up to 1:500 when compared to CM alone; DMSO concentrations greater than 1:500 resulted in cell disruption. Second, the specificity of antigen stimulation was confirmed by a lack of proliferation and cytokine gene expression in cultured PBMCs from patients with allergy in the presence of an allergen (cat dander) to which they were not sensitive (determined by intradermal skin testing). Third, although responses to allergens are impossible to evaluate in nonatopic subjects, the antigen- and mitogen-driven (TT and phytohemagglutinin, respectively) sensitivity of nonatopic subjects to PDE4 inhibitors is similar to that of atopic subjects. Fourth, with respect to both proliferation and cytokine gene expression, culture kinetics were optimized in separate experiments; the incubation times used in this study reflect these data. Finally, the dose response for rolipram in a 2-hour pretreatment protocol is approximately 10-fold higher than that seen if rolipram remains in the culture for the entire 72 to 96 hours. The curves are parallel, however, and the equivalent treatment of both components of each culture condition makes comparative analysis valid.

Fig. 2 depicts similar data utilizing the PBMCs of the same individuals stimulated with TT.

Fig. 2. Proliferative responses to tetanus toxoid. Results are shown for adherent, nonadherent, and dual pretreatment with rolipram at three concentrations. Data are presented as percentage inhibition $\pm$ SEM relative to stimulated, drug-free cultures (mean counts per minute, 17025 ± 3925), corrected for background with medium alone (mean counts per minute, 1369 ± 294). $n = 4$.



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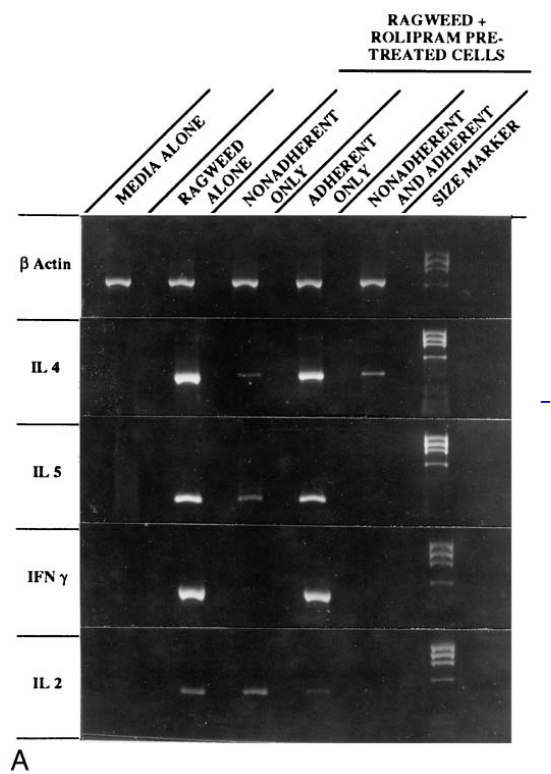
Once again, percent inhibition values, calculated as the percent decrease of the proliferative response relative to antigen-driven, drug-free controls corrected in each case for background values in the absence of antigen, are shown for three different concentrations of rolipram. The percent inhibition of proliferation obtained with nonadherent cell pretreatment is significantly greater than that achieved with adherent cell pretreatment at both 10^{-5} and 10^{-4} mol/L rolipram ($p < 0.003$), and

the nonadherent cell pretreatment and dual pretreatment dose response curves are nearly superimposable. This inhibitory effect is again lost at a concentration of 10⁻⁶ mol/L rolipram. Although a somewhat greater efficacy of rolipram pretreatment in the adherent cell populations in the RW-driven versus TT-driven systems was evident, this did not reach statistical significance (*p* > 0.06). Thus, it is apparent that the primary cellular target for the inhibition of the antigen-driven proliferative response by rolipram in a mixed cell system is the lymphocyte and not the monocyte.

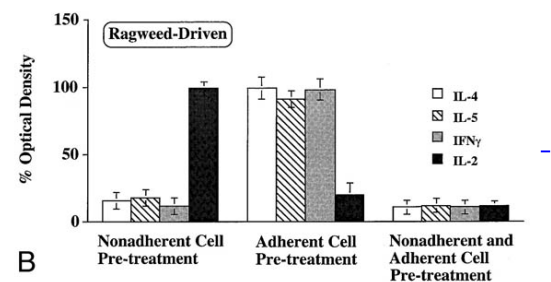
Cytokine gene expression

Fig. 3, A depicts representative results of RT-PCR for the proinflammatory cytokines IL-4, IL-5, IFN- γ , and IL-2 from the RW-driven PBMCs of subject 2.

