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O. AyanlarBatuman
*Thomas Jefferson University*

A. P. Ferrero
*Thomas Jefferson University*

Arturo Diaz
*Thomas Jefferson University*

Sergio A. Jimenez
*Thomas Jefferson University*, Sergio.Jimenez@jefferson.edu

# Regulation of Transforming Growth Factor-β1 Gene Expression by Glucocorticoids in Normal Human T Lymphocytes

**Olcay AyanlarBatuman,* Ann P. Ferrero,* Arturo Diaz,‡ and Sergio A. Jimenez‡**
**Cardeza Foundation for Hematologic Research and the* ‡*Rheumatology Division, Department of Medicine, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107*

## Abstract

**Glucocorticoids (GC) modulate immune function in a number of ways, including suppression of T cell proliferation and other IL-2–mediated T cell functions. These inhibitory effects are similar to those induced by transforming growth factor-β1 (TGF-β1), a cytokine with potent T cell inhibiting activities. We examined the hypothesis that GC effects may be at least partially achieved through modulation of the expression of the TGF-β1 gene in activated T cells. Normal T cells were cultured with or without purified phytohemagglutinin (PHA-p) and 4β-phorbol 12-myristate 13-acetate (PMA) in the presence or absence of the synthetic GC, dexamethasone (100–200 μg/ml). The production of latent and active forms of TGFβ by these cells were analyzed by immunoblotting and bioassays. The steady-state levels of TGF-β1 mRNA were analyzed in total RNA from these cells by Northern hybridizations using a human TGF-β1 cDNA. The results showed that dexamethasone caused an increase in TGFβ production and a dose-dependent two to fourfold increase in TGF-β1 mRNA in activated as well as in unstimulated T cells, 1 h after exposure of the cultures to the steroid. The increase in TGF-β1 mRNA levels by dexamethasone was further potentiated two to threefold by cycloheximide, suggesting that the steroid effect may be due to inhibition of the synthesis of proteins that decrease TGF-β1 gene transcription or the stability of its transcripts. Finally, in vitro nuclear transcription studies indicated the dexamethasone effects on TGF-β1 gene expression to be largely transcriptional. (*J. Clin. Invest.* 1991. 88:1574–1580.) Key words: transforming growth factor β • glucocorticoids • T cell activation • dexamethasone**

## Introduction

Glucocorticoids (GC)[1] are immunosuppressive and can induce lympholysis in vitro and in vivo. Although these properties have resulted in the wide clinical application of GC as antiinflammatory and antineoplastic agents, the mechanisms by which they modulate immune system function are unclear. One possibility, supported by accumulating experimental evidence, is that GC inhibit the synthesis and release of immunoregulatory molecules, such as IL-2 and γ-IFN, by activated T cells (1–5). These lymphokines promote the expansion of antigen-specific helper/inducer or cytotoxic/suppressor T cell clones as well as natural killer cells that are involved in immune regulation and surveillance (6). The steady-state mRNA levels of both IL-2 and γ-IFN are significantly decreased when T cells are activated in vitro with purified phytohemagglutinin (PHA-p) and phorbol myristate acetate (PMA) in the presence of dexamethasone (4). The inhibitory effects of GC on immunoregulatory T cell functions in vivo was demonstrated in studies that showed inhibition of both IL-2 and IL-2 receptor (IL-2R) gene expression in activated T cells from GC-treated patients with pulmonary sarcoidosis (7, 8). Recently, it has been shown that transforming growth factor-β1 (TGF-β1) (9), a cytokine produced by mitogen-activated T cells, B cells, monocytes, and fibroblasts, is a potent inhibitor of multiple T cell functions in vitro (10–17). In common with the other members of the TGFβ gene family, TGF-β1 has profound stimulatory effects on fibroblast chemotaxis and on expression of the genes for the extracellular matrix proteins collagen and fibronectin (18). Furthermore, it has been shown that TGF-β1 can upregulate its own production by these cells (19). Although several target genes regulated by TGF-β1 have been identified in a variety of human cells and cell lines, little is known about the mechanisms that regulate the expression of the TGF-β1 gene.

The immunosuppressive effects of GC and TGF-β1 on T lymphocytes share important similarities. Both inhibit IL-2–mediated T cell functions, such as lectin- and IL-2–induced T cell proliferation and generation of regulatory T cells in vitro (20–22) and in vivo (23, 24). For this reason, we investigated whether GC altered the TGF-β1 gene expression in mitogen-activated human T cells. Our results show that dexamethasone significantly increases the expression of the TGF-β1 gene and its protein product in these cells. Its effect on the TGF-β1 gene is observed as early as 1 h after T cell induction with PHA-p/PMA. The enhancement of the steady-state TGF-β1 mRNA levels in response to cycloheximide and the results of in vitro nuclear transcription studies indicate that dexamethasone may regulate TGF-β1 gene expression at a transcriptional level.

## Methods

*Cells.* PBMC, $2 \times 10^8$–$2.5 \times 10^9$ were isolated by density gradient separation from buffy coat by-products of whole blood donated by healthy volunteers (Interstate Blood Bank, Philadelphia, PA). Buffy coats from 20 different donors, 21–31 yr old, were studied. Adherent cell-depleted T cells were prepared by incubating the PBMC in plastic petri dishes for 4 h and passing the nonadherent cells twice over nylon wool columns (25). Purity of nylon wool nonadherent T cell populations was determined by flow cytometry and histocytochemistry, using an FITC-conjugated anti-pan T cell MAb anti-CD2 (Becton Dickin-

Address correspondence to Olcay AyanlarBatuman, M.D., Cardeza Foundation for Hematologic Research, Jefferson Medical College, Curtis Building, Room 812, 1015 Walnut Street, Philadelphia, PA 19107.

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1. *Abbreviations used in this paper:* GAPD, glyceraldehyde-3-phosphate dehydrogenase; GC, glucocorticoids; PHA-p, purified phytohemagglutinin; TGF-β1, transforming growth factor-β1.

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son Immunocytometry Systems, Mountain View, CA), and a phycoerythrin-conjugated MAb anti-CD14 (Becton Dickinson) which reacts with cells of monocytic origin. Flow cytometric determination of membrane immunofluorescence with two color analysis (26) showed that T cells were > 98% CD2+ and < 1% CD14+ when compared with nonbinding FITC- and phycoerythrin-labeled MAbs; < 1% cells reacted with alpha-naphthyl-acetate esterase. Viability was > 99% as assessed by trypan blue dye exclusion.

