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Immunologic Investigations of T-Cell Regulation of Human IgE Antibody Secretion and Allergic Responses

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The pathophysiology of allergic disease is multifactorial, involving an intricate network of interactions among cells, mediators, and cytokines. Substantial progress has been made in defining the role of antigen-specific T cells and cytokines in the regulation of immunoglobulin E (IgE) synthesis and the atopic diseases. The development of antigen-specific T-cell lines and clones has facilitated efforts to characterize human T-cell subsets and their cytokine repertoires. Molecular methods currently available include techniques for the quantitative analysis of cytokine gene expression and secretion from activated T cells *ex vivo* as well as in tissues. The availability of these newly developed techniques has become essential to the investigation of the pharmacologic regulation of T cells and cytokines both *in vitro* and *in vivo.* Future investigations will contribute to our understanding of the differential regulation of T-cell subsets and their relationships to allergic diseases, ultimately leading to a better understanding of the molecular pathogenesis of allergic diseases and the design of more effective therapeutic interventions.

A mature $CD4^+$ T cell, through its clonally derived T-cell receptor (TcR), recognizes a peptide fragment of an antigen (Ag) only in the context of a membrane-bound class II molecule (in humans, HLA-DR, DQ, or DP) on the antigen-presenting cell (APC; such as macrophage/dendritic cells and B cells) (for review of MHC-restricted recognition of allergens, see Ref. 1). This Ag-specific, MHC-restricted event allows for the induction of the effector functions of T-cell help and the generation of cytokines. Based on the profile of cytokines, two functional subsets of T cells have been described (2): Type 1 T cells (Th1) express IL-2 and IFN-γ, whereas type 2 T cells (Th2) express interleukin 4 (IL-4), IL-5, IL-6, and IL-10. In humans, such dichotomy has been observed from cytokine profiling analyses of Ag-specific T-cell clones of atopic patients (3–6). The molecular basis for and *in vivo* importance of these T-cell subsets are not clearly understood. It has been suggested that cytokines (IL-4, IFNγ, and IL-10) play a role in the functional differentiation of T cells; there is reciprocal regulation of Th1 and Th2 cells by their cytokine products (7).

Our current understanding of the regulation of IgE synthesis has been primarily derived from *in vitro* experiments, together with functional studies of recombinant cytokines. Several studies in humans have indicated that the regulation of IgE is controlled by the reciprocal activity of IL-4 and IFN-γ (8) and requires direct T cell–B cell interactions with both MHC-restricted and non-MHC-restricted events (for example, CD-40–gp39 interactions; Ref. 9). Using an allergen challenge model, the presence

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of infiltrating T cells and the expression of Th2 cytokines at sites of inflammation have been well documented (10–12). An important implication of these studies is that in atopic subjects, allergen induces activation and/or proliferation of cells having the Th2 cytokine profile, and the expression of Th2 cytokines is critical in the induction of IgE synthesis and the initiation of allergic responses. The information gained from these studies has been the basis for designing therapeutic strategies for the modulation of human T-cell activation and cytokine responses by a variety of immunopharmacologic agents. In addition, T cells may play a general role in the regulation of specific immune responses; this is suggested by the importance of T cells as targets in allergen immunotherapy (13, 14).

The role of T cells and cytokines in the regulation of IgE synthesis and allergic diseases has been the subject of several recent reviews (15, 16), to which the reader is referred for more detailed information and discussion. The present review will focus on recent developments concerning the immunologic methods and current immunomodulatory approaches used in the study of Ag-specific T-cell responses and their relationships to atopic diseases.

ANTIGEN-SPECIFIC T-CELL POPULATIONS

Antigen-specific T-cell activation involves an initial cognate cellular interaction and subsequent molecular signals selectively transmitted through a complex series of intracellular phosphorylation/dephosphorylation events. These molecular signals culminate in the induction of specific gene transcription and translation. While mitogen-driven activation can result in both proliferation and cytokine generation, much of the specificity of antigen-driven stimulation is not reproduced by this method (17). For this reason, we and others have focused considerable effort on the establishment of antigen-driven systems for the study of T-cell biology and cytokine pharmacology. While the stimulation of peripheral blood mononuclear cells (PBMCs) with antigen may provide relevant *ex vivo* data, an understanding of differential functional properties of T-cell subsets can be achieved only with the use of antigen-specific, phenotypically characterized T-cell clones.

