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Inhibition of eicosanoid biosynthesis by glucocorticoids in humans

(leukotrienes/prostaglandins/alveolar macrophages/steroid receptors)

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ABSTRACT Therapeutic doses of glucocorticoids are thought to inhibit prostaglandin and leukotriene formation in humans. Several studies in animals, however, have failed to demonstrate modulation of eicosanoid biosynthesis by steroids *in vivo*. We administered prednisone (60 mg/day) to eight healthy volunteers and measured eicosanoid formation by a variety of cell types *in vivo* and *ex vivo*, using sensitive and specific physicochemical assays. We found that the *in vivo* course of prednisone failed to inhibit the synthesis of thromboxane A_2, prostaglandin I_2 (prostacyclin), prostaglanin E_2, and leukotriene E_4 *in vivo* and of leukotriene B_4 *ex vivo*. Biosynthesis of leukotriene B_4, thromboxane B_2, and prostaglandins F_2 and E_2 by macrophage-rich bronchoalveolar lavage cells was strongly suppressed. These findings indicate that therapeutic regimens of glucocorticoids suppress eicosanoid biosynthesis in human macrophages but not in a number of other cell types with steroid receptors, the capacity for eicosanoid formation, and lipocortin-like material.

Eicosanoids have potent proinflammatory actions *in vitro* and *in vivo*. For example, prostaglandins E and I (PGE_2 and PGI_2) are vasodilators and leukotriene B_4 (LTB_4) promotes chemotaxis and diapedesis (1). Glucocorticoids are potent antiinflammatory drugs. Their biochemical mechanism of action, however, remains uncertain (2). Early studies have demonstrated that steroids can suppress the formation of eicosanoids (3) and inhibit the release of arachidonic acid (4) in whole organ and cell culture systems. It has been hypothesized that steroids may act to inhibit the formation of eicosanoids *in vivo* and that this may contribute to their antiinflammatory effects.

Few studies have looked directly for a modulation of eicosanoid biosynthesis in response to steroid administration *in vivo*. Almost all have been performed in animals. These experiments, with rare exception (5), have failed to demonstrate a suppressive effect (6–15), even in the face of unequivocal inhibition of eicosanoid formation *in vitro* (10). We have designed a study to evaluate the effects of therapeutic doses of glucocorticoids on the synthesis of eicosanoids *in vivo* in humans.

METHODS

Subjects. Eight healthy volunteers (6 men, 2 women) aged 22–34 years participated in the 7-day study after giving informed consent. They were nonsmokers and had no history of asthma, allergy, or recent upper respiratory tract infection. They had no contraindications to steroid treatment and took no other medications before the study. Six similar subjects (4 men) participated in the short-term study. The investigation was approved by the Vanderbilt University Committee for the Protection of Human Subjects.

Study Design. In the 7-day study, the eight subjects took 60 mg of prednisone every morning by mouth for 7 days, the equivalent of 8 times the normal total daily production of hydrocortisone. On both of the 2 days immediately before drug treatment and again on both of the final 2 days of this course, heparinized (20 units/ml) whole blood and 24-hr urine collections were obtained. All blood samples were obtained at the same time of day, which at the end of the study was 2 hr after the preceding dose of prednisone. In four of these eight subjects, bronchoalveolar lavage was also performed, initially 1 week before the start and again at the end of the treatment. In the short-term study, the six subjects took 60 mg of prednisone by mouth at 0800 on two consecutive days. Twenty-four-hour urine collections were obtained before and on the first day of drug treatment and heparinized whole blood was obtained 0, 1, 2, 4, 8, 24, and 30 hr after the first dose.

Plasma prednisone and prednisolone concentrations were measured by HPLC (16). The completion of all urine collections obtained in the study was inferred from the close similarity of total creatinine in the four collections obtained from each subject. Prior to storage at −20°C, aliquots (5 ml) were equilibrated with stable-isotope-labeled standards.