*Activation of T cells and culture conditions.* CD2+ lymphocytes were cultured at $2 \times 10^6$/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% defined pooled human AB serum (Gibco), penicillin 100 U/ml, streptomycin (100 $\mu$g/ml) in 25-cm$^2$ tissue culture flasks (Corning Glass Works, Corning, NY) in a humidified 5% $CO_2$ incubator at 37°C. T cells were stimulated with 2 $\mu$g/ml PHA-p (Wellcome Diagnostics, Research Triangle Park, NC) and 30 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) at 37°C for 1–72 h. In some experiments, the human AB serum was omitted from cultures that were harvested at 1 h. Dexamethasone (ESI Pharmaceuticals, Cherry Hill, NJ) (100 $\mu$g/ml–200 $\mu$g/ml) was added to T cell cultures as indicated, the incubations were stopped at appropriate serial time points, and cells were harvested for RNA extraction. In some experiments 30 ng/ml cycloheximide (Sigma), was added to cultures at the times indicated. To quantitate TGF-$\beta$1 levels, supernatants were obtained from T cells cultured in serum-free Aim V medium (Gibco) in the presence or absence of PHA-p (2 $\mu$g/ml)/PMA (30 ng/ml) with or without dexamethasone (20–100 $\mu$g/ml), for 96 h. Supernatants were brought to 1 M final concentration with acetic acid, and were incubated for 10 min–2 h at room temperature to activate latent TGF-$\beta$1. Samples were neutralized to pH 7.0–7.4, lyophilized to dryness, and resuspended in 100 $\mu$l of 0.1% BSA (Sigma) in Dulbecco's modified Eagle's medium (Gibco).

*RNA isolation and Northern hybridizations.* Total RNA was isolated from cells using guanidinium isothiocyanate, according to the method of Chirgwin et al. (27). RNA content of individual samples was equalized by ethidium bromide staining of previously run 1% nondenaturing agarose gels. The samples containing equal amounts of RNA were then separated on 1% agarose-formaldehyde gels and transferred to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, IL). Nick translated, [$\alpha$-$^{32}$P] dCTP-labeled cDNA was hybridized to the filters in 50% formamide, 0.1% SDS, 1× Denhardt's solution, 2× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 50 $\mu$g/ml salmon sperm DNA, with a sp act of $3–4 \times 10^8$ cpm/$\mu$g, at 42°C for 16–20 h. After hybridization, filters were washed twice for 5 min at 22°C in 1× SSC–0.1% SDS, then twice for 30 min at 55°C in 0.1× SSC–0.1% SDS. Autoradiography was performed by exposing to XAR film (Eastman Kodak Co., Rochester, NY) at −70°C. Quantitation of the radioactive band intensities in autoradiographs was performed by scanning laser densitometry and the areas under tracings expressed as a fold increase relative to control samples as indicated.

*DNA probes.* The TGF-$\beta$1 probe is a gel purified 1.05-kb cDNA insert cloned in pSB64, that hybridizes to a 2.5-kb TGF-$\beta$1 mRNA (kindly donated by Dr. Richard Derynck, Genentech) (28). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA in pBR322 that hybridizes to a 1.7-kb GAPD mRNA was used as a control probe.

*Nuclear transcription and isolation of labeled RNA.* Transcription was measured in a nuclear runoff transcription assay (29). For preparation of nuclei, $2 \times 10^8$ cells were suspended in 9 ml of cold buffer A (0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris, pH 7.5, 0.5 mM dithiothreitol). 1 ml of 10% Triton X-100 was added, cells were homogenized with a nuclei homogenizer (Con-Torque power unit; Eberbach Corp., Ann Arbor, MI) three times at 300 rpm and the homogenate centrifuged 5 min at 2,500 rpm. The pellet was washed and resuspended in 50 $\mu$l of buffer B (40% glycerol, 50 mM Tris-HCl buffer at pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA), and were stored at −70°C. For preparation of cDNA filters, plasmids were purified by CsCl centrifugation, plasmid DNA was linearized by digestion with restriction enzymes, and denatured in 0.4 M NaOH at 37°C for 15 min and then neutralized with 0.4 vol of 5 M ammonium acetate. The solution was diluted with 10× SSC and 5 $\mu$g of DNA was applied to nitrocellulose using a dot blot apparatus (Schleicher and Schuell, Keene, NH). Filters were UV crosslinked (Stratagene Cloning Systems, La Jolla, CA) and prehybridized for 4 h at 42°C with continuous shaking. Transcription reactions were carried out in a vol of 100 $\mu$l containing 40 $\mu$l of nuclei in 10 mM Tris, (pH 8.0), 90 mM KCl, 3 mM $MgCl_2$, 2 mM dithiothreitol, 0.04 mM EDTA, 16% glycerol, 1 U/$\mu$l RNAsin (Promega Biotec, Madison, WI), 0.4 mM each of ATP, UTP, and GTP, and 500 $\mu$Ci [$^{32}$P]-CTP (800 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, CA). Incubations were for 20 min at 25°C, and incorporation of [$^{32}$P]-CTP was followed by TCA precipitation of 1-$\mu$l aliquots. Transcription was terminated by addition of 900 $\mu$l of buffer C (100 mM NaCl, 10 mM Tris-HCl buffer at pH 7.5, 2 mM KCl, 1 mM EDTA, and 0.5% SDS). The samples were digested with 100 $\mu$g/ml proteinase K for 60 min at 42°C after the addition of yeast tRNA (100 $\mu$g in 10 $\mu$l $H_2O$). Samples were extracted with phenol/chloroform, adjusted to 10% TCA and 10% saturated sodium pyrophosphate, and nucleic acids precipitated. Samples were then centrifuged for 10 min at 4°C, and pellets were washed with 70% ethanol, dried, and dissolved in 100 $\mu$l buffer with 10 mM Tris, 1 mM EDTA, and 0.1% SDS. An additional 100 $\mu$g of yeast tRNA was added, and nucleic acids were ethanol precipitated from 2.5 M ammonium acetate. The pellets were dissolved in 100 $\mu$l of buffer that contained 20 mM Tris-HCl, 10 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.5, and incubated for 30 min at 37°C with 100 $\mu$g/ml RNAse-free DNAse (Promega) and 1 U/$\mu$l RNAsin. Samples were extracted with phenol/chloroform, and ethanol precipitated from 0.3 M sodium acetate. Labeled transcripts were resuspended in prehybridization buffer, and 5 $\mu$l TCA were precipitated and counted. $8.5–10.5 \times 10^6$ cpm of each sample were adjusted to 400 $\mu$l in the same buffer and hybridized to the filters with appropriate probes with continuous shaking at 42°C for 72 h. The filters were washed for 15 min in 2× SSC and treated with RNaseA (10 $\mu$g/ml in 2× SSC) for 15 min at 37°C; washed in 2× SSC, 0.1%, SDS for 15 min at room temperature and for 15 min at 60°C; dried and exposed to film, then cut out and $^{32}$P content directly determined in a scintillation counter.

