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Differential regulation of interleukin 4 and interleukin 5 gene expression: A comparison of T-cell gene induction by anti-CD3 antibody or by exogenous lymphokines

(mRNA/c-*myc*/c-*myb*/cyclosporine A/cycloheximide)

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ABSTRACT **Murine T helper type 2 clones were stimulated with immobilized anti-CD3 antibody or with recombinant lymphokines to compare the expression of T-cell activation genes induced by these stimuli. Immobilized anti-CD3 antibody, recombinant interleukin 2 (IL-2), and recombinant interleukin 4 (IL-4) all induced proliferation of the T helper type 2 clones 10-5-17 and D10. Proliferation of these clones induced by anti-CD3 antibody was completely inhibited by cyclosporine A, whereas cyclosporine A had little effect on proliferation induced by recombinant IL-2 or recombinant IL-4. Both immobilized anti-CD3 antibody, and recombinant IL-2 induced the expression of the protooncogenes c-*myc* and c-*myb*. Immobilized anti-CD3 antibody also induced expression of the lymphokine genes IL-4, interleukin 5 (IL-5), and granulocyte–macrophage colony-stimulating factor. In contrast, recombinant IL-2 induced IL-5 mRNA expression but did not induce detectable expression of IL-4 or granulocyte–macrophage colony-stimulating factor mRNA. Likewise, recombinant IL-4 induced expression of IL-5 but not IL-4 mRNA. Thus, the IL-4 and IL-5 genes appear to be differentially regulated after stimulation with recombinant lymphokines. Effects of cyclosporine A and the protein synthesis inhibitors cycloheximide and anisomycin on IL-4 and IL-5 gene expression suggest that these genes are activated by different pathways after anti-CD3 stimulation. Cyclosporine A completely inhibited anti-CD3-induced expression of IL-4 mRNA but not of IL-5 mRNA, and protein-synthesis inhibitors completely inhibited induction of IL-5 mRNA but not of IL-4 mRNA. Together, our data show that T-cell receptor-mediated and lymphokine receptor-mediated signals induce different patterns of lymphokine gene expression and provide strong evidence that the IL-4 and IL-5 genes are differentially regulated.**

Proliferation of resting T cells usually requires specific recognition by the T-cell receptor of antigen associated with an appropriate major histocompatibility complex molecule. This specific recognition activates a complex series of events resulting in the expression of lymphokine and lymphokine receptor genes. T-cell proliferation is thought to depend on endogenously produced lymphokines acting through an autocrine pathway (1–3). Resting T cells normally do not proliferate when stimulated with exogenous lymphokines such as interleukin 2 (IL-2) or interleukin 4 (IL-4), and T-cell receptor-mediated signals are necessary to induce lymphokine responsiveness (4, 5). In contrast, antigen-specific murine T-cell lines and clones have been derived that express lymphokine receptors and can proliferate in response to exogenous lymphokines as well as in response to T-cell receptor-mediated signals (6, 7).

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In the present study, murine T helper type 2 (Th2) clones (8, 9) were stimulated through the T-cell receptor/CD3 complex or through lymphokine receptors to compare the patterns of gene expression induced by signals mediated through these receptors. In particular, we examined changes in lymphokine and protooncogene mRNA levels after stimulation of Th2 clones with immobilized anti-CD3 antibody, recombinant IL-2, or recombinant IL-4. Anti-CD3 antibody and exogenous lymphokine both induced expression of the protooncogenes c-*myc* and c-*myb*. Although anti-CD3 antibody induced expression of the IL-4, interleukin 5 (IL-5), and granulocyte–macrophage colony-stimulating factor (GM-CSF) genes, recombinant lymphokine (IL-2 or IL-4) induced expression of the IL-5 gene, but not of the IL-4 gene, suggesting that these genes are differentially regulated. Furthermore, cyclosporine A and protein-synthesis inhibitors have distinct effects on the induced expression of these genes, providing further evidence that the IL-4 and IL-5 genes are differentially regulated.

