Mechanism of Action of Glucocorticosteroids

Inhibition of T Cell Proliferation and Interleukin 2 Production by Hydrocortisone Is Reversed by Leukotriene B_4

James S. Goodwin, Durgaprasadarao Atluru, Stanislaw Sierakowski, and Elias A. Lianos
Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Abstract

The mechanism whereby glucocorticosteroids are immunosuppressive is unknown. One potential mechanism of action of these compounds is inhibition of arachidonic acid metabolism. We found that the inhibition of lymphocyte proliferation by hydrocortisone or dexamethasone was mimicked by nonspecific lipoxygenase inhibitors and also by a specific 5-lipoxygenase inhibitor, but not by a specific cyclooxygenase inhibitor. Mitogen-stimulated cultures of T cells produce $\sim 5 \times 10^{-9}$ M leukotriene B_4 (LTB_4) in 24 h. This production of LTB_4 is completely inhibited by concentrations of hydrocortisone or lipoxygenase inhibitors that inhibit mitogen-induced [^{3}H]thymidine incorporation. The inhibition of lymphocyte proliferation by either hydrocortisone or by the 5-lipoxygenase inhibitor was totally reversed by LTB_4 but not by leukotriene C_4 or leukotriene D_4. LTB_4 had no effect on the inhibition of lymphocyte proliferation by noncorticosteroids such as prostaglandin E_2, histamine, or γ-interferon. The inhibition of interleukin 2 (IL-2) production by hydrocortisone or dexamethasone was also completely reversed by exogenous LTB_4. LTB_4 alone did not cause IL-2 production or cell proliferation when added to resting lymphocytes. Thus, endogenous LTB_4 production appears to be necessary but not sufficient for phytohemagglutinin-induced IL-2 production and lymphocyte proliferation. Glucocorticosteroids inhibit IL-2 production and lymphocyte proliferation by inhibiting endogenous LTB_4 production.

Introduction

While the powerful effects of glucocorticosteroids (hereafter referred to as corticosteroids) on immune function have been recognized almost since their discovery in 1948, the mechanism of action of these drugs (or endogenous hormones) has been unclear. In 1975, Gryglewski et al. (1) and several other groups (2–4) showed that corticosteroids inhibit the release of arachidonic acid from membrane phospholipids and proposed that their antiinflammatory activity is due to this action. This hypothesis was difficult for many investigators to accept because it was already generally accepted that nonsteroidal antiinflammatory agents (NSAIA)[1] work via inhibition of arachidonic acid metabolism, and that the antiinflammatory effects of corticosteroids are clearly different and more powerful than those of NSAIA. The understanding of arachidonic acid metabolism at that time was essentially as a straight line synthetic pathway from membrane phospholipid to arachidonic acid to the prostaglandins. Thus, if corticosteroids and NSAIA inhibit at different points in the same pathway, they should have very similar actions. This problem was clarified when Samuelsson and others (5) identified the lipoxygenase metabolic pathways for arachidonic acid.

This more complex schema of arachidonic acid metabolism allows us to seriously consider the possibility that many of the pharmacologic and perhaps physiologic actions of corticosteroids are indeed via inhibition of arachidonic acid release from membrane phospholipid, because corticosteroids inhibit both cyclooxygenase and lipoxygenase products while NSAIA inhibit only cyclooxygenase products. Clearly, the evidence supporting this concept has grown considerably over the past few years. Danon and Assouline (6) showed that RNA and protein synthesis are required in order for steroids to inhibit prostaglandin production. In 1979 and 1980, Flower and his associates (7, 8) in England and Hirata, Axelrod, and their co-workers (9) at the National Institutes of Health identified a phospholipase A_2–inhibitory glycoprotein, termed macrocortin or lipomodulin, that is synthesized and released by cells upon exposure to corticosteroids. On the other hand, several investigators have failed to find an inhibition by corticosteroids of the endogenous production of arachidonic acid metabolites in vitro (10) and in vivo (11, 12).

In this paper we show that mitogen-stimulated T cells produce substantial amounts of the 5-lipoxygenase product, leukotriene B_4 (LTB_4). Concentrations of hydrocortisone or dexamethasone which inhibit mitogen-induced [^{3}H]thymidine incorporation and interleukin 2 (IL-2) synthesis also inhibit LTB_4 production. In addition, nonspecific lipoxygenase inhibitors and a specific 5-lipoxygenase inhibitor also inhibit the mitogen response. Finally, readdition of LTB_4 in concentrations normally produced to cultures containing hydrocortisone reverses the inhibition by hydrocortisone of IL-2 production and mitogen-induced proliferation of T cells. These findings support the concept that corticosteroids inhibit T cell proliferation via phospholipase A_2 inhibition; specifically, that corticosteroids inhibit mitogen-induced T cell proliferation by suppressing endogenous LTB_4 production.

Methods

Drugs. LTB_4, leukotriene C_4 (LTC_4), and leukotriene D_4 (LTD_4) were kindly supplied by Dr. J. Rokach (Merck-Frosst Laboratories, Dorval,

Received for publication 4 April 1985 and in revised form 16 December 1985.

1. *Abbreviations used in this paper:* FCS, fetal calf serum; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; IL-2, interleukin 2; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; NDGA, nordihydroguaiaretic acid; NSAIA, nonsteroidal antiinflammatory agents; PHA, phytohemagglutinin; PGE_2, prostaglandin E.

J. Clin. Invest.

0021-9738/86/04/1244/07 $1.00
Volume 77, April 1986, 1244–1250

Canada). Various inhibitors of arachidonic acid metabolism and other drugs were examined for their effect on the mitogen response: indomethacin, nordihydroguaiaretic acid (NDGA) (13), hydrocortisone acetate, dexamethasone, histamine, prostaglandin E (PGE_2) (all from Sigma Chemical Co., St. Louis, MO); BW755C (Wellcome Research Laboratories, Beckenham, England) (14); AA861 (Takeda Pharmaceutical, Osaka, Japan) (15, 16), and γ-interferon (Interferon Sciences, Inc., New Brunswick, NJ). The leukotrienes and drugs were dissolved in ethanol and appropriately diluted in RPMI 1640 medium immediately before use. This resulted in ethanol concentrations in the cultures of 10 mg/100 ml to 1 μg/100 ml. Control cultures always contained appropriate amounts of ethanol, which had no measurable effect on the mitogen response.