Antigen-specific human T-cell lines are generated from the PBMCs of study subjects by periodic stimulation with antigen in the presence of APCs and T-cell growth factors, such as IL-2 (18). Antigen-specific T-cell clones are generated from these lines by the limiting dilution technique (usually at 0.3 cell per well), which in principle follows statistics of the Poisson distribution. Autologous irradiated PBMCs or Epstein–Barr virus-transformed B cells are used as APCs for periodic restimulation and expansion of the clonal cell populations. Subcloning and analysis of TcR Vβ gene usage are used to confirm monoclonality. The antigen specificity is confirmed by unresponsiveness of the T cells to an irrelevant antigen (5, 6). The availability of stable, phenotypically characterized, antigen-driven T-cell clones has provided an excellent tool for the study of T-cell clonal diversity, proinflammatory cytokine production, phenotypic heterogeneity to a single allergen, and the differential pharmacologic regulation of the T-cell subsets.

ANALYSIS OF CYTOKINE GENE EXPRESSION

Transcription and secretion of cytokines are dependent on the activation of resting T cells. Several techniques have been used to quantitate cytokine gene expression from activated T cells *ex vivo* or *in situ* from foci of inflammation. These include RNase protection assays, *in situ* hybridization, Northern blotting, and the reverse transcription–polymerase chain reaction (RT-PCR; Ref. 19). Of these techniques, neither Northern blotting nor *in situ* hybridization provides a quantitative assessment of cytokine mRNA; moreover, *in situ* hybridization requires substantial expertise to achieve reproducible results. Two of these techniques, RT-PCR and *in situ* hybridization, have been useful in the study of allergic diseases.

Qualitative and Quantitative RT-PCR

The ability to detect and quantitate multiple cytokine mRNAs from small numbers of target cells makes RT-PCR technology an attractive and powerful tool to assess gene expression (19, 20). In this technique, RNA is extracted from tissues or cells and first-strand cDNA synthesized from an aliquot of RNA (usually 1 μg). Aliquots of cDNA are then subjected to PCR in the presence of cytokine-specific

primers in an automated thermocycler. The products of amplification are analyzed by electrophoresis in agarose gels containing ethidium bromide. The specificity of the amplification is assessed by any or all of the following methods: the expected sizes of amplified fragments, Southern blotting using internal sequence probes, restriction enzyme digestion, or direct sequencing. This simple methodology provides a qualitative assessment of cytokine gene expression.

To quantitate the level of cytokine gene expression, modified RT-PCR-based techniques have been developed. Early methods provided relative quantitation (also referred to as semiquantitative RT-PCR) of cytokine transcripts by comparing the amounts of the amplified products of the target template and a constitutively expressed gene, such as β-actin or HPRT. Using either radioactive incorporation or densitometry measurements of the amplified products, the ratio of target gene product and constitutive gene product was calculated and compared between different test samples. Although informative, this method has inherent shortcomings. Optimal PCR reactions are dependent on various parameters, including primer specificity and stability, the concentrations of magnesium and DNA polymerase, cycling profiles, and the number of amplification cycles. These factors account for much of the variability seen in amplification specificity and efficiency.

The more recent development of "quantitative, competitive RT-PCR (QC-PCR)" (22) has sought to minimize some of these problems. In QC-PCR, an exogenous RNA standard [referred to as the internal standard (IS)], which is created as an allelic size variant of the gene of interest (Refs. 11, 21–23), is amplified in the presence of the same primer set in the same tube with the target RNA. Thus, the amplification of both the IS and the target RNA templates is affected equally by variables during RT-PCR. The difference in fragment sizes of the IS and target mRNA allows the two amplified products to be distinguished on agarose gel. The amount of unknown target mRNA can be extrapolated from the density or radioactivity ratios of varying dilutions of the target product amplified with a known concentration of IS. A significant improvement of this technique involves the use of "polycompetitors" to allow quantitation of multiple target genes with the same internal standard (24). Finally, a modification wherein the amplified DNA is captured covalently onto microtiter plates and quantitated by oligonucleotide hybridization and enzyme-linked immunosorbent assay has recently been described (25).