MATERIALS AND METHODS

Tissue Culture Medium. RPMI 1640 medium supplemented with penicillin at 100 units/ml, streptomycin at 100 μg/ml, 20 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 5% heat-inactivated fetal calf serum was used for maintenance of T-cell clones and for all stimulations and assays.

Antibodies. The 2C11 antibody (10) is a purified hamster monoclonal antibody specific for the ε chain of the murine CD3 complex and was provided by J. A. Bluestone (University of Chicago, Chicago).

Recombinant Lymphokines. Recombinant human IL-2 was from Cetus. Recombinant purified murine IL-4 was from W. E. Paul (National Institutes of Health, Bethesda, MD).

T-Cell Clones. The murine Th2 T-cell clone 10-5-17 is $CD4^+$, $CD8^-$, keyhole limpet hemocyanin (KLH)-specific, and restricted by $I\text{-}A^b$. Generation of this clone has been described (11). Clone 10-5-17 was maintained in tissue culture by stimulation every 10 days with antigen (KLH) and irradiated syngeneic spleen cells. Recombinant human IL-2 (50 units/ml) was added 2 days after antigenic stimulation. Clone D10 was obtained from C. Janeway (Yale University School of Medicine, New Haven, CT) (12). This clone was maintained in culture by weekly stimulation with antigen (conal-

Abbreviations: Th1 and Th2, T helper type 1 and type 2, respectively; IL-2, -4, and -5, interleukin 2, 4, and 5, respectively; GM-CSF, granulocyte–macrophage colony-stimulating factor.

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bumin) and recombinant IL-2 at 50 units/ml. These clones were used in experiments 7–12 days after their last stimulation.

Proliferation Assay. T-cell clones (5×10^4 cells per well) were stimulated in 96-well flat-bottom plates (Falcon 3072, Becton Dickinson Labware) with immobilized anti-CD3 antibody, with recombinant IL-2 (50 units/ml), or with recombinant IL-4 (3000 units/ml) or were maintained without any stimulation (control). Stimulations were performed with or without cyclosporine A at 1.0 μg/ml (Sandoz Pharmaceutical). The anti-CD3 antibody was immobilized onto the plastic surface of each well by adding 0.1 ml of antibody at 2 μg/ml in borate buffer and incubating for 2 hr at room temperature. After 48-hr incubation at 37°C, each well was treated with 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol; Ci = 37 GBq; New England Nuclear) and incubated for an additional 24 hr. Cells were harvested on a PHD cell harvester (Cambridge Technology, Cambridge, MA), and incorporated radioactivity was measured with a liquid scintillation counter (LKB 1212, Rackbeta, Gaithersburg, MD). Results are presented as the mean ± SEM of triplicate wells.

cDNA Probes. The IL-4 probe consisted of the 370-base-pair (bp) *Rsa* I fragment of the murine IL-4 cDNA as isolated by T. Honjo (Kyoto University, Kyoto) and was obtained from S. Ho (Mayo Clinic, Rochester, MN). The IL-5 probe was a 1.7-kilobase (kb) full-length cDNA (13) and was obtained from K. Bottomly (Yale University, New Haven, CT). The GM-CSF probe was a 398-bp *Bam*HI and *Eco*RV fragment of the GM-CSF cDNA (14) and was obtained from M. Uhler (University of Oregon, Portland, OR). The c-*myb* probe was a 500-bp insert of the c-*myb* cDNA (15) and was obtained from D. Kastner (National Institutes of Health). The c-*myc* probe consisted of three *Pst* I fragments of c-*myc* cDNA (16) and was from K. Kelly (National Institutes of Health). The actin probe was a 1.7-kb *Pst* I insert derived from human actin cDNA and was from D. Singer (National Institutes of Health).