Isolation of peripheral blood lymphocytes. Peripheral blood mononuclear cells were isolated on Ficoll-Hypaque density gradients (Pharmacia Fine Chemicals, Piscataway, NJ) from heparinized blood of healthy adult donors. Glass-adherent cells were removed by incubation at 37°C for 1 h on glass petri dishes in RPMI 1640 with 20% fetal calf serum (FCS). T cells were isolated from these nonadherent cells by rosetting with 2-aminoethylisothio-uranium bromide hydrobromide–treated sheep red blood cells followed by centrifugation over Ficoll-Hypaque for 30 min at 300 *g*. The sheep red blood cells were lysed with Tris-NHCl buffer. The cell population, termed T cells, contained 95% T cells, as identified by the pan-T monoclonal reagent Leu-5, and 1–2% peroxidase-positive cells.

In some experiments the T cells were exposed to 500 rads irradiation using a 2560 Maximar III, 250 KV X-ray system (General Electric Co., Milwaukee, WI). After irradiation, cells were washed and resuspended in fresh medium for culture.

Cell cultures. The T cells were cultured in microtiter plates, 10^5 cells in 200 μl of RPMI 1640 supplemented with L-glutamine, 10% FCS, and penicillin-streptomycin. Phytohemagglutinin (PHA) and the drugs (arachidonic acid metabolites and inhibitors of arachidonic acid metabolism) were added directly to the wells upon initiation of cultures. Tritiated thymidine was added to cultures 64 h after addition of mitogens, and at 72 h the cells were harvested on filter paper and counted in a liquid scintillation counter. All cultures were performed in triplicate. Percentage inhibition caused by the various agents was calculated and results expressed as: percent inhibition = $1 - (A - C)/(B - C) \times 100\%$, where A is counts per minute in cultures with drugs, B is counts per minute in cultures without drug, and C is counts per minute in nonstimulated cultures.

Measurement of LTB_4 production. Cultures of T cells, 10^6 cells/ml, were established in 10 × 75-mm culture tubes (2054; Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) with or without sera. After 0 or 24 h, culture media were harvested and processed for leukotriene extraction, high pressure liquid chromatographic separation, and quantification by radioimmunoassay (RIA). Media, 1 ml, were spiked with 1,800–2,000 cpm of purified $^3H[LTB_4]$ (specific gravity 32 Ci/mmol) to calculate recoveries. In a first step, media were extracted using 0.5 vol of isopropanol. They were then acidified with formic acid (5 M to a pH of 3.0–3.5) and a second extraction was performed using 2.5 vol of diethyl ether. After centrifugation and phase separation, the organic phase (isopropanol/diethyl ether) was removed and the aqueous was reextracted with 2.5 vol of diethyl ether. The organic phases were pooled, dried under a nitrogen stream, and reconstituted in 1 ml of methanol/water (3:7). This final solution was filtered through 0.2 μm filters and injected in a high pressure liquid chromatograph consisting of two solvent delivery modules (112; Beckman Instruments, Inc., Fullerton, CA), a gradient controller, and a reverse-phase C_{18} 5 μ cartridge (Nova Pak; Waters Associates, Millipore Corp., Milford, MA) placed in a radial compression module (RCM-100; Waters Associates). Leukotrienes were detected at 280 nm using an in-line absorbance detector (Spectroflow 772; Kratos Analytical Instruments, Ramsey, NJ) interphased with an integrator/printer plotter (SP 4270; Spectra-Physics Inc., Mountain View, CA). Elutions were performed at flow rates of 1 ml/min. An initial isocratic phase (15 min) using methanol/water/acetic acid (65:35:0.02 vol/vol), pH 5.7, adjusted with concentrated ammonium hydroxide (solvent B), was followed by a progressive gradient to 100% methanol over 30 min. LTB_4 and hydroxyeicosatetraenoic acid (HETE) were eluted during the gradient phase, upon completion of which the column was reconstituted with solvent B for 15 min. Elution fractions (1 ml) were collected at 1-min intervals and those corresponding to LTB_4 eluates (retention time 24.72 + 0.12 min) were dried under vacuum and reconstituted in 300 μl of 0.01 M phosphate buffer containing 0.1% bovine gamma globulin for subsequent quantification of LTB_4 by RIA. Recoveries of $^3H[LTB_4]$ added in the media before extraction and chromatographic separation were 85–90%. The RIA employed (Amersham Corp., Arlington Heights, IL) (17) is sensitive to 12.5 pg/ml and has 0.1% cross reactivity with LTC_4, PGE_2, arachidonic acid, 5-hydroxyeicosatetraenoic acid (5-HETE), 11-HETE, and 15-HETE. It has 2% cross reactivity with 12-HETE and 3% cross reactivity with sterioisomers of LTB_4. None of the drugs employed in these studies, except of course LTB_4, interfered with the measurement of LTB in the radioimmunoassay when added directly to the assay.

Measurement of interleukin 2 production. For IL-2 production 10^6 T cells were cultured at 37°C in 1 ml RPMI with 10% FCS in 12 × 75-mm round bottom plastic culture tubes with mitogens and drugs as indicated above. At 48 h the culture supernates were collected and were dialyzed against 500 vol of phosphate-buffered saline for 48 h at 4°C with ten changes of dialysis fluid. The IL-2–dependent cells used were an HT-2 cell line of BALB/C origin (generously provided by Dr. C. W. Spellman, Cancer Research Center, University of New Mexico School of Medicine, Albuquerque, NM). The cell line was maintained in RPMI 1640 with 10% FCS and IL-2 at 60 μ/ml.

HT-2 cells were harvested from petri dishes, washed, and adjusted to a concentration of 10^5 cells/ml with RPMI 1640, 10% FCS, 2 mM glutamine, and antibiotics. Cells were cultured in 100-μl vol in microtiter plates in the presence of 100 μl of IL-2 containing supernates, or those supernates diluted with complete media. After 22 h $[^3H]$thymidine was added and at 26 h cells were harvested and counted in a liquid scintillation counter. All cultures were performed in triplicate. Results were compared with a standard curve generated by adding known amounts of purified IL-2 (Electro-Nucleonics Inc., Silver Spring, MD) to HT-2 cells. In experiments where the effect of corticosteroids, LTB_4, or other drugs was assessed on IL-2 production, control cultures were established where those drugs were added at the end of the culture. This never had an effect on the estimate of IL-2 production.