*TGF-$\beta$1 assay.* The CCL64 assay was performed as described by Tucker et al. (30). In brief, CCL64 cell line obtained from American Type Culture Collection, Bethesda, MD, was maintained in DMEM (Gibco), supplemented with penicillin (100 U/ml), streptomycin (100 $\mu$g/ml), and 10% defined fetal calf serum (Gibco) in a humidified 5% $CO_2$ incubator at 37°C. To assay for TGF-$\beta$1, $6 \times 10^4$ cells/well were grown in 24-well tissue culture plates (3548; Costar Corp., Cambridge, MA) until nearly confluent when media was changed to DMEM 5% FCS. After 24 h, 300 $\mu$l of each supernatant was added to triplicate wells. Unless otherwise stated, each supernatant was tested in dilutions of 1:10, 1:100, 1:1,000, and 1:10,000. After 24 h incubation, the cells were pulsed with [$^3$H] thymidine (2 $\mu$Ci/ml) for 4 h, the medium was removed, and the cell layers were washed with 1 ml of ice cold 10% TCA for 10 min. Cells were solubilized in 250 $\mu$l of 0.25 N NaOH for 10 min and harvested using a microculture harvesting device and counted in liquid scintillation counter to measure [$^3$H] thymidine uptake. For each assay a standard curve was obtained with 4 ng–1 pg/ml of a purified human TGF-$\beta$1 standard (R and D Systems, Inc., Minneapolis, MN). Results were expressed as counts per minute. To neutralize TGF-$\beta$1 activity, a rabbit anti-TGF-$\beta$1 (AB-10-NA, R and D Systems) was added at 1:500 and 1:1,000 dilutions.

*Immunoblot analysis for TGF-$\beta$1.* Equal volume aliquots of the supernatants were slot-blotted on nitrocellulose membranes, using a slot-blot apparatus (Bio-Dot SF; Bio-Rad Laboratories, Richmond, CA) according to manufacturer's instructions. Membranes were then reacted with a rabbit polyclonal antibody to TGF-$\beta$1, (AB-20-PB, R and D Systems) and the bound antibody visualized with horseradish peroxidase-coupled goat anti-rabbit IgG.

## Results

*Stimulation of TGF-$\beta$1 gene expression in activated T cells.* To determine the expression of the TGF-$\beta$1 gene by T cells, other

mononuclear cells that produce TGF-β1 were removed. Since T cells cannot be induced to produce IL-2, to express high affinity IL-2 receptors, or to proliferate by PHA-p alone in the absence of adherent cells (6, 30, 31), PMA was also added to T cell cultures. The phorbol ester is the most effective substitute for adherent cells in activating adherent cell-depleted T cells with mitogen. To confirm the expression of TGF-β1 gene by human T cells, adherent cell depleted-T cells were cultured with or without PHA-p/PMA for 24 h. Total RNA from these cells was isolated and examined by Northern hybridization with the TGF-β1 cDNA. To determine the baseline TGF-β1 mRNA levels, T cells were subjected to RNA extraction immediately after they were isolated from the nylon wool columns. Fig. 1 shows the steady-state levels of TGF-β1 mRNA in resting (*a*), cultured but unstimulated (*b*), and PHA-p/PMA-stimulated (*c*) T cells from one individual. Clearly detectable levels of TGF-β1 mRNA were found in resting T cells that were processed immediately after isolation. T cells that were cultured in the absence of mitogens for 24 h in medium containing 5% human AB serum expressed three to four times more TGF-β1 mRNA (Fig. 1 *b*) than resting T cells. This pattern of response to dexamethasone was consistently observed in all individuals studied. In T cells from the 20 donors, the increase in TGF-β1 mRNA expression after culture for 24 h was 3.8±0.7 (2 SD)-fold compared to resting T cells. Stimulation of T cells for 24 h with PHA-p (2 μg/ml) and PMA (30 ng/ml) resulted in a 16.8±0.9 (2 SD)-fold increase in TGF-β1 mRNA compared to the resting T cells ($P < 0.01$). To rule out the possible upregulation of TGF-β1 mRNA by TGF-β1 that may have been present in the serum used, T cells were also cultured in the absence of serum for 24 h. Steady-state TGF-β1 mRNA levels in T cells cultured with or without serum were not different (data not shown).

*Effect of dexamethasone on steady-state TGF-β1 mRNA in T cells.* To determine the effects of dexamethasone on TGF-β1 steady-state mRNA levels, adherent cell-depleted T cells from 15 individuals were cultured with or without PHA-p/PMA in the presence of dexamethasone. A representative experiment from one individual donor is shown in Fig. 2. Dexamethasone (100 μg/ml or 200 μg/ml), in the absence of mitogens, resulted in a 4.2±0.6 (2 SD)- and 7.1±0.8 (2 SD)-fold increase, respectively, in TGF-β1 mRNA levels compared to T cells cultured without dexamethasone ($P < 0.01$) (Fig. 2 *a*). Dexamethasone induced a concentration-dependent increase in steady-state levels mRNA in PHA-p/PMA treated T cells, as well (Fig. 3). In 15 donors, the increase in TGF-β1 mRNA levels in PHA-p/PMA stimulated T cells after 24 h of incubation with 100 μg/ml and 200 μg/ml dexamethasone was 3.2±0.5 (2 SD)- and 6.2±0.4 (2 SD)-fold respectively. As shown in Fig. 4, the stimulatory effect of dexamethasone was detected as early as 1 h after it was added to T cells. TGF-β1 mRNA levels were two and fourfold greater in T cells induced for 1 h or 2 h with PHA-p/PMA in the presence of 100 μg/ml dexamethasone, compared

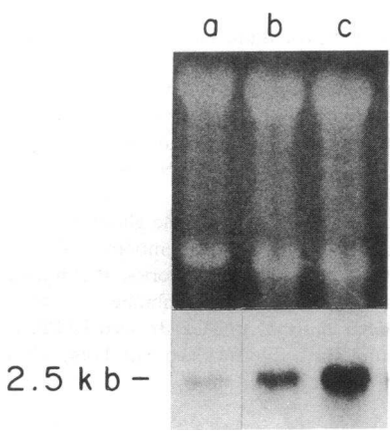


*Figure 1.* Steady-state TGF-β1 mRNA levels in stimulated and unstimulated peripheral blood T cells from one individual. Northern hybridization analyses were performed on total RNA. Cells were either resting (*a*), cultured for 24 h without stimulation (*b*), or exposed for 24 h to PHA-p (2 μg/ml) and PMA (30 ng/ml) (*c*). Upper part of the figure shows the ethidium bromide-stained formaldehyde gel with bands corresponding to 28S and 18S ribosomal RNA. 5 μg of total RNA was loaded in each lane.