Northern (RNA) Blot Analysis. T-cell clones ($15–30 \times 10^6$ cells per group) were stimulated for 1–48 hr in 80-cm^2 plastic tissue-culture flasks (Nunc) with immobilized anti-CD3 antibody, with recombinant IL-2 (50 units/ml), or with recombinant IL-4 (3000 units/ml) or were unstimulated (control). The anti-CD3 antibody was immobilized onto the plastic surface of each flask by adding 15 ml of the antibody at 2 μg/ml in borate buffer (pH 8.5) and incubated for 2 hr at room temperature. In some experiments, stimulations occurred in the presence of cyclosporine A at 0.5–1.0 μg/ml, cycloheximide at 1–20 μg/ml (Sigma), or 2–50 μM anisomycin (Sigma). Total cellular RNA was isolated from each group of cells (17), and 10 μg of RNA per group was separated by electrophoresis in formaldehyde-agarose gels and blotted onto GeneScreen membrane filters (New England Nuclear). Specific cDNA probes were ^{32}P-labeled by nick translation or the random primer method and were hybridized to RNA filters. These filters were exposed at −70°C onto Kodak XAR-2 film in Kodak X-omatic cassettes with Cronex Lightning Plus intensifying screens. The same filters were hybridized sequentially with multiple probes after stripping in 0.1× standard saline citrate (SSC) (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.05% SDS for 10 min at 80°C. The relative density of mRNA bands was measured by using an LKB Gelscan XL densitometer.

RESULTS

Th2 Clones Proliferate in Response to Activation Through Their T-Cell Receptor or Lymphokine Receptors. Immobilized anti-CD3 antibody stimulates proliferation of clones 10-5-17 and D10 in the absence of antigen-presenting cells (Fig. 1). This proliferation can be specifically blocked with an anti-

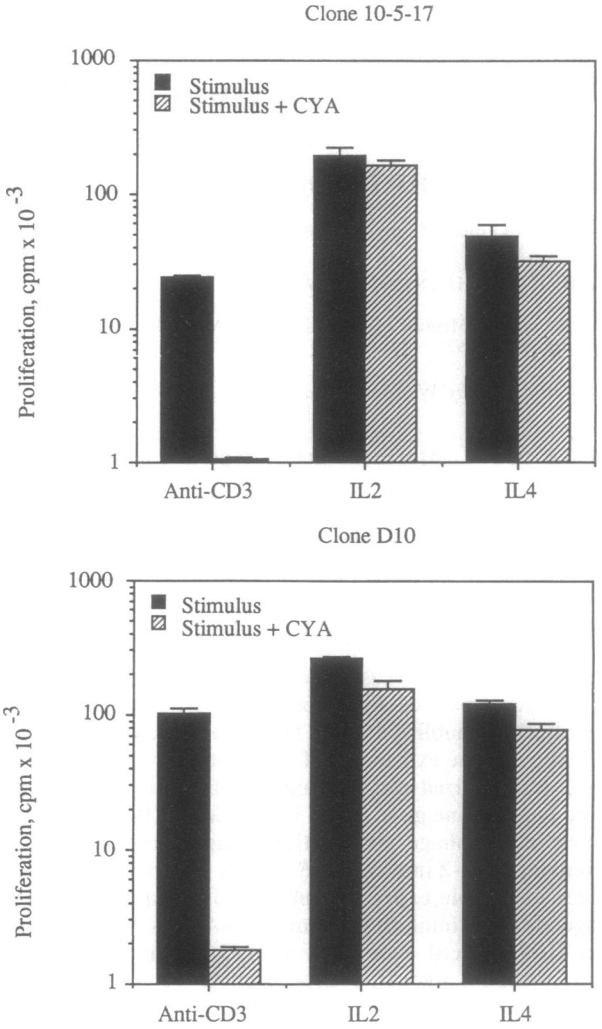

FIG. 1. Anti-CD3 or lymphokine-induced proliferation of Th2 clones. Th2 clones were stimulated with immobilized anti-CD3 antibody, with recombinant IL-2, or with recombinant IL-4 in the presence or absence of cyclosporine A (CYA), as described. Each point represents the mean ± SEM of triplicate wells.