Results

Table I presents the results of experiments examining the effect of hydrocortisone and a variety of inhibitors of arachidonic acid metabolism on $[^3H]$thymidine incorporation into mitogen-stimulated human T cells. As we (18) and others (19) have previously reported, hydrocortisone and dexamethasone in near-physiologic concentrations cause significant inhibition of $[^3H]$thymidine incorporation, while indomethacin (a cyclooxygenase inhibitor) actually enhances $[^3H]$thymidine incorporation (20). BW755 and NDGA, which block both the cyclooxygenase and lipoxygenase pathways and therefore have an effect on arachidonic acid metabolism similar to hydrocortisone (13, 14) also inhibit $[^3H]$thymidine incorporation. AA861, a relatively selective inhibitor of 5-lipoxygenase with less effect on cyclooxygenase or on other lipoxygenase enzymes (15, 16) also caused substantial inhibition of mitogenesis. This suggests that a 5-lipoxygenase metabolite is necessary for normal mitogen-induced proliferation of T cells to occur. It also indirectly suggests that the relevant action of hydrocortisone in inhibiting mitogenesis might be in preventing the synthesis of a 5-lipoxygenase product. Accordingly, we next tested the effect of the addition of various 5-lipoxygenase metabolites of arachidonic acid on the inhibition of mitogenesis by hydrocortisone. The results of experiments

Table I. Effect of Corticosteroids and Other Inhibitors of Arachidonic Acid Metabolism on [^{3}H]Thymidine Incorporation of PHA-stimulated Human T Cells

Drug	cpm (SEM)	Percent change (SEM)
mol/liter		
—	27,581 (3042)	—
Indomethacin (10^{-6})	34,621 (3968)*	+26 (6)
Hydrocortisone (10^{-5})	4,115 (804)*	−86 (5)
Hydrocortisone (10^{-6})	4,072 (1131)*	−85 (6)
Dexamethasone (10^{-6})	3,987 (1401)*	−86 (4)
BW755C (10^{-5})	3,716 (665)*	−87 (6)
NDGA (10^{-5})	2,129 (473)*	−93 (3)
AA861 (10^{-5})	4,121 (1282)*	−83 (5)

Human erythrocyte rosette positive cells were isolated from peripheral blood and cultured for 72 h with 2.5 μg/ml PHA. The drugs were added at the initiation of the cultures. Several concentrations of each drug were employed, but only one concentration is shown in the table. For NDGA, BW755C, and AA861, the concentration shown in the table causes >90% inhibition of 5-lipoxygenase activity (see Table IV). None of the drugs affected cell viability as assessed by trypan blue exclusion at the end of the culture. Data are presented as the mean±SEM for experiments on six different subjects. All cultures were performed in triplicate. * Significantly different from control with $P < 0.01$ by paired t test.

with two subjects are shown in Table II. LTB_4 by itself caused a slight inhibition of the PHA response. More importantly, LTB_4 in low concentrations (10^{-9} and 10^{-10} M) almost completely reversed the inhibition caused by hydrocortisone. In contrast, neither LTC_4 nor LTD_4 had an effect on the PHA response or on the hydrocortisone inhibition of that response. LTC_4 and LTD_4 were tested at many concentrations (only one is shown), all of which were ineffective. A summary of experiments on 10 individuals is shown in Fig. 1. Hydrocortisone at 10^{-6} M caused an average of 72% inhibition of [^{3}H]thymidine incorporation. Adding back various concentrations of LTB_4 largely eliminated that inhibition, with 10^{-10} M LTB_4 giving the optimal effect (3% inhibition compared with control). Similar data was obtained when the PHA response was inhibited by AA861, with any of the three concentrations of LTB_4 employed causing complete restoration of the response. Just as with inhibition by hydrocortisone in Table II, neither LTC_4 nor LTD_4 had any effect on the inhibition of [^{3}H]thymidine incorporation by AA861 (data not shown).

Fig. 2 shows the effect of delaying LTB_4 addition to PHA-stimulated cultures containing hydrocortisone. Addition of LTB_4 up to 8 h after initiation of the culture still resulted in complete reversal of the inhibition by hydrocortisone, while after 16 h of culture, addition of LTB_4 had little effect on hydrocortisone inhibition. Also, when the cells were preincubated with hydrocortisone for 2 h and then washed, addition of LTB_4 after the washing still reversed the inhibition caused by hydrocortisone (data not shown).

Table III shows data suggesting that the reversal of inhibition of T cell proliferation by LTB_4 is specific for corticosteroids. LTB_4 did not reverse inhibition of [^{3}H]thymidine incorporation caused by PGE_2, histamine, γ-interferon, or γ-irradiation, but did reverse the inhibition caused by hydrocortisone or dexamethasone.

Table II. Effect of Adding 5-Lipoxygenase Metabolites of Arachidonic Acid on the Inhibition of Mitogen-induced Proliferation by Hydrocortisone

PHA (2.5 ug/ml)	Hydrocortisone	Arachidonic acid metabolite	Subject 1	Subject 2
			cpm	*cpm*
+	—	—	18,389	20,069
+	—	LTB_4 10^{-10}	15,672	17,788
+	—	LTB_4 10^{-9}	15,210	17,297
+	—	LTC_4 10^{-9}	18,140	20,247
+	—	LTD_4 10^{-9}	18,493	19,941
+	10^{-7} M	—	10,391	14,401
+	10^{-7} M	LTB_4 10^{-10}	16,476	19,617
+	10^{-7} M	LTB_4 10^{-9}	17,526	18,507
+	10^{-7} M	LTC_4 10^{-9}	10,240	13,970
+	10^{-7} M	LTD_4 10^{-9}	10,174	14,472
+	10^{-6} M	—	4,995	6,766
+	10^{-6} M	LTB_4 10^{-10}	16,269	20,331
+	10^{-6} M	LTB_4 10^{-9}	15,106	18,507
+	10^{-6} M	LTC_4 10^{-9}	5,004	7,120
+	10^{-6} M	LTD_4 10^{-9}	4,874	6,720

Results of experiments with two subjects are shown. The counts per minute given represent the mean of triplicate cultures. The standard deviation of the triplicate cultures was always within ±10% of the mean counts per minute. In both subjects the addition of LTB_4 alone caused a slight inhibition of PHA-stimulated [^{3}H]thymidine incorporation, while both 10^{-7} M and 10^{-6} M hydrocortisone caused substantial inhibition. Strikingly, addition of LTB_4 to the cultures containing hydrocortisone restored the counts per minute almost to control levels. LTC_4 and LTD_4 had essentially no effect in this system over a wide concentration range (10^{-8}–10^{-12} M; only the values for 10^{-9} M are given).