Whole blood leukocyte stimulation *ex vivo* was performed as described (17). Heparinized whole blood was divided into aliquots in polypropylene tubes and stimulants were added as follows: freshly opsonized zymosan A (100 and 250 μg/ml), the formylated tripeptide fMet-Leu-Phe (1 and 2 μM) in the presence of cytochalasin B (5 μg/ml), anti-human IgE (1 and 2 μg/ml), or no added stimulant (17). After incubation at 37°C in a shaking water bath for 30 min, the plasma was immediately collected by centrifugation and aliquots were equilibrated with 5 ng of $^2H_4$-labeled LTB_4.

Bronchoscopy and Bronchoalveolar Lavage. Bronchoalveolar lavage cells (BALCs) were obtained as described (18). Total recovery of BALCs ranged from 4.7 to 8.0 × 10^6 cells, of which 83–93% were alveolar macrophages, and cell viability as assessed by trypan blue exclusion was 93–100%. The recovery of BALCs, percentage of macrophages, and percentage of viable cells were unaltered by steroid therapy. BALCs were immediately processed without washing in order to minimize loss of any steroid effects that had been induced *in vivo*. Cells were pelleted at 400 × *g* for 15 min and resuspended in RPMI 1640 containing fetal bovine serum (10%), L-glutamine (2 mM), penicillin (200 units/ml), and gentamicin (40 μg/ml). Cells were seeded in 24-well plastic culture plates (Costar) at 200,000 cells per 0.4 ml per well. Some wells were devoted to a simultaneous *in vitro* study and either buffer alone or hydrocortisone phosphate (final concentration, 0.1, 1, or 10 μM) was added to these. Cells were preincubated at 37°C in a 5% CO_2/100% humidity atmosphere for 1.5, 4.5 or 7.5 hr followed, without an exchange of medium, by addition of either no stimulus or freshly op-

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Abbreviations: LT, leukotriene; PG, prostaglandin; Tx, thromboxane; BALC, bronchoalveolar lavage cell.

sonized zymosan A (100 μg/ml) (19). Incubation was continued with the stimulant for 4 hr. The culture media were then collected and centrifuged and the supernatants were equilibrated with eicosanoid standards for quantitative analysis.

Derivatization and Extraction of Eicosanoids. Urinary 2,3-dinorthromboxane B_2 (2,3-dinor-TxB_2) and 11-dehydro-TxB_2 (20), PGI-M (21), and PGE-M (22) and LTB_4 released *ex vivo* (17) were analyzed by gas chromatography/mass spectrometry. Eicosanoids were measured in BALC supernatants by a modification of methods previously described (23). Urinary LTE_4 was measured by HPLC–RIA (18).

RESULTS

Plasma Prednisone and Prednisolone. To confirm compliance with the protocol and demonstrate effective bioconversion of prednisone to its active form, prednisolone, we measured plasma prednisone and prednisolone on both of the 2 days before treatment and again on both of the final 2 days of treatment. The later samples were drawn 2 hr after ingestion of the daily dose of prednisone. Neither prednisone nor prednisolone was detectable in any subject on control days. Prednisone was again not detected on the final 2 days and prednisolone ranged from 340 to 650 ng/ml (mean ± SD, 524 ± 101).

Blood Leukocyte Count and Differential. The total blood leukocyte count increased from 6.3 ± 0.8 to 8.9 ± 1.6 × 10^3 per μl after 7 days of steroid treatment. Neutrophils changed from 61 ± 6% to 72 ± 7% and monocytes from 7 ± 1% to 5 ± 1%. In the short-term study, at 0, 1, 2, 4, 8, 24, and 30 hr the leukocyte counts were 4.6 ± 0.3, 4.7 ± 0.4, 5.8 ± 0.6, 9.7 ± 1.6, 7.6 ± 0.6, 6.6 ± 0.6, and 8.5 ± 0.4 × 10^3 per μl; the neutrophils were 54 ± 4%, 55 ± 5%, 81 ± 3%, 92 ± 1%, 90 ± 2%, 58 ± 2%, and 91 ± 1%; the monocytes were 7.5 ± 0.5%, 6.3 ± 0.6%, 2.0 ± 0.3%, 1.3 ± 0%, 1.2 ± 0.2%, 7.5 ± 0.8%, and 0.6 ± 0.2%.