We have used QC-PCR to study cytokine gene expression in T cells from peripheral blood and bronchoalveolar lavage samples of subjects with airway inflammation following allergen challenge (11, 21–23). We demonstrated enhanced expression of mRNA for the Th2-cytokine, IL-5, in the mononuclear population of asthmatic lung lavage samples. We have also used semiquantitative RT-PCR to analyze the modulation of cytokine transcripts in PBMCs and to study the expression of cytokine transcripts in buccal epithelium, human mast cells, and endothelial cells (26–28). Thus, analysis of cytokine mRNA from small numbers of a variety of cultured cells is possible using PCR-based techniques. Figure 1 shows an example of QC-PCR technique.

In Situ hybridization

An additional tool useful in the analysis of cytokine gene expression is *in situ* hybridization. In this technique, cytokine mRNAs can be identified within the cells of tissue sections. When combined with immunocytochemistry, it is a powerful tool to identify the cell sources of the cytokine mRNAs. The methodology involves fixing target tissue to glass slides, incubating with hybridization buffer containing the radiolabelled probe of interest (or the probe in a "sense" direction as a negative control), and visualization by autoradiography (29). Using this technique, Robinson *et al.* demonstrated an increased expression of mRNAs for IL-2, IL-4, IL-5, and GM-CSF in the lung lavages of asthmatic patients. They also demonstrated that in patients with asthma, IL-4 and IL-5 were expressed predominantly by the T cells (10). Using *in situ* hybridization, Varney *et al.* showed that successful grass pollen immunotherapy was associated with an increase in the number of IL-2- and IFN-γ-stained cells (30). Finally, Durham *et al.* demonstrated that the number of IFN-γ-stained cells increased in the nasal biopsies of patients treated with grass pollen immunotherapy (31). Thus, *in situ* hybridization has provided significant insights into the pathogenesis of allergic inflammation.

ANALYSIS OF CYTOKINE PROTEIN SECRETION

While the assessment of proinflammatory cytokine gene expression from T cells may provide a pre-

cise and highly sensitive indication of cellular activation states, transcriptional and posttranscriptional regulatory events may influence the ultimate outcome of cellular activation. For this reason, gene expression data are often combined with direct or indirect measurements of cytokine protein generation. Currently, the three primary methods used for cytokine protein measurement are bioassays, enzyme-linked immunosorbent assays (ELISA), and flow cytometry with intracellular cytokine staining.

Bioassays

Bioassays for the assessment of cytokine production have been established in many laboratories for many cytokines. Bioassays detect the biological activity of the cytokine through a growth-promoting effect on a dependent cell line; nonfunctional cytokine will not be detected by this method. However, bioassays may be subject to interference by additional growth factors not related to the cytokine of interest but present in the sample undergoing analysis. While these assays often require considerable maintenance, they provide an inexpensive and highly sensitive measurement of cytokine production. For example, Gleich and colleagues have developed an eosinophil survival assay for the measurement of IL-5 with a sensitivity of 22 fmol and a usable range of two orders of magnitude (32). With rare exceptions, these assays have been supplanted by other techniques.

Enzyme-Linked Immunosorbent Assay

ELISA assays for the detection of many cytokines and other molecular targets are available commercially. These assays boast sensitivities as low as 0.1 pg. They offer the advantages of high precision, species specificity, negligible cross-reactivity with closely related molecular structures, and a high resistance to interference by serum proteins or other incidental factors. Using commercially available