The fact that exogenous LTB_4 reversed the inhibition of mitogenesis caused by hydrocortisone or lipoxygenase inhibitors suggested that endogenous LTB_4 production might be a necessary

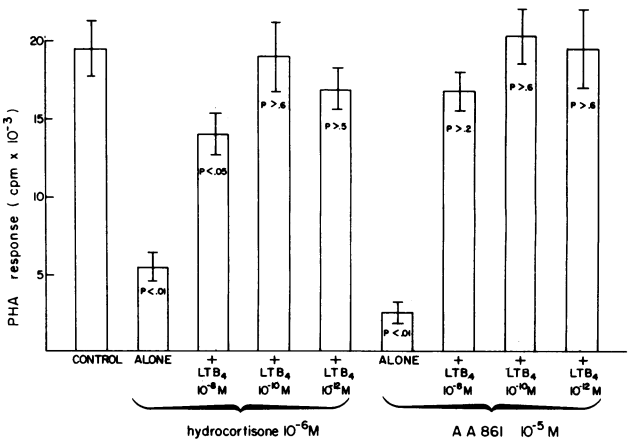

Figure 1. Effect of LTB_4 on inhibition of PHA-stimulated [^{3}H]thymidine incorporation by hydrocortisone or AA861. Data are expressed as the mean±SEM for experiments on 10 subjects. P values are for paired t tests comparing the counts per minute of the drug-treated cultures with the control responses. Both hydrocortisone and AA861 caused substantial (>70%) inhibition of [^{3}H]thymidine incorporation and this suppression was completely eliminated by LTB_4 at 10^{-10}.

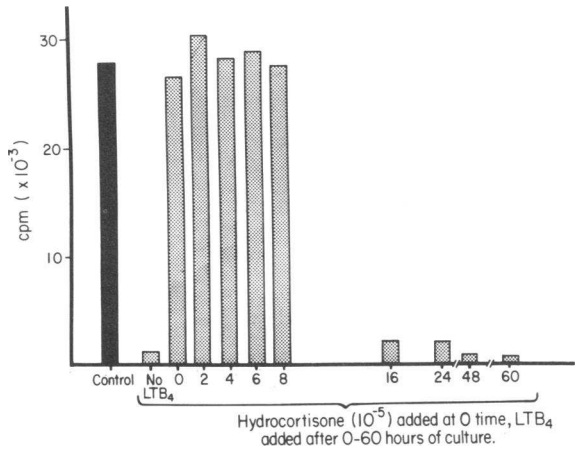

Figure 2. Effect of delaying addition of LTB_4 for various times after culture initiation on reversal of hydrocortisone-induced inhibition of lymphocyte proliferation. Mean counts per minute from two experiments is shown. Hydrocortisone (10^{-5} M) caused >90% inhibition of [^{3}H]thymidine incorporation. This inhibition was completely reversed by addition of LTB_4 (10^{-10} M) at the beginning of the culture or 2, 4, 6, or 8 h after beginning the culture. If LTB_4 addition was delayed for 18 h or longer it had little or no effect on hydrocortisone inhibition of T cell proliferation.

component of mitogen-stimulated T cell proliferation. Accordingly, we measured LTB_4 levels in these cultures.

Fig. 3 shows a representative high performance liquid chromatography (HPLC) analysis of the supernates of T cells cultured without (*A*) or with (*B*) PHA for 24 h. In the supernates from the T cells cultured with PHA there was a large peak at the retention time comparable to LTB_4. Elution fractions corresponding to this peak were collected and assayed for immunoreactive LTB_4 using an RIA. These results are presented in Table IV and show that the LTB_4 peak seen in Fig. 3 contained substantial amounts of immunoreactive LTB_4. The 1710 pg of LTB_4 produced by the 10^6 T cells corresponds to an LTB_4 concentration of 5×10^{-9} M. No measurable LTB_4 was produced by unstimulated T cells. Addition of hydrocortisone at 10^{-5} or 10^{-6} or of specific or nonspecific lipoxygenase inhibitors completely eliminated LTB_4 production (Table IV) and inhibited by >90% the production of other arachidonic acid metabolites (5-HETE, 12-HETE, and 15-HETE; data not shown). Hydrocortisone at 10^{-7} and 10^{-8} M caused lesser inhibition of LTB_4 production (Table IV). This parallels the less potent inhibition of lymphocyte proliferation by 10^{-7} M hydrocortisone (Table II).

Table III. Lack of Effect of LTB_4 on Inhibition of T Cell Proliferation Caused by Inhibitors Other Than Corticosteroids

Inhibitor	LTB_4	Experiment 1	Experiment 2
—	−	23,057	22,294
—	+	17,778	16,342
Hydrocortisone (10^{-6})	−	13,600	14,293
Hydrocortisone (10^{-6})	+	20,724	22,296
Dexamethasone (10^{-6})	−	13,168	11,390
Dexamethasone (10^{-6})	+	21,188	20,105
PGE_2 (10^{-6})	−	13,156	15,952
PGE_2 (10^{-6})	+	11,377	17,686
Histamine (10^{-4})	−	12,444	12,976
Histamine (10^{-4})	+	11,945	14,618
Interferon (100 μ/ml)	−	14,350	13,824
Interferon (100 μ/ml)	+	13,931	14,682
Irradiation (500 rad)	+	6,588	6,846
Irradiation (500 rad)	−	7,146	7,747

Results of experiments with two subjects are shown. Just as in Table II, LTB_4 (10^{-10} M) by itself caused a mild inhibition of [^{3}H]thymidine incorporation, but reversed the greater degree of inhibition caused by hydrocortisone or dexamethasone. In contrast, LTB_4 had essentially no effect on the inhibition of [^{3}H]thymidine incorporation caused by PGE_2, histamine, interferon, or γ-irradiation. All cultures contained PHA at 2.5 μg/ml.