After prednisone administration for 1 week, excretion of the dinor metabolites of TxA_2 (164 ± 22 vs. 161 ± 19 ng/day) and of PGI_2 (200 ± 16 vs. 225 ± 16 ng/day) and excretion of PGE-M (4.3 ± 0.9 vs. 7.1 ± 2.1 μg/day) was not suppressed or changed significantly. In fact, a slight increase in PGE-M occurred in all but one of the subjects, which was not statistically significant for the group as a whole. To examine the possibility of a steroid effect on the metabolic disposition of eicosanoids, in the 2-day study we measured two different urinary metabolites of TxA_2, dinor-TxB_2 (270 ± 43 vs. 245 ± 46 ng/day) and 11-dehydro-TxB_2 (390 ± 110 vs. 357 ± 136 ng/day) and found no change in either measurement. Urinary LTE_4 (49 ± 12 ng/day) also remained unchanged with prednisone treatment (45 ± 18 ng/day).

LTB_4 Release by Whole Blood Leukocytes *ex Vivo*. Basal LTB_4 release on the 2 control days in the absence of added stimulant was 24 ± 5 pg per 10^6 leukocytes. On the control days, stimulation with anti-IgE increased the LTB_4 release by 1.5- to 2-fold above basal, fMet-Leu-Phe by 20- to 40-fold, and opsonized zymosan by 20- to 40-fold. After prednisone administration for 1 week, the LTB_4 release induced by each of these stimulants was not changed. In the 2-day study, we examined the possibility of an acute steroid effect on LTB_4 release by blood leukocytes. In the hours following prednisone ingestion, there was actually a 3- to 4-fold rise in LTB_4 released per 10^6 leukocytes, which returns to baseline after 24 hr (Fig. 1).

Eicosanoid Release by BALCs *ex Vivo*. Eicosanoid formation in alveolar macrophage-rich BALCs was assessed in response to stimulation for 4 hr by either no stimulant or opsonized zymosan added after three different prestimulus *ex vivo* incubation times. This was measured once before and once at the end of the prednisone treatment.

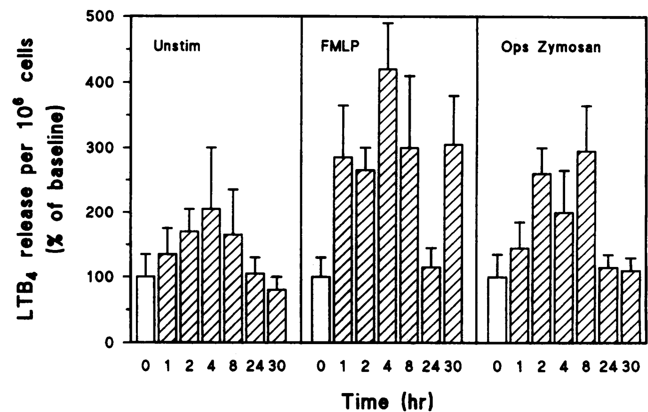

FIG. 1. *Ex vivo* LTB_4 accumulation in stimulated whole blood early after prednisone administration. Prednisone (60 mg) was given at 0 and 24 hr. Whole blood was obtained at the times shown and aliquots were stimulated as described in *Methods*. Results are shown for unstimulated aliquots and for aliquots stimulated with fMet-Leu-Phe (FMLP) at 1 μM or opsonized zymosan at 100 μg/ml; results for alternative doses of stimulants were similar. Results are expressed as a percentage of the value obtained at time zero (open bar). A transient rise in LTB_4 release *per cell* coincided with the steroid-induced changes in leukocyte count and differential and returned to baseline at 24 hr.