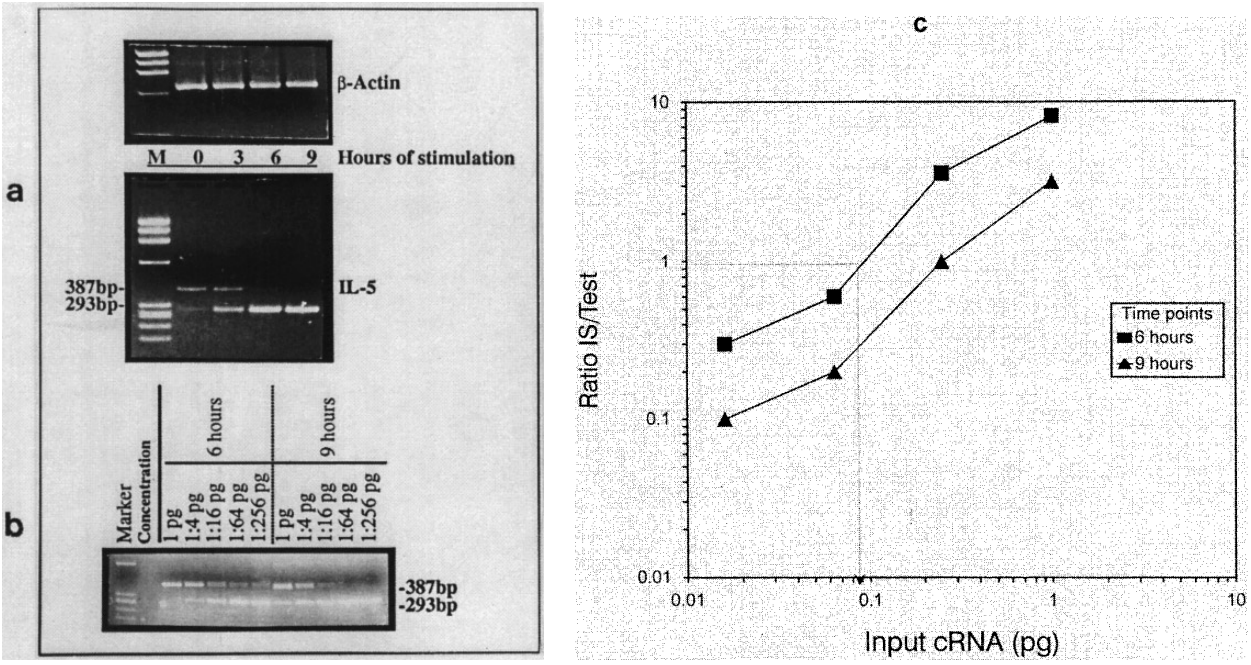

FIG. 1. Quantitative analysis of interleukin 5 mRNA expression. (a) Total cellular RNA from antigen-stimulated PBMCs was reverse-transcribed with an oligo(dT) primer and subjected to PCR. (Top) Constitutive expression of β-actin at each of four time points. (Bottom) The increasing competition of native IL-5 transcript (293-bp fragment) with a constant amount of IS (387-bp fragment). (b) A fixed amount of total cellular RNA from the 6- and 9-h time points is subjected to QC-PCR with varying amounts of the IS competitor, as shown. (c) Quantitation of the individual bands in (b) (by radioactive incorporation or densitometry measurements) is depicted graphically. The concentration of IL-5 in the test sample is determined by the amount of IS at the IS/test sample ratio of 1. In this experiment, low levels of IL-5 mRNA are present at 3 h and increase at 6 h (0.1 pg/200 ng RNA) and 9 h (0.3 pg/200 ng total RNA). Experimental variability is typically less than 5% (22).

ELISA kits for various cytokines, we have conducted a study to characterize the cytokine profiles of a short ragweed allergen (Amb a 1)-specific T-cell clones from two allergic subjects and one nonallergic subject (6). The clones studied were uniformly $CD3^+$ $CD4^+$ $CD8^-$ by flow cytometry. We demonstrated that while the clones from the atopic subjects were predominantly Th0 with lesser numbers of Th1 and Th2 clones, clones from the nonatopic subject were uniformly of the Th1 phenotype. Detailed analysis of cytokine profiles from these clones allowed us to show uniformly high levels of IL-13 (>4 ng/ml/2×10^5 cells) production, regardless of cellular phenotype (Fig. 2). These levels did not correlate with levels of IL-4 production and implicate IL-13 directly in the generation of chronic allergic inflammation.

While the majority of ELISA assays employ a standard "sandwich" method for the measurement of free ligand, a number of modified ELISA assays have recently been developed. One such modification uses a biotinylated cytokine competitor to detect both free and bound fractions of the target cytokine (33, 34). Another assay allows the culture of cytokine-producing cells directly in the presence of the primary anti-cytokine antibody. This enables cumulative detection of secreted cytokine, obviating the influence of autocrine consumption (35, 36). Finally, a variation on the sandwich ELISA methodology using a biotinylated oligonucleotide with subsequent PCR as the detection system has been employed to improve sensitivity to the 10 fg range (37).