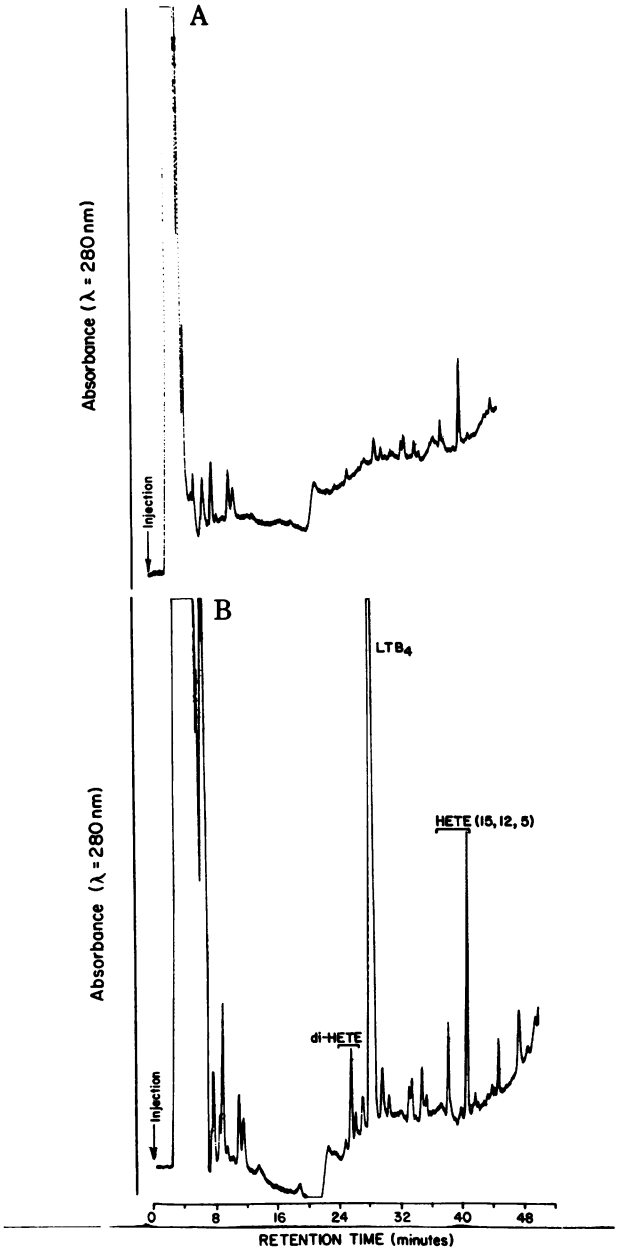

Figure 3. HPLC analysis of supernates of T cells cultured alone (*A*) or with PHA (*B*). The major peak in the supernate from the PHA-stimulated T cell culture had a retention time identical to LTB_4 and corresponded to the peak of immunoreactive LTB_4 by RIA (see Table IV). There were additional smaller peaks with retention times identical to 12-HETE and 5-HETE in the HPLC analysis of the PHA-stimulated culture that were lacking in the control cultures.

Table IV. LTB_4 Levels in PHA-stimulated T Cell Cultures

Cells	PHA	Duration of culture	Drug	LTB_4
		h	*mol/liter*	*pg/10^6 cells*
T cells	−	0	—	<100
+	−	24	—	<100
+	+	0	—	<100
+	+	24	—	1710±160*
+	+	24	hydrocortisone (10^{-5})	<100
+	+	24	hydrocortisone (10^{-6})	<100
+	+	24	hydrocortisone (10^{-7})	580 + 90*‡
+	+	24	hydrocortisone (10^{-8})	840 + 210*‡
+	+	24	NDGA (10^{-5})	<100
+	+	24	BW755C (10^{-5})	<100
+	+	24	AA861 (10^{-5})	<100

10^6 T cells were incubated in 1 ml media with or without PHA (2.5 μg/ml). Cultures were harvested immediately or after 24 h, and the supernates were removed and stored frozen for later analysis for LTB content. LTB_4 concentration was determined by RIA of samples eluting with the retention of LTB_4 after HPLC (see Methods). The data are given as picograms of LTB_4 per 10^6 cells; that is, per 1 ml supernate of 10^6 cells cultured in 1 ml media. The T cell preparations contained 1–2% monocytes as identified by peroxidase staining and >95% T cells, as identified by erythrocyte rosetting and staining with pan-T monoclonal reagents. Data represent the mean±SEM for four experiments on cells from different individuals.

* Different from cultures without PHA by paired *t* test, $P < 0.001$.

‡ Different from values of cells cultured with PHA without hydrocortisone, $P < 0.01$ by paired *t* tests.

Table V. Inhibition of IL-2 Production in Mitogen-stimulated Lymphocytes by Corticosteroids Is Reversed by LTB_4

PHA	Inhibitor	Arachidonic acid metabolite	IL-2
		mol/liter	*U/ml*
−	−	—	<20
+	−	—	2,937±460*
+	Hydrocortisone 10^{-5} M	—	<20
+	+	LTB_4 (10^{-8})	4,384±842*
+	+	LTB_4 (10^{-10})	2,840±52*‡
+	+	LTB_4 (10^{-12})	576±116*‡
+	+	LTB_4 (10^{-14})	212±45*‡
+	+	LTC_4 (10^{-8})	<20
+	+	PGE_2 (10^{-8})	<20
+	Dexamethasone 10^{-5} M	—	<20
+	+	LTB_4 (10^{-10})	3,430±283*
+	AA861	10^{-5} M	<20
+	+	LTB_4 (10^{-10})	3,482±109*
−	−	LTB_4 (10^{-8})	<20

Data represent the mean±SEM from six experiments in different donors. 10^6 T cells were cultured for 48 h in one ml media, and the supernates were dialyzed and assayed for IL-2 activity as described in the methods section.

* Different from cultures with PHA + hydrocortisone, $P < 0.01$ by paired *t* test.

‡ Different from cultures with PHA + hydrocortisone, $+10^{-8}$ M, LTB_4, $P < 0.01$ by paired *t* test.