In BALCs obtained on control days, the spontaneous release of the three cyclooxygenase products increased linearly with increasing time in culture (Fig. 2, open bars) and maintained approximately constant relative proportions of 17:2.3:1 for $TxB_2/PGF_2/PGE_2$. In contrast, LTB_4 accumulation declined progressively with increasing time in culture. Stimulation by opsonized zymosan added for 4 hr after prestimulus culture times of 1.5, 4.5, or 7.5 hr (Fig. 3, open bars) increased the release of the three cyclooxygenase products by 2- to 3-fold above the corresponding values for spontaneous release and again maintained their relative proportions unchanged. LTB_4 accumulation after zymosan stimulation increased 3- to 4-fold above spontaneous release.

After prednisone administration *in vivo*, there was a profound reduction of eicosanoid release *ex vivo* by the BALCs. Release of the three cyclooxygenase products was reduced to only 12–19% of control values for zymosan-stimulated cells (Fig. 3, solid bars). A very similar reduction, to 11–20% of control, occurred for spontaneous release (Fig. 2, solid bars). In contrast, release of LTB_4 was reduced only to 25% of control for zymosan-stimulated cells (Fig. 3, solid bars) and to 63% of control for unstimulated (spontaneous) release (Fig. 2, solid bars). No measurable recovery from these degrees of inhibition occurred for any eicosanoid, even after 7.5 hr in culture before the addition of stimulant (Figs. 2 and 3, solid bars).

In separate experiments conducted at the same time as the above, BALCs were exposed *in vitro* to hydrocortisone added to the wells during the incubation period that preceded the addition of either opsonized zymosan or buffer control. In BALCs obtained prior to prednisone administration (control BALCs), release of the three cyclooxygenase products showed a clear time- and concentration-dependent suppression for both zymosan-stimulated (Fig. 3, hatched bars) and spontaneous release (Fig. 2, hatched bars). At the longest incubation time (7.5 hr plus 4 hr stimulation), the suppressive effect had not yet reached the level we observed in the suppressed BALCs obtained after 1 week of prednisone administration *in vivo* (see above). This is in contrast to LTB_4 release, where the suppressive effect on zymosan-stimulated LTB_4 observed in prednisone-suppressed BALCs had yet to appear under these *in vitro* conditions, while suppression of spontaneous LTB_4 seemed already to have reached the level