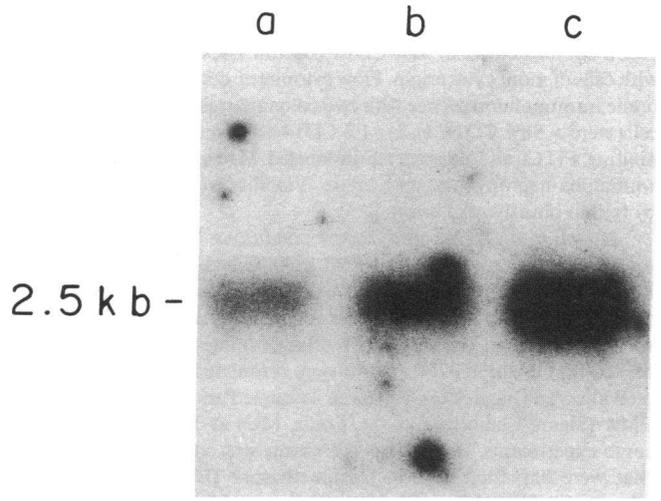


*Figure 2.* Effect of dexamethasone on TGF-β1 mRNA levels in unactivated T cells from one individual. Northern hybridization analyses were performed on total RNA from T cells that were either cultured for 24 h without stimulation (*a*), or treated for 24 h with dexamethasone 100 μg/ml (*b*), or 200 μg/ml (*c*). 5 μg of total RNA was loaded in each lane.

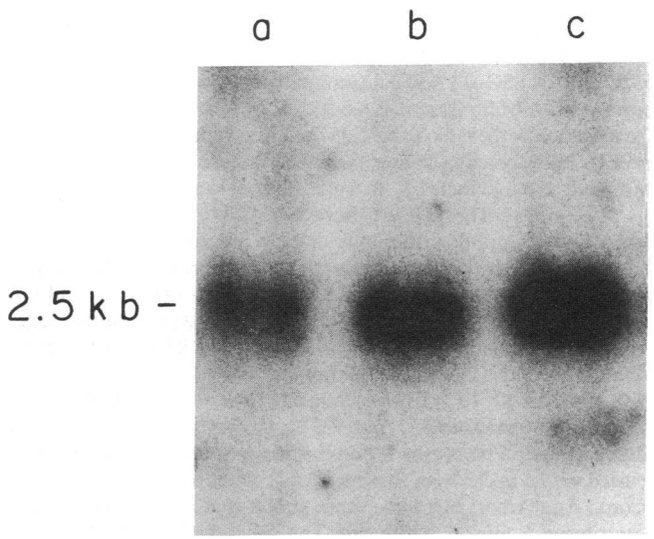


*Figure 3.* Effect of dexamethasone on TGF-β1 mRNA levels in activated T cells from one individual. Cells were either treated for 24 h with PHA-p (2 μg/ml)/PMA (30 ng/ml), in the absence of dexamethasone (*a*), or in the presence of 100 μg/ml dexamethasone (*b*), or 200 μg/ml dexamethasone (*c*). 5 μg of total RNA was loaded in each lane.

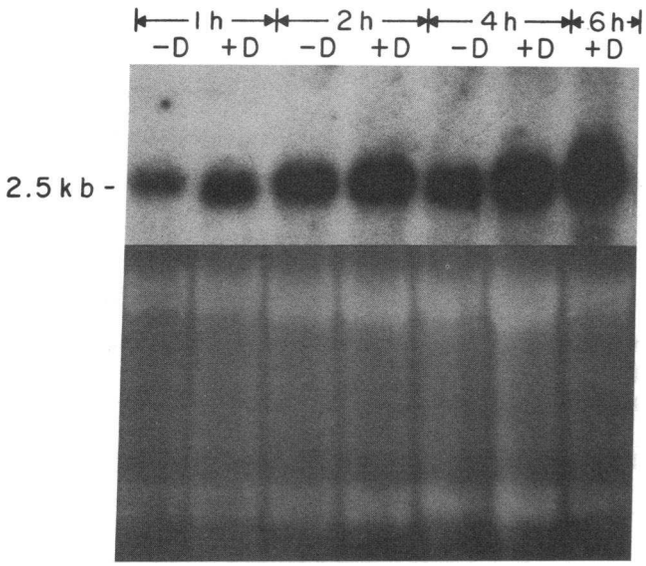


*Figure 4.* Time course of dexamethasone action on the levels of TGF-β1 mRNA in T cells from one individual activated with PHA-p/PMA. Northern hybridization analyses were performed on total RNA from T cells treated with PHA-p (2 μg/ml)/PMA (30 ng/ml) for 1, 2, or 4 h in the presence or absence of dexamethasone (100 μg/ml) and 6 h in the presence of dexamethasone (100 μg/ml). 5 μg of total RNA was loaded in each lane. Lower part of the figure shows the ethidium bromide-stained formaldehyde gel with bands corresponding to 28S and 18S ribosomal RNA.

to those that did not receive dexamethasone. Similar results were obtained in experiments with T cells from four additional individuals. The dexamethasone-induced increase in TGF-β1 mRNA levels reached a peak at 4–6 h and persisted for at least 24 h, as long as dexamethasone and the mitogens were present in the culture medium (data not shown).

We next investigated whether the presence of dexamethasone at the initiation of T cell activation was necessary for its stimulatory effect on TGF-β1 mRNA levels. As illustrated in Fig. 5, when dexamethasone (100 μg/ml) was added as late as 12 h after T cell stimulation by PHA-p/PMA it caused a two-fold increase in TGF-β1 mRNA levels over the subsequent 12 h (Fig. 5, *a* and *b*). In contrast, when dexamethasone was added to cultures 20 h after PHA-sp/PMA, it was ineffective in elevating TGF-β1 mRNA levels in T cells (Fig. 5 *c*). Similar results were obtained in experiments with T cells from seven additional individuals.