Corticosteroids have been shown to inhibit IL-2 production, and exogenous IL-2 reverses the inhibition of mitogen-induced T cell proliferation by corticosteroids (46–49). To explore the relationship between LTB_4 and IL-2, we examined the effect of hydrocortisone and LTB_4 on IL-2 production by mitogen-stimulated T cells. As shown in Table V, inhibition of IL-2 production by hydrocortisone is reversed by exogenous LTB_4. There is a clear dose-response relationship, with an effect as low as 10^{-14} M LTB_4. PGE_2 or LTC_4 did not affect the inhibition of IL-2 production by hydrocortisone.

Discussion

In order to conclude that the effects of corticosteroids in a given physiologic system are due to inhibition of arachidonic acid metabolism, several criteria should be met. First, one should demonstrate that corticosteroids do indeed inhibit arachidonic acid metabolism in the system under study. Second, one should be able to duplicate the effect of corticosteroids with drugs which inhibit all the metabolic pathways for arachidonic acid. If so, then it might also be possible to find a more specific inhibitor of arachidonic acid metabolism, such as a specific cyclooxygenase inhibitor or a specific inhibitor of one of the lipoxygenase enzymes, that duplicates the effects of corticosteroids in the physiologic system. Third, one should be able to reverse the effect of corticosteroids by adding back one or more specific arachidonic acid metabolites to the system, such as PGE, LTC_4, 15-hydroperoxyeicosatetraenoic acid, etc.

In this present communication we found that, while exogenous LTB_4 by itself caused slight inhibition of T cell proliferation, LTB_4 added in the first 8 h of culture completely overcame the much more dramatic inhibition caused by hydrocortisone or dexamethasone. LTB_4 is normally produced in mitogen-stimulated T cell cultures and this production is completely inhibited by concentrations of hydrocortisone that inhibit [^{3}H]thymidine incorporation. Other drugs that inhibit LTB_4 production, such as specific and nonspecific lipoxygenase inhibitors, also inhibit the mitogen response, and exogenous LTB_4 can reverse that inhibition. We conclude that endogenous LTB_4 production early in the culture is necessary for mitogen-stimulated T cell proliferation and that corticosteroids inhibit T cell proliferation by reducing endogenous LTB_4 production. Addition of LTB_4 to mitogen-stimulated T cells cultured without hydrocortisone has little effect, presumably because there are already optimal levels of LTB_4 produced endogenously.

LTB_4 is produced by enzymatic hydrolysis of leukotriene A_4 which is labile epoxide resulting from oxidation of arachidonic acid at the 5 position (5). LTB_4 has powerful chemoattractant and aggregating properties for neutrophils (21, 22). There have been few reports suggesting a role for LTB_4 in cellular immune responses. Several laboratories have reported modest (23–25) or no (26) inhibition of mitogen-stimulated proliferation of human T cells by LTB_4 or by LTC_4 (27). In addition, Rola-Plezczynski et al. (23) and we (28) have found that LTB_4 causes induction of suppressor T cells. Several years ago Kelly et al. (29) reported that nonspecific lipoxygenase inhibitors such as NDGA inhibited mitogen-induced proliferation. Johnson and Torros (30) found that LTB_4, LTC_4, or LTD_4 could replace the requirement for IL-2 for interferon production by murine splenocytes. In another

study, we found that LTB_4 can act synergistically with suboptimal levels of IL-2 in stimulating proliferation of IL-2 responsive cells (31).

While LTB_4 has been identified as a major arachidonic acid metabolite of polymorphonuclear leukocytes (32), monocyte-macrophages (33–35), basophils (36), and eosinophils (37), it has not been well accepted that purified T cells produce LTB_4 or, indeed, any arachidonic acid metabolites (35, 38–42). Parker et al. (43) reported in 1979 that lymphocytes metabolized arachidonic acid to 5-HETE, but their lymphocyte preparations contained substantial numbers of monocytes and some neutrophils. Goetzl (44) reported that human T cells (E-rosette positive) stimulated with concanavalin A or a calcium ionophore produce substantial amounts of 5-lipoxygenase metabolites, including LTB_4, but others have disputed this claim (35, 38, 45), arguing that the LTB_4 produced comes from contaminating monocytes in the T cell preparation. We view this as an unlikely explanation for our results for several reasons. First, LTB_4 was produced only in conjunction with the T cell mitogen PHA and not by resting cells (Fig. 3 and Table IV). Second, the percentage of contaminating monocytes in our preparations was <2%; in further experiments (Atluru, D., E. Lianos, and J. S. Goodwin, manuscript submitted for publication) we found that adding back increasing numbers of monocytes to the T cells, to a final concentration of 20% monocytes, did not increase the LTB_4 produced upon PHA stimulation. We feel that previous efforts to determine if lymphocytes produce LTB_4 may have failed because the investigators added high concentrations of exogenous arachidonic acid to their cell cultures (35). We have found that exogenous arachidonic acid from 10^{-5} to 10^{-9} M inhibits LTB_4 and 5-HETE production by PHA-stimulated T cells by >90% (45a). Laclos and his colleagues (46) have recently reported a similar phenomenon of inhibition of 5-lipoxygenase in human neutrophils by exogenous arachidonic acid.

There is a seeming discrepancy between the amount of the LTB_4 required to restore T cell proliferation or IL-2 production in cultures containing hydrocortisone (Fig. 1 and Table V) and the amount of the LTB_4 production actually produced in the cultures not containing hydrocortisone (Table IV). Much more LTB_4 is produced than would appear necessary to maintain IL-2 production and T cell proliferation. We feel part of this discrepancy is due to the fact that we measured LTB_4 production at 18 h, whereas the effect of endogenous LTB_4 is early, at least within the first 8 h of culture, based on the data in Fig. 2. The early LTB_4 production in the first minutes of the culture is below the sensitivity of our assay, but, by extrapolation, is presumably in the range (10^{-10}–10^{-12} M) that causes restoration of IL-2 production and T cell proliferation when added to PHA-stimulated cultures containing hydrocortisone.