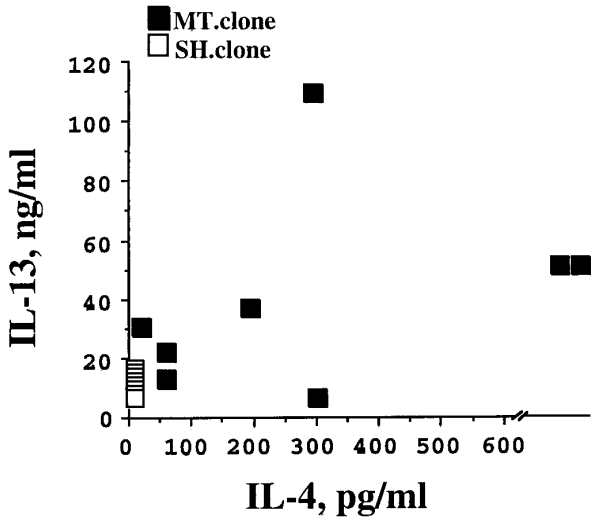

FIG. 2. IL-4 and IL-13 secretion in Amb a 1-specific T cells. Amb a 1-specific T-cell clones were derived from an atopic subject (MT) and a nonatopic subject (SH). All T-cell clones (eight from subject MT, solid squares; eight from subject SH, open squares) were stimulated with Amb a 1 (10 mg/ml) in the presence of autologous APCs. Culture supernatants were collected 24 h after stimulation; IL-4 and IL-13 from these supernatants were quantitated by ELISA.

Flow Cytometry with Intracellular Cytokine Staining

The most recent development in the area of cytokine protein detection has been the development of intracellular cytokine staining of fixed and permeabilized cells with labeled monoclonal antibodies and subsequent detection by flow cytometry. Such methods usually employ an agent such as momensin to inhibit secretion of cytokine protein, enhancing intracellular accumulation and facilitating staining. This methodology offers the advantages of detection of multiple cytokines simultaneously on a single-cell level and the characterization of cytokine production profiles of cell populations. Prussin and colleagues have used flow cytometric techniques to identify distinct Th0, Th1, and Th2 subsets in human $CD4^+$ $CD27^-$ lymphocytes and have demonstrated the utility of this technique in studying increased IL-5 production characteristic of hypereosinophilic states (38, 39). However, reliable cytokine detection has only been achieved with the use of mitogenic stimuli; the feasibility of this assay in detection of cytokine production in antigen-driven T-cell populations remains to be determined.

PHARMACOLOGIC MODULATION OF T-CELL RESPONSES

It is clear that significant progress has been made in the development of cellular and molecular tools for characterizing antigen-induced activation of T cells; these new developments have facilitated pharmacologic studies of T cell and cytokine responses in disease states. For example, the generation of proinflammatory cytokines may be downregulated by a variety of therapeutic agents for the atopic diseases. Studies from our laboratory and others have documented the efficacy of corticosteroids, nonselective and selective phosphodiesterase (PDE) inhibitors, and peptide immunotherapy in the downregulation of Th2 cytokines.

Glucocorticoids

Glucocorticoids are potent antiinflammatory agents that affect a variety of immune cells. In lymphocytes, glucocorticoids bind a specific cytoplasmic receptor; this complex translocates to the nucleus, where it is felt to induce the transcription of a number of regulatory genes including the IκBα gene. The product of this gene prevents nuclear translocation of NF-κB, a *trans*-acting regulatory protein for proinflammatory cytokines including IL-4. Thus, corticosteroids may downregulate allergic inflammatory responses (40).

We have used antigen-driven PBMCs and antigen-specific T-cell clones of the Th0, Th1, and Th2 subtypes to conduct a detailed analysis of differential kinetics and efficacy of corticosteroids (41). Our results demonstrated a strong dose-dependent downregulation of PBMC responses to a variety of antigens (IC_{50} for dexamethasone = 3 nM); moreover, significant downregulation of antigen-driven responses could be achieved even with the addition of dexamethasone 36 h after initial antigen stimulation. Analysis of cytokine protein production from ragweed (Amb a 1)-stimulated Th1 and Th2 clones revealed dose-dependent downregulation of cytokines from both T-cell subsets with a panel of corticosteroids (dexamethasone, budesonide, and hydrocortisone). Of note, IFN-γ production showed a significantly greater sensitivity to inhibition with 10^{-7} M budesonide and 10^{-6} M hydrocortisone (P = 0.02 and 0.03, respectively) than did IL-4 production. Differences in IFN-γ and IL-4 production in cultures with lower doses of corticosteroids were not significant (P uniformly > 0.1) (Fig. 3a). IL-13 secretion showed similar, dose-dependent inhibition from either Th1 or Th2 clones with any of the three corticosteroids; no significant differences between the phenotypes were observed (P uniformly > 0.3) (Fig. 3b). Thus, the clinical efficacy of corticosteroids may be due to their downregulatory effects on T-cell-derived proinflammatory cytokine production.