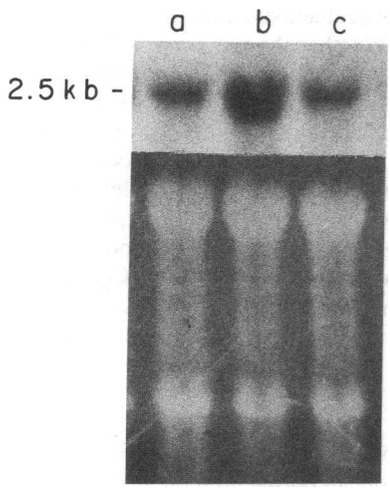


*Figure 5.* Effect of time of addition of dexamethasone on TGF-β1 mRNA levels in activated T cells from one individual. Northern hybridization analyses were performed on total RNA from T cells treated with PHA-p (2 μg/ml) and PMA (30 ng/ml) for 24 h in the absence of dexamethasone (*a*). To identical cultures, dexamethasone (200 μg/ml) was added at 12 h (*b*), or 24 h (*c*). Lower part of the figure shows the ethidium bromide-stained formaldehyde gel with bands corresponding to 28S and 18S ribosomal RNA. 5 μg total RNA was loaded in each lane.

*Effect of cycloheximide on dexamethasone-stimulated expression of TGF-β1 gene by activated T cells.* We next investigated whether dexamethasone-induced stimulation of TGF-β1 mRNA expression by PHA-p/PMA activated T cells required de novo protein synthesis. In experiments not shown, we found that 30 μg/ml cycloheximide caused > 97% inhibition of $^{14}$C-leucine incorporation into newly synthesized protein in PHA-p/PMA-activated T cells. Therefore, nonactivated T cells and T cells activated with PHA-p/PMA in the presence or absence of dexamethasone (100 μg/ml) were cultured with cycloheximide (30 μg/ml) for 6 h. As shown in Fig. 6, unstimulated (Fig. 6, *a* and *b*), as well as PHA-p/PMA-activated T cells cultured in the absence (Fig. 6, *c* and *d*) or in the presence (Fig. 6, *e* and *f*) of dexamethasone showed increased steady-state TGF-β1 mRNA levels after incubation with cycloheximide for 6 h. In contrast, the steady-state mRNA levels for GAPD, used as a control, did not change. Similar results were obtained in experiments with T cells from five additional normal donors.

*Effect of dexamethasone on TGF-β1 gene transcription.* To explore the level of regulation of the expression of TGF-β1 gene that is modulated by dexamethasone, in vitro nuclear

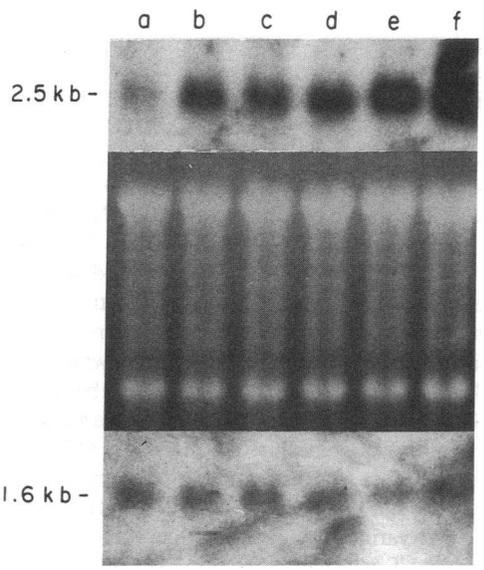


*Figure 6.* Effect of cycloheximide of TGF-β1 mRNA levels in activated and unactivated T cells from one individual. Northern hybridization analyses were performed on total RNA from T cells that were cultured in serum-free medium for 6 h without PHA-p/PMA (*a*), with cycloheximide (*b*), with PHA-p/PMA (*c*), with PHA/PMA and cycloheximide (*d*), with PHA-p/PMA and dexamethasone (100 μg/ml) (*e*), and PHA-p/PMA, cycloheximide, and dexamethasone (*f*). Lower part of the figure shows the ethidium bromide-stained formaldehyde gel with bands corresponding to 28S and 18S ribosomal RNA, respectively. 5 μg of total RNA was loaded in each lane. Lowest part of the figure shows the same blot rehybridized with a [$^{32}$P]-labeled GAPD cDNA probe that hybridizes to a 1.6-kb mRNA, as described in Methods.

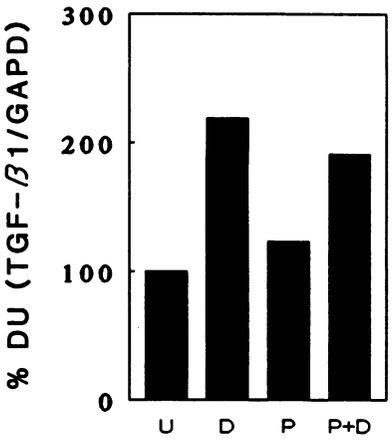


*Figure 7.* Dexamethasone stimulation of TGF-β1 gene transcription. Results of three experiments are shown. T cells were cultured for 6 h in the absence or presence of dexamethasone (200 μg/ml). The in vitro nuclear run-off assay was performed as described in Methods. The signals were quantitated by laser densitometry. Plasmid vector without insert was used to estimate the background level, which was virtually zero. Values are expressed as percent of control and are normalized by comparing the intensities of signals from the experimental conditions to those obtained from control hybridization with GAPD cDNA.