Fig. 3. Cytokine gene expression of ragweed-driven PBMC cultures. **A**, Representative result from a single experiment in which PBMCs from an atopic subject were cultured. Normalization by β actin gene expression at subsaturating cycle number is depicted in the *top row*. RT-PCR products for IL-4 (*row 2*), IL-5 (*row 3*), IFN- γ (*row 4*), and IL-2 (*row 5*) are shown following subsaturating amplification of cultures under the conditions listed at the top of the figure. Unstimulated and drug-free control cultures are shown in *columns 1 and 2*, respectively. The DNA size marker is shown in *column 6*. **B**, Densitometry data are depicted for all subjects for each cytokine and each culture condition. The numbers represent percentage optical density $\pm$ SEM of PCR amplification product bands normalized to stimulated, drug-free controls and with background values subtracted. *n* = 4.



A



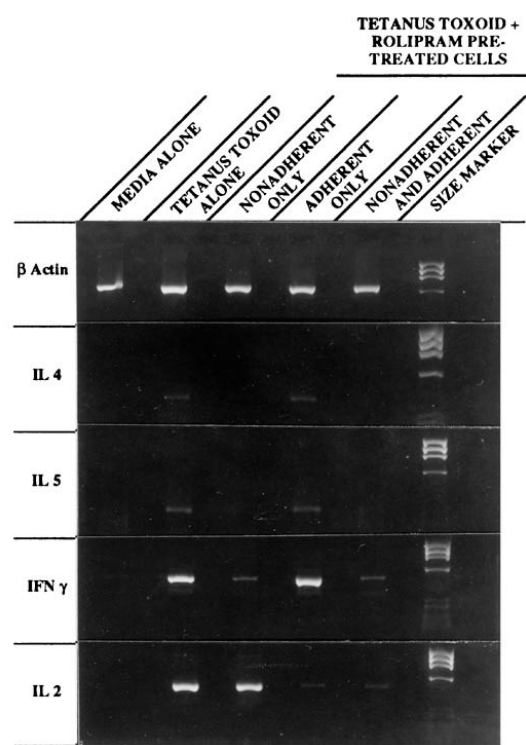
B

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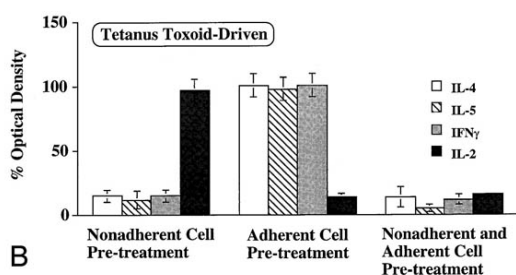
Successful normalization of messenger RNA for each of the culture conditions is evident from the equivalent band densities of the PCR products for the constitutive marker gene β actin, shown in the top row. The first two columns depict gene expression in fractionated cells in the absence of antigen and in RW-driven, fractionated cells under drug-free conditions, respectively. A marked downregulation of gene expression for IL-4, IL-5, and IFN- γ is apparent in both the nonadherent cell pretreatment and dual pretreatment groups ($p < 0.012$ and $p < 0.008$ for all of the cytokines in each group, respectively), once again implicating the lymphocyte as the primary cellular target for PDE4 inhibitors; pretreatment of the adherent cell population was ineffective in downregulating gene expression for these cytokines ($p > 0.3$). Interestingly, downregulation of IL-2 gene expression was consistently ascribable to the adherent cell pretreatment and dual pretreatment groups ($p < 0.01$ and $p < 0.002$, respectively); pretreatment of the nonadherent cell population was ineffective in downregulating gene expression for IL-2 ($p > 0.3$), thus implicating the monocyte as the primary cellular target for downregulation of IL-2 gene expression by PDE4 inhibitors. Finally, no consistent synergy was apparent for gene expression of any of the cytokines in the dual pretreatment population when compared to gene expression of the primary cellular target for that cytokine. A quantitative representation of these data, depicted as percent inhibition relative to RW-driven, drug-free controls, is shown in Fig. 3, B ■ for the entire group of four subjects.

Fig. 4, A shows representative results of RT-PCR for the same proinflammatory cytokines from the TT-driven PBMCs of the same subject.