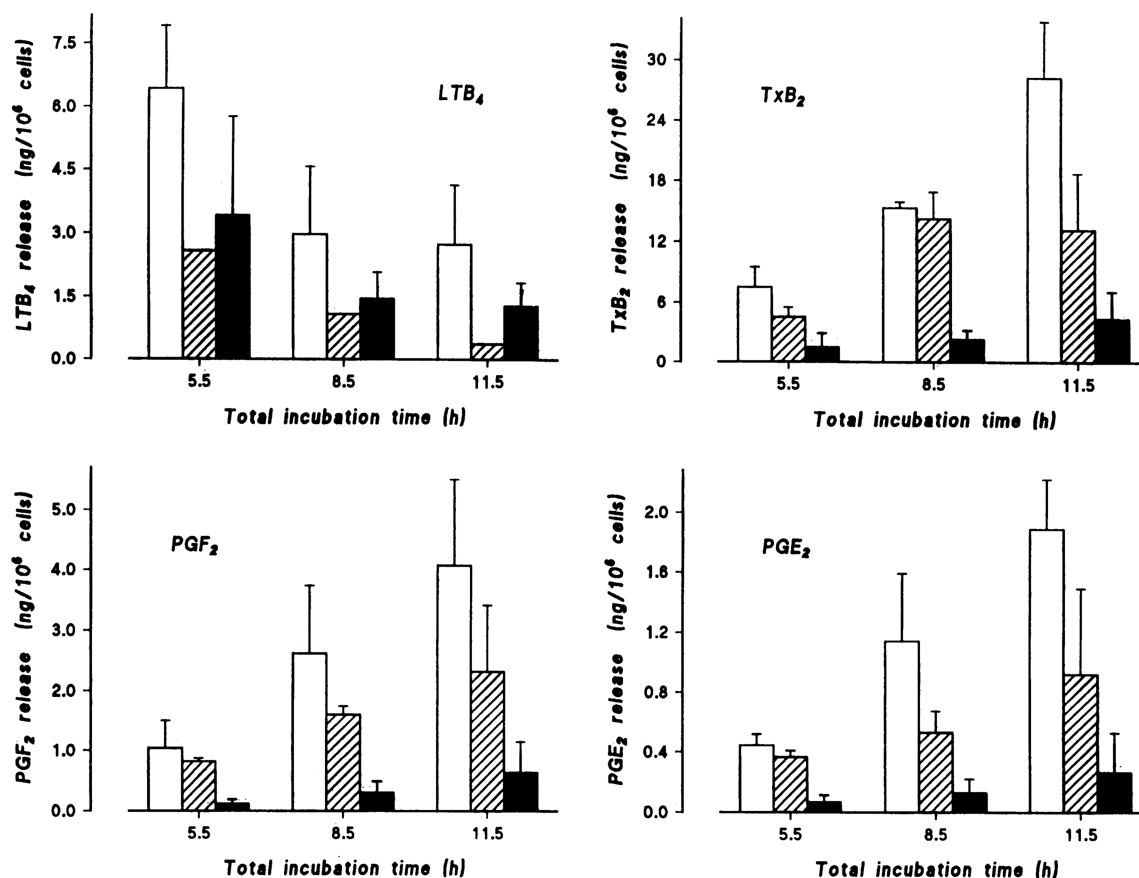

FIG. 2. Spontaneous extracellular accumulation of four eicosanoids released by BALCs. Conditions were as described for Fig. 3 except that buffer rather than zymosan was added 4 hr before the end of the incubations. Open bars show control release, solid bars show the effect of the *in vivo* prednisone treatment, and hatched bars show the effect of 10 μM hydrocortisone added at the beginning of the incubations.

found in the cells obtained *ex vivo* during steroid treatment. In BALCs obtained after prednisone administration *in vivo*, the addition of hydrocortisone to the culture medium had little or no further inhibitory effect on any of the four eicosanoids.

DISCUSSION

We undertook this study to determine the effects of steroid treatment at antiinflammatory doses on eicosanoid formation in humans. The excretion of principal urinary metabolites of three eicosanoids was measured as an index of the daily systemic biosynthesis of their parent eicosanoids. Cyclooxygenase inhibitors dose-dependently inhibit excretion of these compounds (24). This suggested that a similar suppression by steroid administration should have been readily detectable by this approach. Under physiologic conditions, urinary Tx-M derives principally from platelets (20). Although glucocorticoids have been shown to inhibit TxA_2 synthesis in U-937 cells (a monocyte-derived cell line) by a mechanism dependent upon steroid binding (25), steroid receptors have not been reported in platelets, which lack a significant capacity for new protein synthesis. Thus, the lack of a prednisone effect on Tx-M is unsurprising and consistent with similar findings reported in patients in septic shock receiving intravenous steroids (11). PGI_2 is a major product of endothelial cells in culture (26) and vascular stimulation results in marked augmentation of PGI-M excretion *in vivo* (27). PGE_2 is the predominant cyclooxygenase product of microvascular endothelial cells in culture (28). Both PGI_2 and PGE_2 are formed at the site of vascular injury in humans (29). Therefore, as endothelial cells have been reported to possess steroid receptors (30) and are susceptible to steroid inhibition of eicosanoid release in culture (31), the failure of steroid treatment to depress excretion of metabolites of these eicosanoids was unexpected. In fact, a small paradoxical increase was seen in PGE-M excretion, similar to findings reported in rabbits after dexamethasone treatment (9). Excretion of LTE_4 is increased coincident with allergen-evoked bronchospasm in allergic volunteers, and inhibition of LT formation has been speculated to contribute to the efficacy of steroids in the treatment of asthma (18). In the short-term study, steroid administration did not alter LTE_4 excretion in our healthy volunteers.