*Table I. Effect of Supernatants from Human T Lymphocytes on the Proliferation of CCL64 Cells**

| Sample added | [$^3$H]-thymidine uptake | |
|---|---|---|
| | *cpm (×10$^{-3}$)* | |
| Aim V media | 53.15±2.8 (100)‡ | |
| Aim V media + Dex (50 μg/ml) | 37.82±2.3 (71) | |
| T Cell supernatants | Acidified | Nonacidified |
| Unstimulated | 38.7±2.3 (73) | 44.7±2.1 (80) |
| PHA-p/PMA | 20.4±1.3 (38) | 22.3±4.2 (40) |
| PHA-p/PMA + Dex (50 μg/ml) | 9.6±0.9 (18)§ | 17.4±1.8 (31) |
| PHA-p/PMA + Dex + anti-TGFβ-1 | 44.5±0.5 (80)‖ | ND |
| TGFβ-1 standard | 7.9±1.3 (6.8) | ND |
| TGFβ-1 standard + anti-TGFβ-1 | 32.5±1.7 (58) | ND |

* Supernatants from T cells cultured under various experimental conditions were added to preconfluent cultures of CCL64 cells and the [$^3$H]-thymidine incorporation into macromolecular DNA was determined as described in Methods. Each supernatant was tested in triplicate and the values shown are the averages of [$^3$H]-thymidine incorporation expressed as cpm × $10^{-3}$±2 SD. Data presented in this table represent results obtained with 1:10,000 dilution of the T cell supernatants as well as conditioned media. TGF-β1 standard was used at a final concentration of 0.04 ng/ml. In certain experiments the anti-TGFβ antibody AB-10-NA (R and D Systems) was added as described in Methods. ND, not tested. ‡ Numbers in parentheses indicate percent proliferation of CCL64 cells. § $P < 0.01$ compared to cpm of samples cultured without dexamethasone. ‖ $P < 0.01$ compared to cpm of respective samples that were cultured without antibody.

transcription assays were performed with nuclei isolated from T cells that had been incubated for 6 h with PHA-p/PMA in the presence or absence of dexamethasone (100 μg/ml). The results of three experiments summarized in Fig. 7, show that the rate of transcription of the TGF-β1 gene is increased over twofold by 100 μg/ml dexamethasone, while stimulation with mitogen did not affect the transcription rate. However, addition of dexamethasone to cells incubated with mitogens, resulted in an increase in the T cell TGF-β1 gene transcription rate beyond that seen with dexamethasone alone. These observations suggested that dexamethasone and mitogens stimulate TGF-β1 mRNA expression by different mechanisms.

*Effect of dexamethasone on production of TGF-β1 by T cells.* To ascertain whether the dexamethasone-induced increase in steady-state TGF-β1 mRNA levels is accompanied by a concomitant increase in the production of TGF-β1 protein, we determined the biological activity of TGF-β1 in T cell supernatants. For this purpose, T cells were cultured without serum in the presence or absence of PHA-p/PMA with or without dexamethasone. The collected supernatants were assayed for TGF-β1 biologic activity in the CCL64 bioassay which uses a lung epithelial cell line (MV1 Lu) that is known to be growth inhibited by TGF-β1 (26). As seen in Table I, whereas supernatants from unactivated T cells inhibited proliferation of CCL64 cells by 27%, supernatants from activated T cells resulted in a 62% inhibition. Although the levels of TGF-β1 produced by T cells showed a large variation, acidified supernatants conditioned by unactivated T cells had ~ 8 pg/ml TGF-β1-like biologic activity; supernatants from PHA-activated T cells had twofold higher levels of ~ 16 pg/ml dexamethasone treated-activated T cells had the highest levels of ~ 30 pg/ml. We realize the shortcomings of this biological assay and are in the process of setting up a more direct and sensitive ELISA assay to confirm our present observations. The supernatants from T cells that were activated in the presence of dexamethasone induced a further inhibition to 82%. To confirm that the inhibition of CCL64 proliferation was indeed due to TGF-β1, the supernatants were treated with a specific anti-TGF-β1 antibody. The antibody significantly reduced the inhibitory effects of supernatants from T cells activated in the presence of dexamethasone indicating that these samples contained TGF-β1 activity which was blocked by the addition of an anti-TGF-β1 antibody (Table I). Although the majority of TGF-β1 activity was obtained after acidification of supernatants, unacidified supernatants also contained TGF-β1 activity, although to a lesser extent compared to acid-activated samples, i.e., 69% vs. 82% after acidification. Whether the majority of dexamethasone-induced TGF-β1 is in latent form will be tested by experiments in which we shall compare antibody neutralization of acidified versus nonacidified supernatants. The presence of TGF-β1 in supernatants of activated peripheral blood lymphocytes and T cells was further demonstrated by immunoblot analysis. A representative example from four experiments is seen in Fig. 8 and shows that both peripheral blood lymphocytes and T cells produce immuno-detectable TGF-β1 that appears to be highest in concentration in dexamethasone-treated T cell supernatants.

## Discussion

In this study we examined the regulation of TGF-β1 gene expression by dexamethasone in normal human T cells activated by PHA-p and PMA. The results show that dexamethasone significantly enhances the expression of the TGF-β1 gene and the production of the corresponding protein by activated T

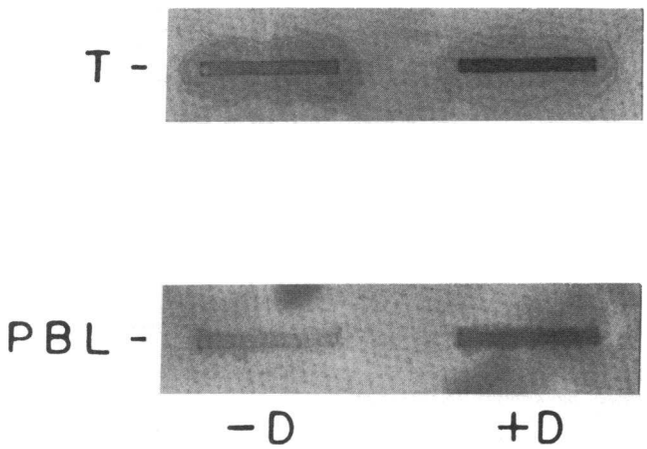


*Figure 8.* Dexamethasone-mediated stimulation of TGF-β1 production by activated lymphocytes. Immunoblot analysis was performed with supernatants from pHA-p/PMA-treated PBL and T cells of one individual, that were cultured with or without dexamethasone (100 μg/ml), and were visualized with a rabbit anti-TGFβ-1 antibody as described in Methods.

cells in a dose and time dependent fashion. Although the biological significance of this finding is unknown, the increased synthesis of an immunosuppressor cytokine could be one mechanism by which GC mediate immunosuppression. Since TGF-β1 is a potent inhibitor of T cell proliferation, its increased synthesis by activated T cells could be a biologic mechanism by which T cells limit the extent of their proliferative response to antigenic stimulation.