Phosphodiesterase Inhibitors

Phosphodiesterase (PDE) isozymes catalyze the degradation of intracellular cyclic nucleotides; their inhibition results in the upregulation of steady-state cyclic nucleotide levels with activation of specific protein kinase isoforms. Elevations of cAMP have been shown to downregulate immunologic responses to both mitogenic and antigenic stimuli. Phosphodiesterase enzymes may be classified into seven distinct families based on substrate specificity, inhibitor sensitivity, and sequence homology; PDE3, PDE4, and PDE7 have been shown to specifically regulate cAMP levels (42). We have investigated the efficacy of selective inhibitors of PDE3 and PDE4 in antigen-specific T-cell responses. In PBMCs, inhibitors of the PDE4 isozymes cause dose-dependent downregulation of T-cell proliferative responses and gene expression of proinflammatory cytokines. The low number of antigen-specific target cells in these assays precludes adequate analysis of cytokine secretion (43, 44). However, using phenotypically characterized T-cell clones, we have demonstrated dose-dependent downregulation of IL-4 and IL-13 protein production by PDE4 inhibitors (Fig. 4); PDE3 inhibitors, while capable of elevating intracellular cAMP, are ineffective in modulating cytokine protein production, perhaps due to compartmentalization of cyclic nucleotide-mediated signals (45). Demonstration of clinical efficacy for these agents awaits the results of ongoing phase 2 trials.

Peptide Immunotherapy

Despite nearly a century of use and proven efficacy in the treatment of allergic rhinitis and allergic asthma, the precise mechanism of immunotherapy remains unclear. A number of alterations in immune function have been documented during immunotherapy, including a decline in antigen-specific IgE levels, an increase in antigen-specific IgG levels, and a diminution of antigen-specific lymphocyte responsiveness (46). Recently, using mitogen-stimulated short-term T-cell lines, a number of investigators have provided evidence to suggest a phenotypic shift in responder T cells from a Th2 population to a Th1 population during immunotherapy. These investigators have advanced the hypothesis that this shift accounts for the clinical benefit seen with immunotherapy (13, 14). We have recently completed an *ex vivo* study of antigen-specific T-cell responses from participants in a randomized, double-blind, placebo-controlled study of two different doses of a peptide immunotherapy product (47). In this study, subjects were immunized with a combination of two recombinant peptides containing the major epitopes of Fel d 1, the major allergen of cat dander extract. Short-term T-cell lines were derived from each subject before and after immunotherapy, using cat dander extract, the recombinant peptides, or tetanus toxoid

(an irrelevant antigen). While antigen-specific T-cell proliferative responses were unchanged by immunotherapy, peptide-induced IL-4 secretion was downregulated by immunotherapy. Moreover, this downregulation was greater in the high-dose treatment cohort than the low-dose treatment cohort; this effect correlated closely with clinical outcomes in the treatment groups. While an inverse trend for IFN-γ production was noted, this did not reach statistical significance. Finally, significant changes in IL-4 and IFN-γ production were not evident in the cell lines derived with either the more nonspecific cat dander extract or the irrelevant tetanus toxoid antigen. These data support the hypothesis of T-cell phenotypic shift as a mechanism for the efficacy of immunotherapy and underscore the high degree of antigen specificity of this treatment modality.

CONCLUSION

Advances in cellular and molecular methods have provided essential tools for examining the role of antigen-specific T cells and cytokines in the regulation of IgE synthesis and the pathogenesis of allergic diseases. These and other newly developed techniques will be crucial in our effort to define the differential regulatory roles of Th1 and Th2 cells in allergic and immunologic diseases. However, it is