IL-4 antibody implicating IL-4 as an autocrine growth factor in this system (data not shown). In contrast to resting T cells that do not proliferate efficiently in response to exogenous lymphokines alone, these Th2 clones proliferate in response to the exogenous lymphokines IL-2 and IL-4 (Fig. 1). The proliferation of these clones induced by immobilized anti-CD3 antibody is completely inhibited by cyclosporine A at 1.0 μg/ml, whereas the proliferation induced by IL-2 or IL-4 is much less affected (Fig. 1), suggesting that the activation pathways induced by these stimuli are not identical.

Immobilized anti-CD3 Antibody and Exogenous Lymphokines Induce Different Patterns of Gene Expression. Because clones 10-5-17 and D10 proliferate in response to exogenous lymphokines as well as to immobilized anti-CD3 antibody, these clones were used to compare changes in gene expression induced by these stimuli. Clone 10-5-17 was stimulated with immobilized anti-CD3 antibody or recombinant IL-2, and the expression of several T-cell activation genes, including lymphokine genes and protooncogenes, was followed over time by Northern blot analysis (Fig. 2). Both anti-CD3 antibody and recombinant IL-2 induced expression of the protooncogenes c-*myc* and c-*myb*. Immobilized anti-CD3 antibody also induced expression of the lymphokine genes IL-4, IL-5, and GM-CSF. In contrast, recombinant IL-2 induced expression of IL-5 mRNA but did not induce de-

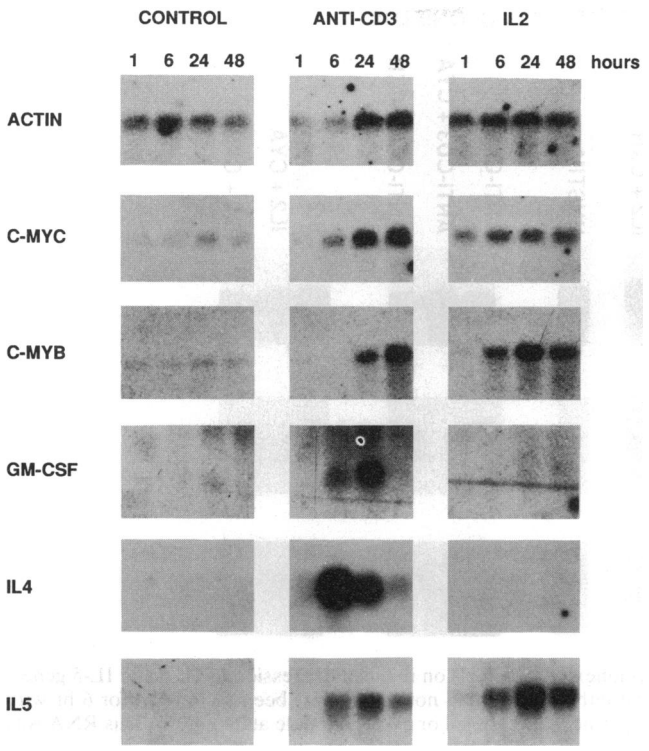

FIG. 2. Northern blot showing time course of expression of T-cell activation genes after stimulation with anti-CD3 antibody or with recombinant IL-2. Total cellular RNA was prepared from clone 10-5-17 cells stimulated with culture medium (control), anti-CD3 antibody, or recombinant IL-2 for the indicated time. This RNA was used to prepare Northern blots that were hybridized with the indicated probes.