Corticosteroids have been shown to inhibit IL-2 production, and exogenous IL-2 reverses the inhibition of mitogen-induced T cell proliferation by corticosteroids (47–50). Thus, either LTB_4 or IL-2 reverses the inhibition of T cell proliferation caused by corticosteroids. As shown in Table V, exogenous LTB_4 restores IL-2 production by PHA-stimulated T cells cultured with hydrocortisone. Thus, we conclude that in T cell proliferation, LTB_4 production is a necessary step in IL-2 production. Corticosteroids, by inhibiting LTB_4 production, also inhibit IL-2 production, resulting in decreased T cell proliferation. The effect of corticosteroids on T cell proliferation can be overcome by adding LTB_4, thus restoring endogenous IL-2 production to normal, or by adding IL-2 directly. Our findings suggest that specific 5-lipoxygenase inhibitors or specific LTB_4 antagonists might possess immunosuppressive properties similar to corticosteroids, while, because of their specificity, they might not share many of the other physiologic effects of corticosteroids.

Acknowledgments

We are grateful to Dr. Rokach of Merck-Frosst, Dorval, Canada for his generosity in supplying us with leukotrienes and to Dr. M. Nishikawa and his colleagues at Takeda Chemical Industries, Osaka, Japan for supplying the AA861.

This work was supported by grants AG01245-06, AM34672-01, and AM34793-01 from the United States Public Health Service, and by grant 312-12-03 from the American Heart Association.

References

1. Gryglewski, R. J., B. Panczenko, R. Korbut, L. Grodzinska, and A. Ocetkiewica. 1975. Corticosteroids inhibit prostaglandin release from perfused mesenteric blood vessels in rabbits. *Prostaglandins.* 10:343–354.

2. Kantrowitz, F., D. R. Robinsin, M. B. McGuire, and L. Levin. 1975. Corticosteroids inhibit prostaglandin production by rheumatoid synovia. *Nature (Lond.).* 258:737–739.

3. Floman, Y., and U. Zor. 1976. Mechanism of steroid action in inflammation: inhibition of prostaglandin synthesis and release. *Prostaglandins.* 12:403–411.

4. Tashjian, A., E. Yoelkel, J. McDonough, and L. Levin. 1975. Hydrocortisone inhibits prostaglandin production by mouse fibrosarcoma cells. *Nature (Lond.).* 258:739–741.

5. Samuelsson, B. 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science (Wash. DC).* 220: 568–578.

6. Danon, A., and G. Assouline. 1978. Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. *Nature (Lond.).* 273:552–553.

7. Flower, R. J., and G. J. Blackwell. 1978. Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. *Nature (Lond.).* 278:456–458.

8. Blackwell, G. J., R. Carnuccio, M. Di Rose, R. J. Flower, L. Parente, and P. Perrico. 1980. Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature (Lond.).* 287:147–149.

9. Hirata, F., E. Schiffman, K. Yenkatasubramanian, D. Salomen, and J. Axelrod. 1980. A phospholipase A2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. USA.* 77: 2533–2538.

10. Chandrabose, K. A., E. G. Lapentina, C. J. Schmitges, M. I. Siegel, and P. Cuatrecasas. 1978. Action of corticosteroids in regulation of prostaglandin biosynthesis in cultured fibroblasts. *Proc. Natl. Acad. Sci. USA.* 75:214–217.

11. Gold, E. W., O. D. Fox, and P. R. Edgar. 1978. The effect of long term corticosteroid administration on lipid and prostaglandin levels. *J. Steroid Biochem.* 9:313–316.

12. Naray-Fejes-Toth, A., G. Fejes-Toth, C. Fischer, and J. C. Frolich. 1984. Effect of dexamethasone on in vivo prostanoid production in the rabbit. *J. Clin. Invest.* 74:120–123.

13. Hamberg, M. 1976. On the formation of thromboxane B2 and 12L-hydroxy-5,8,10,14-eicosatetranoic acid (12 ho-20:4) in tissues from the guinea pig. *Biochim. Biophys. Acta.* 431:651–659.

14. Higgs, G. A., R. J. Flower, and J. R. Vane. 1979. A new approach to anti-inflammatory drugs. *Biochem. Pharmacol.* 28:1949–1958.

15. Yoshimoto, T., K. Yokoyama, K. Ochi, and S. Yamamoto. 1983. A selective inhibitor of arachidonate 5-lipoxygenase, AA861. *In* Perspectives in Prostaglandin Research. Y. Shiokawa, M. Katori, and Y. Mizughima, editors. Experta Medica, Amsterdam. 72–79.

16. Yoshimoto, T., C. Yokoyama, K. Ochi, S. Yamamoto, Y. Maki, Y. Ashida, S. Terao, and M. Sheraishi. 1982. 2,3,5-trimethyl-6 (12-hy-

droxy-5, 10-dodecadiynyl)-1,4-benzoquinone (AA861). A selective inhibitor of the 5-lipoxygenase reaction and the biosynthesis of slow-reacting substance of anaphylaxis. *Biochim. Biophys. Acta.* 713:470–477.

17. Salmon, J. A., P. Simmons, and R. M. J. Palmer. 1982. A radioimmunoassay for leukotriene B_4. *Prostaglandins.* 24:225–234.

18. Goodwin, J. S., R. P. Messner, and R. C. Williams. 1979. Inhibition of T cell mitogenesis: effect of mitogen dose. *Cell. Immunol.* 45: 303–309.

19. Robertson, A. J., J. H. Gibbs, R. C. Potts, R. A. Brown, M. Browning, and J. S. Beck. 1981. Dose-related depression of PHA-induced stimulation of human lymphocytes by hydrocortisone. *J. Immunopharmacol.* 3:21–29.

20. Goodwin, J. S., A. D. Bankhurst, and R. P. Messner. 1977. Inhibition of T cell mitogenesis by prostaglandins: existence of a prostaglandin producing suppressor cell. *J. Exp. Med.* 146:1719–1734.

21. Ford-Hutchinson, A. W., M. A. Bray, M. V. Doig, M. E. Shipley, and J. H. Smith. 1980. Leukotriene B: a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature (Lond.).* 286:264–266.

22. Goetzl, E. J., L. L. Brindley, and D. W. Goldman. 1983. Enhancement of human neutrophil adherence by synthetic leukotriene constituents of the slow-reacting substance of anaphylaxis. *Immunology.* 50:35–44.

23. Rola-Plezczynski, M., P. Boreat, and P. Sirois. 1982. Leukotriene B4 induces human suppressor lymphocytes. *Biochem. Biophys. Res. Commun.* 198:1531–1537.