Fig. 4. Cytokine gene expression of tetanus toxoid-driven PBMC cultures. **A**, Results from the tetanus toxoid portion of the same experiment as shown in Fig. 3. Normalization by β actin gene expression at subsaturating cycle number is depicted in the top row. RT-PCR products for IL-4 (row 2), IL-5 (row 3), IFN- γ (row 4), and IL-2 (row 5) are shown following subsaturating amplification of cultures under the conditions listed at the top of the figure. Unstimulated and drug-free control cultures are shown in columns 1 and 2, respectively. The DNA size marker is shown in column 6. **B**, Densitometry data are depicted for all subjects for each cytokine and each culture condition. The numbers represent percentage optical density $\pm$ SEM of PCR amplification product bands normalized to stimulated, drug-free controls and with background values subtracted. $n = 4$.



A



B

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Once again, successful normalization of messenger RNA for each of the culture conditions is evident from the equivalent band densities of the PCR products for the constitutive marker gene β actin, shown in the top row. The first two columns depict gene expression for control conditions analogous to those in Fig. 3, A. ■ When compared to the RW-driven study, an identical pattern of sensitivity for proinflammatory cytokine gene expression in both the adherent cell pretreatment population and the nonadherent cell pretreatment population is evident. Specifically, whereas IL-4, IL-5, and IFN- γ are downregulated exclusively in the nonadherent cell and dual pretreatment groups, IL-2 is downregulated exclusively in the adherent cell and dual pretreatment groups; no synergy in the dual pretreatment group is apparent. Once again, a quantitative representation of these data, depicted as percent inhibition relative to ragweed-driven, drug-free controls, is shown in Fig. 4, B ■ for the entire group of four subjects.

Surface marker expression

In an effort to discern whether the differential efficacy of PDE4 inhibitors on the lymphocytes was due to a cognate or a noncognate mechanism, the modulation of expression of a battery of cell-surface costimulatory molecules was assessed on both the lymphocytes and the monocytes. The effects of ragweed and rolipram treatment, individually and in combination, were investigated using PBMCs and monocytes purified by elutriation. No differences in the density of surface expression of any of the following molecules were noted: CD3, CD11a, CD14, CD25, CD29, CD49d, CD54, CD58, CD69, CD71, CD80, CD86, and HLA-DR.

Discussion

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The ability of an elevated level of intracellular cAMP to downregulate a variety of immune responses is well described.^{9 13} By use of a variety of antigen- and mitogen-driven mixed cell systems, this efficacy has been ascribed to isoforms of the PDE4.^{14 17 20 21} In this report, we have provided data on both antigen-driven proliferation and cytokine gene expression, implicating the responder T cells as the primary cellular target for the effects of immune downregulation by PDE4 inhibitors. Furthermore, by using flow cytometry, we have shown that neither antigen stimulation nor exposure to a PDE4 inhibitor is capable of modulating surface expression of a large panel of second-signal and adhesion molecules on either lymphocytes or monocytes. Because the single time point used in the flow cytometric studies matches the time point at which effects on cytokine gene expression are maximal, we feel these data are significant; a more rigorous exclusion of efficacy would require assessment of additional markers at multiple time points. Although the panel investigated is by no means exhaustive, these data lend credence to the hypothesis that the mechanism for downregulation of immune responses by PDE4 inhibitors on their target lymphocytes involves changes in noncognate cellular interactions. Indeed, separate studies by Wu and colleagues and Cook and colleagues have shown that epidermal growth factor-mediated signal transduction in fibroblasts may be abrogated by elevations of intracellular cAMP; the mechanism for this signal blockade involves cAMP-induced hyperphosphorylation of Raf-1 on serine 43.^{25 26} However, Dent and coworkers have shown that treatment of Raf-1 with either serine/threonine or tyrosine phosphatases is able to inactivate Raf-1 completely with blockade of signal transduction.²⁷ Thus a specific phosphorylation state of key signaling intermediates may be necessary for proper signal

transduction. Coghlan and colleagues have recently shown the association of the regulatory subunit of protein kinase A and protein phosphatase 2B with a common membrane-anchoring protein.²⁸ This colocalization on the inner aspect of the cell membrane may position these opposing regulators of signaling intermediates, such as Raf-1, for rapid regulation of specific protein phosphorylation states. Therefore a direct effect of intracellular cAMP on antigen-driven signal transduction in T lymphocytes may account for the efficacy of PDE4 inhibitors in this mixed cellular system.

A number of other observations may be made from these data. First, the efficacy of the PDE4 inhibitor on both proliferation and cytokine gene expression, on a percent basis compared to controls, is nearly identical for the RW- and TT-driven systems. Second, while gene expression for cytokines characteristic of both TH1 and TH2 responses are present in both antigen-driven systems, the relative quantities are different. Specifically, a greater level of gene expression for IL-4 and IL-5 is present in the RW-driven system, whereas IL-2 gene expression is greater in the TT-driven system. Interestingly, IFN- γ is expressed at high levels in both systems. Thus, although discrete TH1 and TH2 specificity is not apparent in this mixed cell system with RW and TT, phenotypic bias is present. Further analysis of the potential differential effects of PDE4 inhibitors on TH1 and TH2 cells must await the use of antigen-specific T-cell clones.