We chose an *ex vivo* whole blood model of stimulated leukocytes to assess the effect of *in vivo* administration of prednisone on the lipoxygenase pathway. In this model, leukocytes remain in the whole blood environment with drug concentrations closely approximating those present *in vivo* after therapeutic dosing. While the individual contributions by neutrophils and monocytes to total LTB_4 release cannot be assessed, they are probably substantial (32) and dependent on the stimulus applied, for both monocytes (33) and neutrophils (34), whereas lymphocytes synthesize no LTB_4 (32). We found that after 1 week of treatment with prednisone, there was no reduction in stimulated LTB_4 release. This was particularly surprising, because both neutrophils and monocytes participate in inflammatory reactions, bear steroid receptors (35), and contain lipocortin-staining granules (36–38). However, in a previous study of fractionated leukocytes, the prior administration of a single dose of dexamethasone has also been reported to have no effect on stimulated LTB_4 release (14). In the short-term study we demonstrated that coincident with the acute prednisone-induced leukocytosis, neutrophilia, and monocytopenia after 4–8 hr, there was actually a marked enhancement of the LTB_4 released per leukocyte. Our whole blood model precludes the ability to

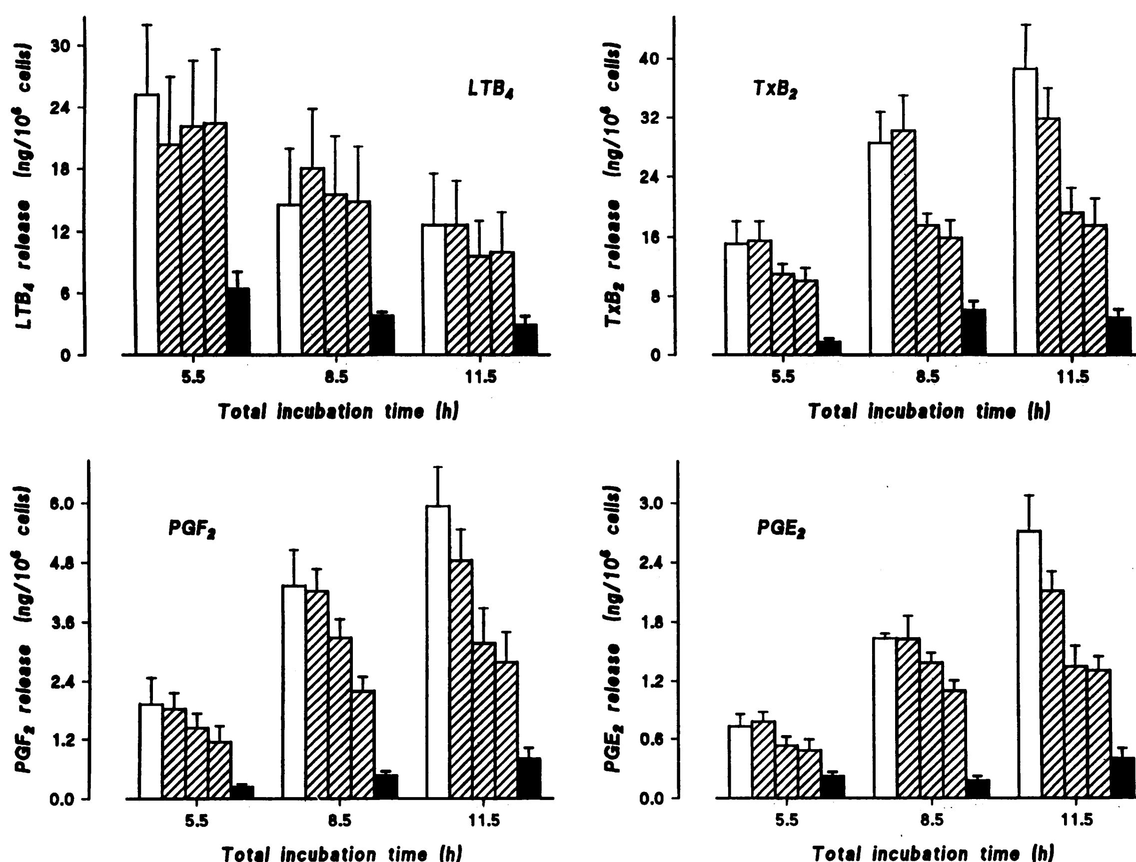

FIG. 3. Zymosan-stimulated extracellular accumulation of four eicosanoids released by BALCs. BALCs were obtained by bronchoalveolar lavage of four of the eight study subjects, once before and once at the end of the prednisone treatment, and cultured for 1.5, 4.5, or 7.5 hr. Open bars show eicosanoid accumulation on control days for each of the three prestimulus times as mean ± SEM for the study group. Solid bars show the effect of *in vivo* prednisone treatment and hatched bars show the effect of three concentrations of hydrocortisone (0.1, 1, and 10 μM, shown from left to right) added to control cells at the beginning of the prestimulus incubation period. The addition of hydrocortisone to cells obtained after prednisone treatment (data not shown) did not cause any further significant change from the results shown by solid bars.

discriminate selective effects on individual types of leukocytes. However, the increased LTB_4 in the face of marked monocytopenia encouraged us to examine an alternative calculation of our data as if all LTB_4 were derived from the neutrophil fraction. The results of this speculation suggest that LTB_4 release per neutrophil may be increased to 1.5- to 2.5-fold above baseline 4–8 hr after steroid ingestion, whereas it is diminished to ≈50% after 7 days.