24. Payan, D. G., and E. J. Goetzl. 1983. Specific suppression of human T lymphocyte function by leukotrienes B4. *J. Immunol.* 131: 551–560.

25. Gualde, N., D. Atluru, and J. S. Goodwin. 1985. Effect of lipoxygenase metabolites of arachidonic acid on proliferation of human T cells and T cell subsets. *J. Immunol.* 134:1125–1129.

26. Guttery, J. E., A. Tilden, D. K. Herron, P. Gallagher, S. R. Bakers, and E. W. Ades. 1984. Leukotrienes (LTB_4 and LTD_4): In vitro effects on human lymphocyte proliferation and transformation. *J. Clin. Lab. Immunol.* 13:151–153.

27. Webb, D. R., I. Nowowiejski, C. Healy, and T. J. Rogers. 1982. Immunosuppressive properties of leukotriene D_4 and E_4 in vitro. *Biochem. Biophys. Res. Commun.* 104:1617–1623.

28. Atluru, D., and J. S. Goodwin. 1984. Control of polyclonal immunoglobulin production from human lymphocytes by leukotrienes. *J. Clin. Invest.* 74:1444–1451.

29. Kelly, J. P., M. C. Johnson, and C. W. Parker. 1979. Effect of inhibitors of arachidonic acid metabolism on mitogenesis in human lymphocytes. *J. Immunol.* 122:1563–1572.

30. Johnson, H. M., and B. A. Torres. 1984. Leukotrienes: positive signals for regulation of γ-interferon production. *J. Immunol.* 132:413–422.

31. Atluru, D., and J. S. Goodwin. 1986. Leukotriene B_4 causes proliferation of interleukin-2 dependent T cells in the presence of suboptimal levels of interleukin-2. *Cell. Immunol.* In press.

32. Borgeat, P., and B. Samuelsson. 1979. Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. USA.* 76:2148–2153.

33. Humes, J. L., S. Sadowski, M. Galavadge, M. Goldenberg, E. Subers, R. J. Bonney, and F. A. Kuehl. 1982. Evidence for two sources of arachidonic acid for oxidative metabolism by mouse peritoneal macrophages. *J. Biol. Chem.* 257:1591–1599.

34. Bray, M. A., A. W. Ford-Hutchinson, and M. J. H. Smith. 1981. *In* SRS-A and Leukotrienes. P. J. Piper, editor. Wiley, Chicester, West Sussex, United Kingdom. 253–265.

35. Goldyne, M. E., G. F. Burrish, P. Poubelle, and P. Borgeat. 1984. Arachidonic acid metabolism among human mononuclear leukocytes: lipoxygenase-related pathways. *J. Biol. Chem.* 259:8815–8819.

36. Levine, L. 1983. Inhibitions of the A-23187-stimulated leukotriene and prostaglandin biosynthesis of rat basophil leukemia cells by nonsteroidal anti-inflammatory drugs, anti-oxidants, and calcium blockers. *Biochem. Pharmacol.* 32:3023–3031.

37. Goetzl, E. J., P. F. Weller, and F. F. Sun. 1980. The regulation of human eosinophil function by endogenous mono-hydroxy-eicosatetranoic acids. *J. Immunol.* 124:926–935.

38. Goldyne, M. D. 1983. Eicosanoids and immunoregulation. *Recent. Adv. Clin. Immunol.* 3:9–21.

39. Dy, M., M. Astoin, M. Rigaud, and J. Hamburer. 1980. Prostaglandin release in the mixed lymphocyte culture: effect of pre-sensitization by a skin allograft; nature of the PG-producing cell. *Eur. J. Immunol.* 10:121–127.

40. Bray, M. A., R. G. Powell, and P. M. Lydyard. 1981. Prostaglandin generation by separated human blood mononuclear cell fractions. *Int. J. Immunopharmacol.* 3:377–383.

41. Kennedy, M. S., J. D. Stobo, and M. E. Goldyne. 1980. In vitro synthesis of prostaglandins and related lipids by populations of human peripheral blood mononuclear cells. *Prostaglandins.* 20:135–141.

42. Bankhurst, A. D., E. Hastain, J. S. Goodwin, and G. T. Peake. 1981. The nature of the prostaglandin-producing mononuclear cell in human peripheral blood. *J. Lab. Clin. Med.* 97:179–190.

43. Parker, C. W., W. F. Stenson, M. G. Huber, and J. P. Kelley. 1979. Formation of thromboxane B_2 and hydroxyarachidonic acids in purified human lymphocytes in the presence and absence of PHA. *J. Immunol.* 122:1572–1581.

44. Goetzl, E. J. 1981. Selective feedback inhibition of the 5-lipoxygenation of arachidonic acid in human T-lymphocytes. *Biochem. Biophys. Res. Commun.* 101:344–350.

45. Decker, D. M., and M. S. Kennedy. 1984. Arachidonic acid metabolism of the monocyte cell line U937. *Clin. Res.* 32:39 (Abstr.)

45a. Atluru, D., E. Lianos, and J. S. Goodwin. Arachidonic acid inhibits 5-lipoxygenase in human T cells. *Biochem. Biophys. Res. Commun.* In press.

46. Laclos, B. F., P. Braquet, and P. Borgeat. 1984. Characteristics of leukotrienes and hydroxyeicosatetranoic acid synthesis in human leukocytes: effect of arachidonic acid concentration. *Prostaglandins Leukotrienes Med.* 13:47–52.

47. Gillis, S., G. R. Crabtree, and K. A. Smith. 1979. Glucosteroid inhibition of T cell growth factor production. *J. Immunol.* 123:1624–1632.

48. Arya, S. K., F. Wong-Staal, and R. C. Gallo. 1984. Dexamethasone-mediated inhibition of human cell growth factor and γ-interferon messenger RNA. *Immunology.* 133:273–280.

49. Randazzo, B., T. Hirschberg, and H. Hirschberg. 1984. Inhibition of the antigen-activated T cell response by methylprednisolone is caused by inhibition of interleukin-2 production. *Int. J. Immunopharmacol.* 6: 419–429.

50. Larsson, E. L. 1980. Cyclosporin A and dexamethasone suppress T cell responses by selectively acting on distinct sites of the triggering process. *J. Immunol.* 124:2828–2833.