Stimulation and subsequent activation of T cells by specific antigens or by mitogens requires two signals: (*a*) the occupation of the T cell antigen receptor, an effect which can be mimicked by PHA-p; and (*b*) an effect of accessory cells, mimicked by agents that activate protein kinase C, such as phorbol esters (6, 31, 32). In response, multiple T cell genes are sequentially activated, over a period of several days (33), resulting in blastogenesis within 12 h, cell division by 24–48 h, with subsequent differentiation into immunocompetent T cells. Our time course experiments demonstrate that dexamethasone exercises its effect on TGF-β1 gene expression only during the first 12 h after activation, a time period corresponding to the blast transformation stage of T cell activation. Dexamethasone has other effects as well. For example, dexamethasone is known to inhibit gene expression of IL-2 and γ-IFN, both of which are expressed during the initial 2 h of T cell activation with PHA-p and PMA (4). We have reported that dexamethasone significantly decreases cell surface expression of the high affinity IL-2 receptor (IL-2R) in PHA-p/PMA activated T cells in a similar time frame (34), suggesting that during the initial 12 h of T cell induction, expression of several genes important in the regulation of T cell activation can be modulated by dexamethasone. More recently it was found that TGF-β1 can inhibit the progression of activated T cells from the $G_1$ to the S phase of the cell cycle and can inhibit IL-2R generation in murine T cell lines (35). Taken together, these observations raise the possibility that inhibition of T cell proliferation and IL-2R generation in vivo may be mediated or potentiated by TGF-β1, and thus, that TGF-β1 may play a pivotal role in some human diseases. However, although TGF-β1 can regulate expression of the IL-2 receptor in T cell lines, whether it alters IL-2 gene expression in human T cells is not clear (36). Thus, it is unlikely that TGF-β1 alone is responsible for the inhibitory effects of GC on T cell functions. Physiologic variations of plasma GC levels make it difficult to assess the role of endogenous GC in modulating TGF-β1 expression. It would be interesting to compare TGF-β1 expression by T cells during periods of normal and elevated plasma GC levels, such as during stress when IL-2R expression and T cell activation are both decreased (37, 38).

The superinduction of TGF-β1 mRNA caused by cycloheximide suggests that de novo protein synthesis is required for TGF-β1 mRNA degradation in T cells (39). Whether dexamethasone inhibits a short half-lived ribonuclease(s) that degrades TGF-β1 mRNA or it acts by a posttranslational mechanism that is coupled to TGFβ-mRNA degradation is not known. Since cycloheximide would inhibit new TGF-β1 synthesis by PHA-p/PMA-stimulated T cells, upregulation of its mRNA by the cytokine itself cannot account for the cycloheximide-induced increase in steady state mRNA levels for TGF-β1.

Promoters that contain positive and negative regulatory regions, upstream and downstream of the transcriptional start site of the TGF-β1 gene, have been identified in humans (40, 41). The positive regulatory regions of the upstream promoter for TGF-β1 contain binding sites for known transcription factors including NF-1, SP1, and AP1 that could mediate transcriptional regulation by GC. Corticosteroid-receptor complexes are known to regulate the expression of a variety of genes by stimulating the activity of transcription factor binding sites, including NF-1, SP1, as well as AP-1 either by direct binding or indirectly, by cooperation with other binding factors (42). The in vitro nuclear transcription assays described here indicate that the increase in TGF-β1 gene expression induced by dexamethasone occurs mostly at a transcriptional level whereas stimulation by mitogens appears to occur at least in part post-transcriptionally. This is not surprising since it is known that TGF-β1 steady-state mRNA levels can be modulated through multiple pathways. Regulation of the TGF-β1 gene is post-transcriptional in certain glioblastoma and neuroblastoma cell lines (22), and transcriptional in fibroblast cell lines in which TGF-β1 induces its own gene activation (19), and is translational in an osteoblast-like cell line in response to fibroblast growth factor (43).

Both TGF-β1 and GC affect the functions of a wide variety of cells. Whether the dexamethasone effect on TGF-β1 expression might be found in B cells, steroid responsive-leukemia cells, or in other hemopoietic cells, and, if so, whether this is a biologically relevant effect, remains to be answered by future studies.

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## References

1. Zacharchuk, C. M., M. Mercep, P. K. Chakraborti, S. S. Simons, Jr., and J. D. Ashwell. 1990. Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. *J. Immunol.* 4037–4045.

2. Parrillo, J. E., and A. S. Fauci. 1979. Mechanisms of glucocorticoid action on immune processes. *Annu. Rev. Pharmacol. Toxicol.* 19:179–201.

3. Gillis, S., G. R. Crabtree, and K. A. Smith. 1979. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J. Immunol.* 123:1624–1631.

4. Arya, S. K., F. Wong-Staal, and R. C. Gallo. 1984. Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon mRNA. *J. Immunol.* 133:273–276.

5. Robb, R. J., and R. M. Kutny. 1987. Structure-function relationship for the IL-2 receptor system. *J. Immunol.* 139:855–862.

6. Smith, K. A. 1988. Interleukin-2: inception, impact, and implications. *Science (Wash. DC).* 24:1169–1176.

7. Pinston, P., C. Sartini, J. M. Quernheim, and R. Crystal. 1987. Corticosteroid therapy suppresses spontaneous interleukin-2 release and spontaneous proliferation of lung T lymphocytes of patients with active pulmonary sarcoidosis. *J. Immunol.* 139:755–760.

8. Konishi, K., D. R. Moller, C. Saltini, M. Kirby, and R. G. Crystal. 1988. Spontaneous expression of the IL-2 receptor gene and presence of functional IL-2R on T lymphocytes in the blood of individuals with active pulmonary sarcoidosis. *J. Clin. Invest.* 82:775–781.

9. Frolik, C. A., L. L. Dart, C. A. Meyers, E. M. Smith, and M. B. Sporn. 1983. Purification and initial characterization of a type beta transforming growth factor from human placenta. *Proc. Natl. Acad. Sci. USA.* 80:3676–3680.

10. Roberts, A. B., and M. B. Sporn. 1990. The transforming growth factor-betas. *In* Peptide Growth Factors and Their Receptors. M. B. Sporn and A. B. Roberts, editors. Springer-Verlag, Heidelberg.

11. Massague, J. 1990. The transforming growth factor-β family. *Annu. Rev. Cell Biol.* 6:597–641.

12. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlek, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.* 163:1037–1050.

13. Kehrl, J. H., A. B. Roberts, L. M. Wakefield, S. Jakowlew, M. B. Sporn, and A. S. Fauci. 1986. Transforming growth factor β is an important immunoregulatory protein for human B lymphocytes. *J. Immunol.* 137:3855–3860.

14. Espevik, T., I. S. Figari, G. E. Ranges, and M. A. Palladino. 1988. Transforming growth factor-β1 (TGF-β1) and recombinant human tumor necrosis factor-alpha reciprocally regulate the generation of lymphokine-activated killer cell activity. *J. Immunol.* 140:2312–2316.