In contrast to these observations, we observed a simultaneous profound inhibition of eicosanoid release by BALCs. Both cyclooxygenase and lipoxygenase products were reduced, though somewhat unequally. In control BALCs, obtained before prednisone administration, the released amounts of the four eicosanoids were consistent with those previously reported for human alveolar macrophages (39) and LTB_4 release per cell was substantially greater than that from blood leukocytes measured simultaneously.

Hydrocortisone added to cultured BALCs obtained prior to prednisolone administration also suppressed eicosanoid formation in a time- and dose-dependent fashion. However, even the longest drug incubation that we employed (7.5 hr) did not match the profound effect of the week-long course of prednisone on eicosanoid formation by BALCs *ex vivo*. This was especially true for LTB_4. The time to maximal effect of steroid suppression appears to be substantial. This is the case for cultured rat alveolar macrophages (18), but not rat peritoneal macrophages (40). Once induced, suppression in our own study was also long-lasting. BALCs obtained during prednisone administration showed no measurable recovery after culture *ex vivo* as long as 7.5 hr.

Under all the conditions in macrophage-rich BALCs the three cyclooxygenase products always appeared in approximately constant proportions to one another, whereas the kinetics of LTB_4 accumulation followed an independent course. Thus, a constant duration of stimulation by zymosan (4 hr) resulted in increasingly large accumulations of cyclooxygenase products when applied after increasing prestimulus times in culture, whereas the opposite was true for LTB_4. Furthermore, we also found that the inhibitory effects of both prednisone *in vivo* and hydrocortisone in culture were significantly ($P < 0.01$) less on the stimulated formation of LTB_4 (to 25 ± 1.2% control) than on the other three metabolites (to 16 ± 2.0% control). This is unlike findings reported for rat alveolar macrophages in culture (41) and rat gastric tissue *in vivo* (13) but similar to observations with human alveolar macrophages and murine macrophages in culture (42). Stimulated fibroblast cyclooxygenase activity is inhibited by glucocorticoids (43). Perhaps direct effects on BALC cyclooxygenase account for the greater suppression of prostanoid formation than of LTB_4 that we observed.

In summary, our results show that inhibition by glucocorticoids of eicosanoid release *in vivo* occurs in some, but not all, types of eicosanoid-synthesizing cell types. Our findings show that one cannot predict the degree of this cellular suppression of eicosanoid formation by such potential determinants as suppressibility by steroids in culture, the presence of membrane steroid receptors, the enzymatic capacity to synthesize arachidonate metabolites, or the presence of intracellular granules that contain lipocortin-like material. It remains to be determined what effects glucocorticoids might

have on eicosanoid formation in humans in the presence of inflammation.

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