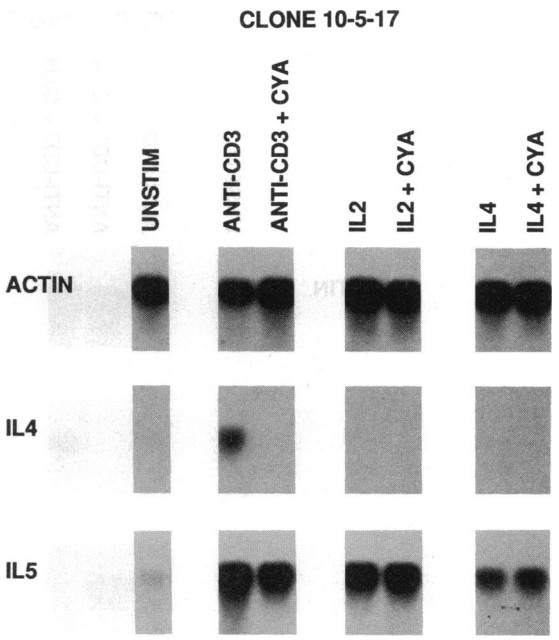

FIG. 3. Northern blot showing the effect of cyclosporine A (CYA) on induced expression of IL-4 and IL-5 genes. Total cellular RNA was prepared from clone 10-5-17 that had or had not (UNSTIM) been stimulated for 6 hr with anti-CD3 antibody, recombinant IL-2, or recombinant IL-4 in the presence or absence of cyclosporine A at 1 μg/ml. This RNA was used to prepare Northern blots that were hybridized with the indicated probes.

tectable IL-4 mRNA or GM-CSF mRNA expression, suggesting that these lymphokine genes are differentially regulated after stimulation with recombinant IL-2. These results were consistent in five independent experiments. Because Th2 clones use IL-4 as an autocrine lymphokine, the effect of exogenous recombinant IL-4 on IL-5 gene expression was also examined (Fig. 3). Stimulation of clone 10-5-17 with recombinant IL-4 induced expression of IL-5 mRNA but did not induce detectable expression of IL-4 mRNA, suggesting that the IL-4 and IL-5 genes are also differentially regulated after stimulation with IL-4.

Cyclosporine A and Cycloheximide Differentially Affect IL-4 and IL-5 Gene Expression. Because the IL-4 and IL-5 genes appear to be expressed coordinately after anti-CD3 stimulation and differentially after lymphokine stimulation, experiments were done to assess the expression of these genes in the presence of cyclosporine A or the protein-synthesis inhibitors, cycloheximide or anisomycin. As mentioned, cyclosporine A is thought to inhibit T-cell activation by preventing expression of lymphokine genes (18, 19). Cyclosporine A completely inhibited expression of IL-4 mRNA induced by immobilized anti-CD3 antibody in clones 10-5-17 and D10 (Figs. 3 and 4). In contrast, cyclosporine A only partially inhibited anti-CD3-induced IL-5 mRNA expression in these clones. Cyclosporine A did not inhibit IL-5 mRNA expression induced by recombinant IL-2 or recombinant IL-4 in clone 10-5-17 and only partially inhibited IL-2-induced IL-5 mRNA expression in clone D10. The protein-synthesis inhibitors cycloheximide (Fig. 4) or anisomycin (data not shown) did not inhibit or only partially inhibited anti-CD3-induced IL-4 gene expression 6 hr after stimulation, and neither cycloheximide nor anisomycin inhibited IL-4 gene expression 4 hr after anti-CD3 stimulation (data not shown). In contrast, cycloheximide and anisomycin completely inhibited IL-5 gene expression induced by anti-CD3 or IL-2. These effects of cyclosporine A and protein-synthesis inhibitors were confirmed in three independent experiments and strongly suggest that the IL-4 and IL-5 genes are differentially regulated.

DISCUSSION

T-cell receptor-mediated stimulation of Th2 clones is thought to trigger an intracellular activation pathway that leads to the expression of several activation genes, including IL-4. IL-4 then acts as an autocrine growth factor to stimulate expression of additional activation genes, perhaps through a second intracellular activation pathway, which ultimately leads to cellular proliferation. Unlike resting T cells, many murine helper T-cell clones proliferate in response to exogenous lymphokines, bypassing the requirement for T-cell receptor-mediated signals (4–7). Because the Th2 clones 10-5-17 and D10 proliferate in response to immobilized anti-CD3 antibody, as well as to recombinant IL-2 or recombinant IL-4 (Fig. 1), these clones were used to compare changes in gene expression induced by T-cell receptor or lymphokine receptor-mediated signals. Although expression of a given gene may be regulated at a number of levels, including transcription and mRNA degradation, in this discussion "gene expression" refers to the level of specific mRNA detectable by Northern blot analysis.