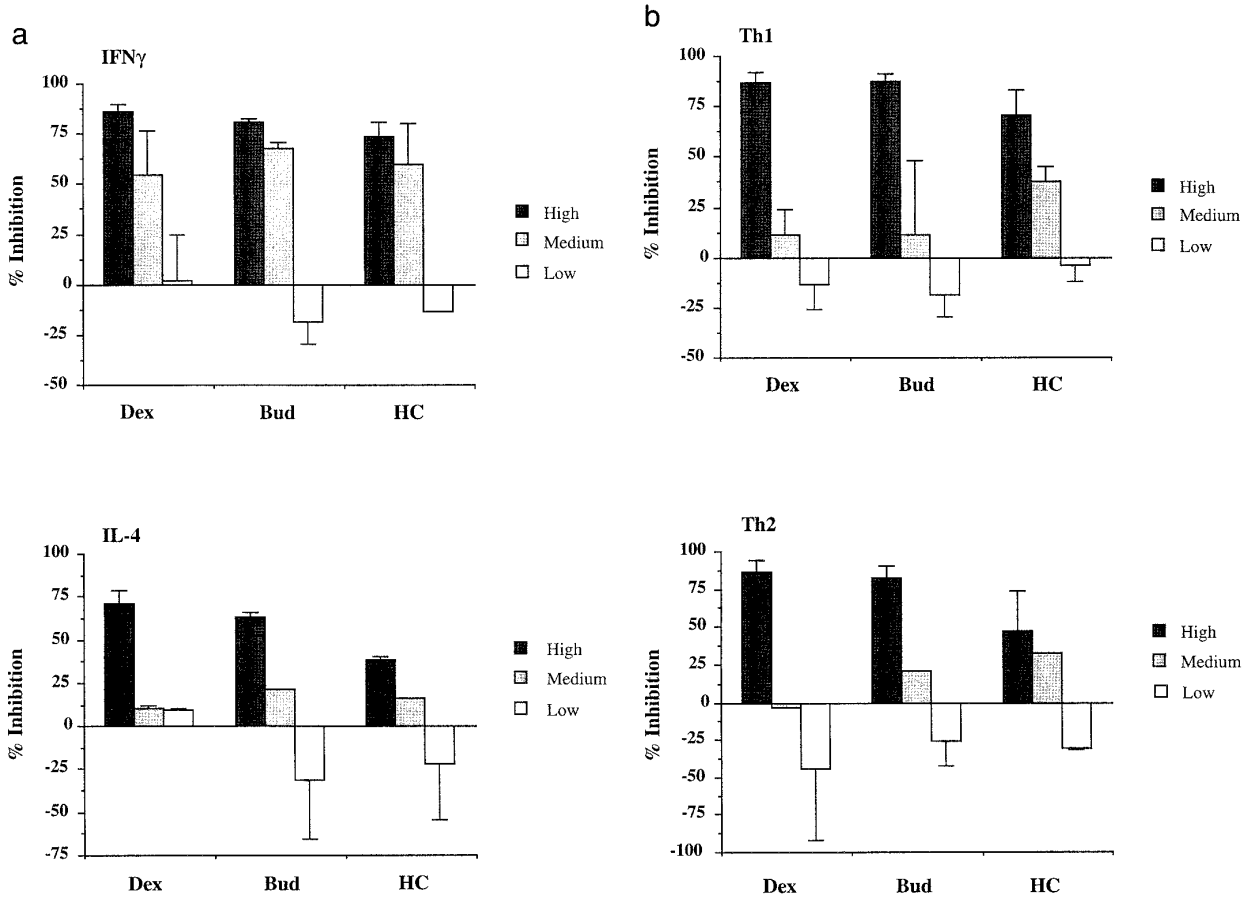

FIG. 3. (a) Modulation of IFN-γ (top) and IL-4 (bottom) secretion by corticosteroids. Cytokine protein, measured by ELISA, is shown for Th1 (top) and Th2 (bottom) clones stimulated in the presence of three concentrations of each of three steroids (Dex, dexamethasone 10^{-7}, 10^{-9}, and 10^{-10} M; Bud, budesonide 10^{-7}, 10^{-9}, and 10^{-10} M; HC, hydrocortisone 10^{-6}, 10^{-8}, and 10^{-9} M). The data are presented as the percentage of inhibition ± SEM relative to stimulated, steroid-free control cultures. (b) Modulation of IL-13 secretion by corticosteroids. IL-13 protein, measured by ELISA, is shown for Th1 and Th2 clones stimulated under the same culture conditions indicated in a. The data are presented as the percentage of inhibition ± SEM relative to stimulated, steroid-free cultures.

imperative that the results obtained from studies of long-term cultured T cells be compared to results from a more "physiologic" system, such as the mixed cell population of a disease-targeted organ. An improved understanding of disease pathogenesis and the contribution of various cell types may be achieved with quantitative or *in situ* PCR in combination with immunohistochemistry or ELISA for