15. Stoeck, M., C. Ruegg, S. Miescher, S. Carrel, D. Cox, V. von Fliedner, and S. Alkan. 1989. Comparison of the immunosuppressive properties of milk growth factor and transforming growth factors β1 and β2. *J. Immunol.* 143:3258–3265.

16. Bristol, L. A., F. W. Ruscetti, D. T. Brody, and S. K. Durum. 1990. IL-1α induces expression of active transforming growth factor-β in nonproliferating T cells via a post-transcriptional mechanism. *J. Immunol.* 145:4108–4114.

17. Lucas, C., L. N. Bald, B. M. Fendly, M. Mora-Worms, I. S. Figari, E. J. Patzer, and M. Palladino. 1990. The autocrine production of transforming growth factor-β1 during lymphocyte activation: a study with a monoclonal antibody based ELISA. *J. Immunol.* 145:1415–1422.

18. Varga, J., J. Rosenbloom, and S. A. Jimenez. 1987. Transforming growth factor-β (TGF-β) causes a persistent increase in steady state levels of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. *Biochem. J.* 247:597–604.

19. Van Obberghen-Schilling, E., N. S. Roche, K. C. Flanders, M. B. Sporn, and A. B. Roberts. 1988. Transforming growth factor β1 positively regulates its own expression in normal and transformed cells. *J. Biol. Chem.* 263:7741–7746.

20. Saxon, A., R. H. Stevens, S. J. Ramer, P. J. Clements, and D. T. Y. Yu. 1978. Glucocorticoids administered in vivo inhibit human suppressor T lymphocyte function and diminish B lymphocyte responsiveness to in vitro immunoglobulin synthesis. *J. Clin. Invest.* 61:922–930.

21. Grayson, J., N. J. Dooley, I. R. Koski, and R. M. Blaese. 1981. Immunoglobulin production induced in vitro by glucocorticoid hormones: T cell-dependent stimulation of immunoglobulin production without B cell proliferation in cultures of human peripheral blood lymphocytes. *J. Clin. Invest.* 68:1539–1547.

22. Bodmer, S., K. Strommer, K. Frei, C. Siepl, N. De Triblet, I. Heid, and A. Fontana. 1989. Immunosuppression and transforming growth factor-β in glioblastoma. *J. Immunol.* 143:3222–3229.

23. Tuchinda, M., R. W. Newcomb, and B. L. DeVald. 1972. Effect of prednisone treatment on the human immune response to keyhole limpet hemocyanin. *Int. Arch. Allergy Appl. Immunol.* 42:533–544.

24. Fontana, A., K. Frei, S. Bodmer, E. Hofer, M. H. Schreier, M. A. Palladino, Jr., and R. M. Zinkernagel. 1989. Transforming growth factor-β inhibits the generation of cytotoxic T cells in virus-infected mice. *J. Immunol.* 143:3230–3234.

25. Ayanlar-Batuman, O., E. Ebert, and S. P. Hauptman. 1985. Impaired IL-2 production and responsiveness in patients with chronic lymphocytic leukemia of B cell variety. *Blood.* 67:279–284.

26. Ayanlar-Batuman, O., J. Shevitz, U. C. Traub, S. Murphy, and D. Sajewski. 1989. Lymphocyte interleukin 2 production and responsiveness are altered in patients with the primary myelodysplastic syndrome. *Blood.* 69:494–500.

27. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry.* 18:5294–5299.

28. Derynck, R., J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn, and D. V. Goeddel. 1985. Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. *Nature (Lond.).* 316:701–705.

29. McKnight, G. S., and R. D. Palmiter. 1979. Transcriptional regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. *J. Biol. Chem.* 254:9050–9058.

30. Tucker, R. F., G. D. Shipley, H. L. Moses, and R. W. Holley. 1984. Growth inhibitor from BSC-1 cells closely related to platelet type beta transforming growth factor. *Science (Wash. DC).* 226:705.

31. Greene, W. C. 1987. The human interleukin-2 receptor: a molecular and biochemical analysis of structure and function. *Clin. Res.* 135:439–450.

32. Robb, R. J., and R. M. Kutny. 1987. Structure-function relationship for the IL2 receptor system. *J. Immunol.* 139:855–862.

33. Crabtree, G. R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science (Wash. DC).* 243:355–360.

34. Ayanlar-Batuman, O. 1990. Glucocorticoids decrease IL-2 receptor α mRNA levels and exert a dose-dependent protein expression in human T cells. *Blood.* 76:794A.

35. Ruegemer, J. J., S. N. Ho, J. A. Augustine, J. W. Schlager, M. P. Bell, D. J. McKean, and R. T. Abraham. 1990. Regulatory effects of transforming growth factor-β on IL-2- and IL-4-dependent T cell-cycle progression. *J. Immunol.* 144:1767–1776.

36. Wahl, S. M., D. A. Hunt, H. L. Wong, S. Dougherty, N. McCartney-Francis, L. M. Wahl, and L. Elingsworth. 1988. Transforming growth factor-β is a potent immunosuppressive agent that inhibits IL-1-dependent lymphocyte proliferation. *J. Immunol.* 140:3026–3032.

37. Munck, A., P. M. Guyre, and N. J. Holbrook. 1984. Physiological functions of glucocorticoids in stress and their relationship to pharmacological actions. *Endocr. Rev.* 5:25–44.

38. Ayanlar-Batuman, O., D. Sajewski, J. E. Ottenweller, D. L. Pitman, and B. H. Natelson. 1990. Effects of repeated stress on T cell numbers and functions in rats. *Brain Behav. Immun.* 4:105–117.

39. Brawerman, G. 1989. mRNA decay: finding the right targets. *Cell.* 57:9–10.

40. Kim, S.-J., A. Glick, M. B. Sporn, and A. B. Roberts. 1989. Characterization of the promoter region of the human transforming growth factor-β1 gene. *J. Biol. Chem.* 264:402–408.

41. Scotto, L., P. I. Vaduva, R. E. Wager, and R. K. Assoian. 1990. Type β1 transforming growth factor gene expression. *J. Biol. Chem.* 265:2203–2208.

42. Schule, R., M. Muller, C. Kaltschmidt, and R. Renkawitz. 1988. Many transcription factors interact synergistically with steroid receptors. *Science (Wash. DC).* 242:1418–1420.

43. Noda, M., and R. Vogel. 1989. Fibroblast growth factor enhances type β1 transforming growth factor gene expression in osteoblast-like cells. *J. Cell Biol.* 109:2529–2535.