Several studies by Hanifin and coworkers have addressed the function and characterization of cAMP-PDE from patients with atopic dermatitis. These investigators first described significant elevations of cAMP-PDE activity in leukocytes from atopic patients in 1982.²⁹ This activity was localized to the monocyte fraction, rather than the lymphocyte fraction, in these patients.⁶ Despite this elevated activity, the isozyme found in patients with atopic dermatitis was consistently more sensitive to a variety of both nonselective and PDE4 selective inhibitors.³⁰ Recently, this same group has used chromatofocusing to separate this distinct monocyte cAMP-PDE activity.³¹ This enzyme was cytosolic and cAMP specific and was inhibited by standard PDE4 inhibitors, despite an apparent calcium/calmodulin dependence. The investigators suggested that this abnormal cAMP-PDE activity may be etiologic for atopic disease. We feel that the data presented here complement and extend the findings of Hanifin and coworkers. First, their studies have focused primarily on activation-independent functional characterization of cAMP-PDE in subjects with atopic dermatitis. Our studies extend these findings to antigen-driven functional characterization of mixed cell responses and their modulation by PDE4 inhibitors. Although an abnormal cAMP-PDE activity that is more sensitive to PDE4 inhibitors may be present in monocytes, it is apparent from our data that targeting the lymphocyte PDE4 results in specific downregulation of antigen-driven proliferation and expression of proinflammatory cytokines. Li et al.³¹ have shown that low concentrations of recombinant human IL-4 and IFN- γ may act synergistically to upregulate monocyte-specific cAMP-PDE. The ability to downregulate cytokine gene expression in a mixed cellular system through the activity of PDE4 inhibitors on target lymphocytes may normalize the monocyte cAMP-PDE and thus prove therapeutic at the molecular level. Finally, the efficacy of adherent cell pretreatment in the downregulation of IL-2 gene expression may be explained, in part, by the inhibition of this abnormal monocyte PDE activity. However, despite the downregulation of IL-2, it is apparent from our data that sufficient lymphocyte activation and proliferation may still occur. Previous studies from our laboratory have shown that supraphysiologic concentrations of exogenous IL-2 are incapable of reversing the PDE4 effect in antigen-driven PBMCs.²⁰ Finally, recent studies by Prussin et al.³² confirm the divergence of IL-2 and IFN- γ coexpression and suggest that memory status may be a

more important determinant for IL-2 production than is the T cell subset. Taken together, these data suggest that the IL-2 concentration in the microenvironment of the activated T lymphocytes is adequate and that the consequences of this activation may only be modulated by the targeted exposure of antigen-specific responder lymphocytes to the PDE4 inhibitor.

As in our previous studies, we have used PBMCs from atopic individuals who showed lymphocyte responses to both RW and TT.^{20 21} We have investigated the modulation of proinflammatory cytokines at the level of gene expression by using a sensitive semiquantitative RT-PCR assay. Although values for the absolute changes in messenger RNA transcripts are not obtained by this method, this assay does provide highly reproducible estimates of the relative differences in messenger RNA transcripts between treatment conditions. We feel that precise messenger RNA quantitation by competitive RT-PCR would add little to the conclusions.²² Although correlation to cytokine protein production is desirable, we have previously shown that cytokine protein levels in an antigen-driven system are below the limits of detection for standard ELISAs.²¹ This is likely due to the low number of antigen-specific responder lymphocytes in PBMCs. Furthermore, direct measurements of cAMP may be difficult to interpret in such a mixed cellular system. Both of these issues will be addressed in future studies employing nontransformed, antigen-specific TH1 and TH2 clones.

In conclusion, we have shown that the primary cellular targets for the modulation of antigen-driven responses by PDE4 inhibitors in a mixed cellular system are the responder lymphocytes. The precise mechanism for this modulation remains unknown but most likely involves alterations in noncognate cellular functions. These data serve to focus future investigations concerning the mechanisms of cAMP modulation of immune responses on alterations in lymphocyte signal transduction.

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Gregory V. Marcotte, MDT^a, Christine M. Braun, BA^a, Philip S. Norman, MD^a, Christopher F. Nicodemus, MD^b, Anne Kagey-Sobotka, PhD^a, Lawrence M. Lichtenstein, MD, PhD^a, David M. Essayan, MD^a

Baltimore, Md., and Waltham, Mass.

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