As shown in Fig. 2, immobilized anti-CD3 antibody and recombinant IL-2 induce different patterns of expression of T-cell activation genes in clone 10-5-17. Both stimuli induce expression of the protooncogenes c-*myc* and c-*myb*. Because expression of these protooncogenes is associated with proliferation, this result is consistent with the finding that these stimuli both induce proliferation. Immobilized anti-CD3 antibody and recombinant IL-2, however, induce different patterns of lymphokine gene expression. Immobilized anti-CD3 antibody induces expression of the lymphokine genes IL-4, IL-5, and GM-CSF. In contrast, recombinant IL-2

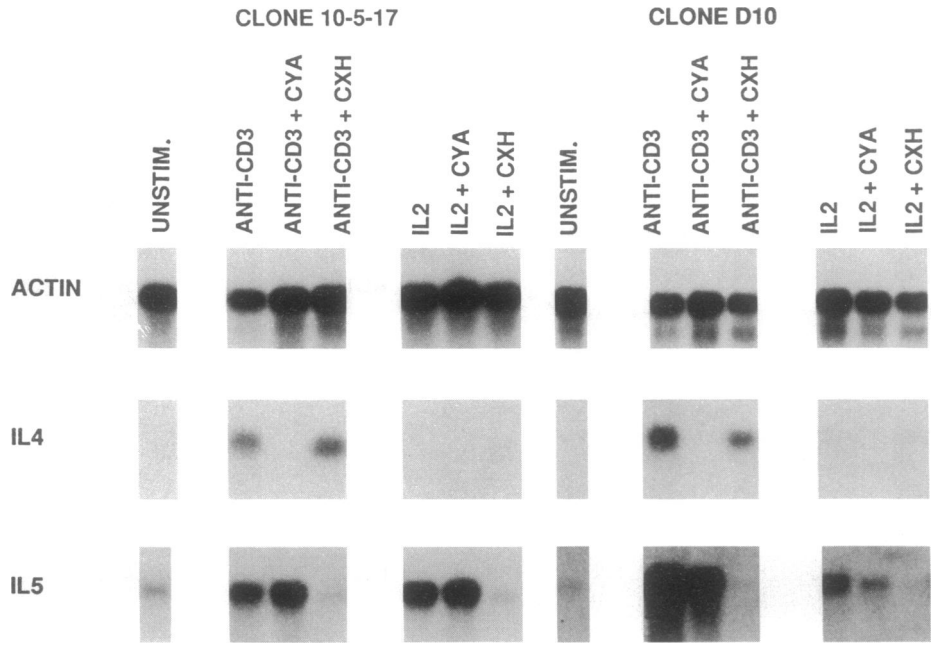

FIG. 4. Northern blot showing the effect of cyclosporine A (CYA) or cycloheximide (CXH) on induced expression of IL-4 and IL-5 genes. Total cellular RNA was prepared from clone 10-5-17 or clone D10 cells that either had or had not (UNSTIM) been stimulated for 6 hr with anti-CD3 antibody or recombinant IL-2 in the presence or absence of cyclosporine A at 1 μg/ml or cycloheximide at 20 μg/ml. This RNA was used to prepare Northern blots that were hybridized with the indicated probes.

induces expression of IL-5 mRNA but no detectable expression of IL-4 or GM-CSF mRNA. Furthermore, IL-4, which is the autocrine growth factor used by Th2 clones, also induces IL-5 mRNA expression but not IL-4 mRNA expression (Fig. 4). IL-2 stimulation not only induces IL-5 mRNA expression but also induces IL-5 secretion in clone 10-5-17 as shown by ELISA (data not shown).