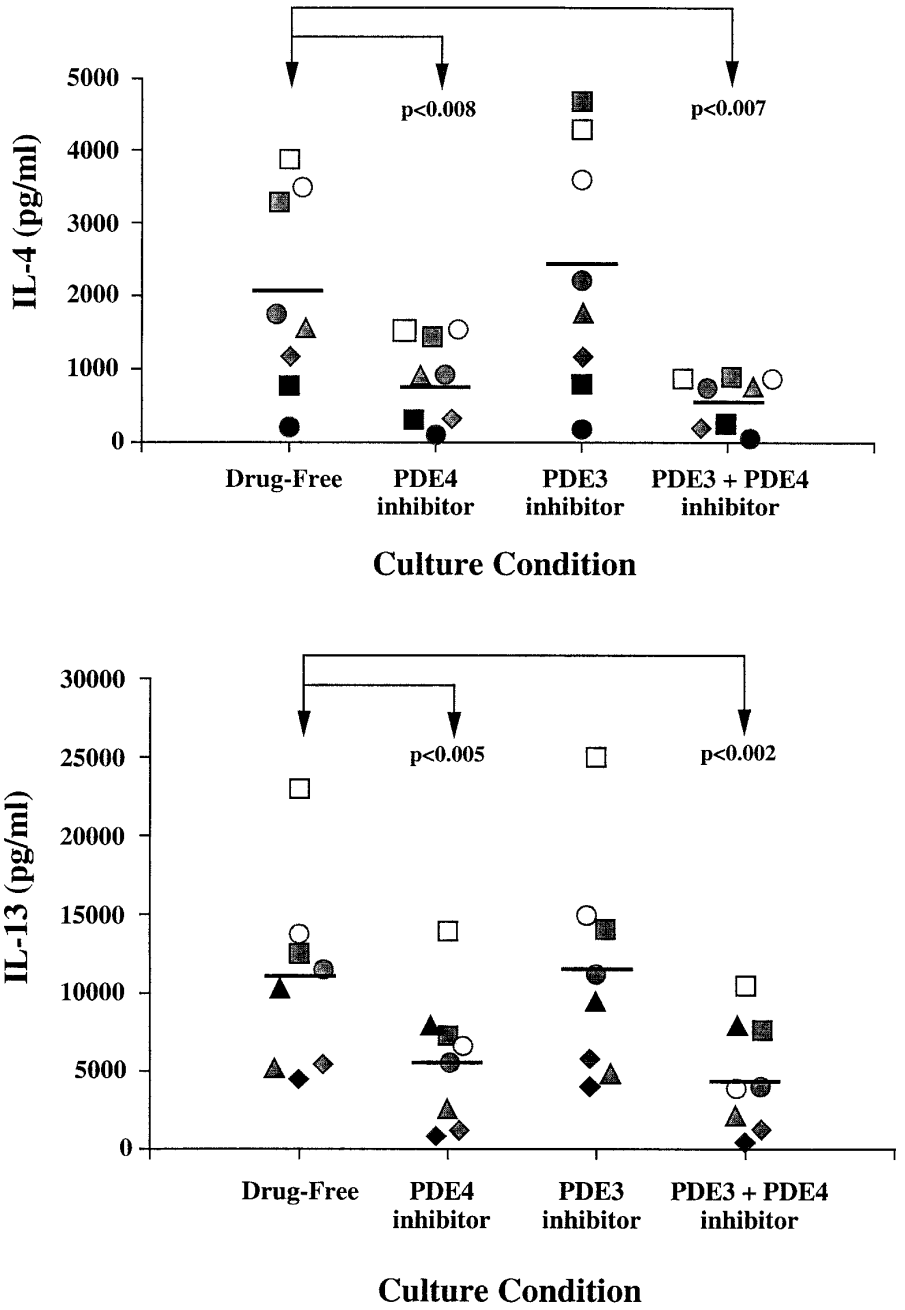

FIG. 4. Modulation of IL-4 (top) and IL-13 (bottom) secretion by PDE inhibitors. Cytokine protein, measured by ELISA, is shown for each of eight T-cell clones under culture conditions without PDE inhibitors (Drug-Free), with rolipram 10^{-5} M (PDE4 inhibitor), with siguazodan 10^{-5} M (PDE3 inhibitor), or with both. Shaded symbols, Th0; black symbols, Th1; and white symbols, Th2. Mean values in pg/ml are shown by the horizontal bars.

cell-associated or secreted proteins; such innovative approaches will assist in the molecular classification of various diseases and provide a powerful tool for monitoring the efficacy of therapeutic interventions.

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