T-cell clones that have been stimulated through their T-cell receptor do not normally secrete only a single lymphokine but, rather, secrete several lymphokines in a defined pattern. For instance, murine T helper type 1 (Th1) clones characteristically secrete IL-2, lymphotoxin, interferon γ, IL-3, and GM-CSF after antigen or lectin stimulation; whereas Th2 clones characteristically secrete IL-4, IL-5, IL-3, and GM-CSF (8, 9). Although both T helper type 1 and Th2 clones express the IL-3 and GM-CSF genes, the genes encoding IL-2, lymphotoxin, and inteferon γ are regulated such that they are expressed only in Th1 clones and not in Th2 clones. Similarly, the IL-4 and IL-5 genes are regulated such that they are expressed only in Th2 clones and not in Th1 clones. Thus, it appears that the IL-2, lymphotoxin, interferon γ, IL-3, and GM-CSF genes are coordinately expressed in Th1 clones, and the IL-4, IL-5, IL-3, and GM-CSF genes are coordinately expressed in Th2 clones after antigen receptor-mediated stimulation.

Because the IL-4 and IL-5 genes appear to be coordinately expressed after anti-CD3 stimulation and differentially expressed after stimulation with recombinant IL-2 or recombinant IL-4 (Fig. 2), the pharmacologic inhibitors cyclosporine A and cycloheximide were used to dissect the activation pathways leading to the expression of these genes. Cyclosporine A is thought to block T-cell activation by preventing transcription of autocrine lymphokine genes (18, 19). It has been suggested that cyclosporine A mediates its effect by altering the binding of regulatory DNA-binding proteins (20). As shown in Fig. 1, cyclosporine A completely inhibits proliferation of clone 10-5-17 induced by immobilized anti-CD3 antibody but has little effect on proliferation induced by recombinant IL-2 or recombinant IL-4. Because proliferation induced by exogenous lymphokines is relatively unaffected by doses of cyclosporine A that completely inhibit anti-CD3-induced proliferation, it appears that those activation events critical for proliferation that occur downstream from lymphokine/lymphokine receptor interaction are less sensitive to cyclosporine A. Cyclosporine A completely blocks expression of IL-4 mRNA induced by immobilized anti-CD3 antibody in clone 10-5-17 and D10. However, IL-5 mRNA expression induced by immobilized anti-CD3 antibody is only partially blocked. Furthermore, IL-5 mRNA expression induced by recombinant IL-2 or recombinant IL-4 is not blocked by cyclosporine A in clone 10-5-17, and IL-5 mRNA expression induced by recombinant IL-2 in clone D10 is only partially blocked by cyclosporine A. Therefore, at least two pathways for IL-5 gene expression appear to exist. Because recombinant IL-4 induces IL-5 gene expression, anti-CD3 stimulation probably induces IL-5 gene expression through an autocrine IL-4 pathway. However, an IL-4 independent pathway also exists because cyclosporine A completely blocks anti-CD3-induced proliferation and IL-4 gene expression, whereas anti-CD3-induced IL-5 gene expression continues in the presence of cyclosporine A. Although cyclosporine A completely inhibits anti-CD3-induced IL-4 mRNA expression but not IL-5 mRNA expression, the protein-synthesis inhibitors cycloheximide and anisomycin completely inhibit anti-CD3-induced IL-5 mRNA expression but not IL-4 mRNA expression, suggesting that IL-5 gene expression depends more upon protein synthesis than does IL-4 gene expression. Although IL-4 and IL-5 genes are coordinately expressed after T-cell receptor-mediated stimulation, the effects of cyclosporine A and protein-synthesis inhibitors show that the pathways by which each of these genes is expressed after T-cell receptor-mediated stimulation differ.

Numerous T-cell receptor-independent pathways for lymphokine gene induction have been described. For example, antibodies directed against the CD28 molecule, in combination with phorbol 12-myristate 13-acetate, induce lymphokine gene expression in human peripheral blood T cells. In contrast to the T-cell receptor-mediated pathway for lymphokine gene induction, which is blocked by cyclosporine A,

the CD28 pathway is cyclosporine A-resistant (21). Another cyclosporine A-resistant pathway has been described whereby lymphokine gene expression in murine T-cell clones is induced by IL-2 and syngeneic or allogeneic spleen cells in the absence of antigen (22). Thus, lymphokine genes appear to be regulated by signals transmitted through the T-cell receptor, lymphokine receptors, and other T-cell surface molecules. The results presented here indicate that the IL-4 and IL-5 genes may be coordinately or differentially expressed, depending on the stimulus.

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