Alterations in Cyclic AMP Metabolism in Human Bronchial Asthma

III. LEUKOCYTE AND LYMPHOCYTE RESPONSES TO STEROIDS

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ABSTRACT On the basis of serial studies the responsiveness of leukocytes and lymphocytes from asthmatic donors to catecholamines was increased during high dose corticosteroid therapy. Similar changes were observed in the cells of normal control subjects given 200 mg of hydrocortisone intravenously. The increase in responsiveness did not appear to be due to changes in lymphocyte subpopulations although this may be a contributing factor. In an effort to elucidate the basis for the improved response, in vitro effects of glucocorticoids on lymphocyte cyclic AMP concentrations were investigated. Glucocorticoids (prednisolone succinate, hydrocortisone, hydrocortisone phosphate, and hydrocortisone succinate) stimulated cyclic AMP accumulation in asthma and normal control lymphocytes, increases occurring within the first 2 min of incubation. In the absence of theophylline, responses were regularly obtained at 10 μM hydrocortisone and usually at 1 μM hydrocortisone but not at submicromolar steroid concentrations. Theophylline potentiated the cyclic AMP response to glucocorticoids and also increased the percentage of positive responses in the 0.01–1.0 μM corticosteroid range. Combinations of 1 μM hydrocortisone and 1 μM epinephrine were sometimes additive or synergistic but in many instances higher glucocorticoid concentrations were needed to obtain augmentation of the catecholamine response. The in vitro glucocorticoid effects may not fully explain their potentiating action in vivo.

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INTRODUCTION

The mechanism of action of glucocorticoids in bronchial asthma has long been a subject of controversy. Even in massive doses, corticosteroids appear to have little direct bronchodilator action and in the absence of concurrent sympathomimetic amine therapy are of limited benefit (1). But in individuals who have failed to respond to large amounts of corticosteroids given alone, small amounts of beta adrenergic drugs have produced dramatic improvement, suggesting that glucocorticoids can amplify catecholamine responsiveness. Evidence for a catecholamine potentiating action of corticosteroids has been obtained in recent studies in experimental animals in vivo and in vitro (2, 3). Ordinarily the improved catecholamine response appears to involve an enhancement of cellular enzyme responsiveness to the modulation effects of cyclic AMP (3, 4) but a direct effect on cyclic AMP accumulation has also been obtained, particularly in tissues from adrenalectomized animals (5, 6). We have previously demonstrated that peripheral blood leukocytes and lymphocytes from individuals with bronchial asthma have a decreased cyclic AMP response to beta adrenergic drugs by comparison with cells from normal control subjects (7, 8). In the present report the ability of corticosteroids to alter adrenergic responsiveness in these cells has been evaluated.

METHODS

Hydrocortisone, hydrocortisone-21-phosphate, hydrocortisone-21-hemisuccinate, and prednisolone-21-hemisuccinate were obtained from the Sigma Chemical Co., St. Louis, Mo. Stock 10 mM corticosteroid solutions were freshly prepared in 95% ethanol and dilutions were made in 0.1 M NaCl. The maximal ethanol concentration used with cells was 1% (vol:

TABLE I

Serial Studies of the Leukocyte Cyclic Response to Isoproterenol in Individuals with Bronchial Asthma. Results Before and After Corticosteroid Therapy.

	Cyclic AMP before corticosteroids		Corticosteroid dose/24 h (hydrocortisone equivalents)	Duration therapy	Cyclic AMP after corticosteroids	
	Buffer	10 mM Isoproterenol			Buffer	10 mM Isoproterenol
	pmol/10^7 cells			*h*	*pmol/10^7 cells*	
1. L. G.	5	15	100	24	5	18
2. M. R.	7	19	140	72	6	33
3. R. Z.	6	23	100	24	9	28
4. B. H.	5	11	200	36	7	28
5. M. H.	3	7	150	24	4	12
6. E. B.	6	17	150	72	6	21

Blood specimens obtained after initiation of corticosteroid therapy were collected at least 12 h after the preceding steroid dose. 4×10^6 leukocytes were incubated for 30 min at 37° in 0.5 ml Gey's solution in the presence and absence of 10 mM isoproterenol. The average percentage of lymphocytes prior to steroid therapy was 31%, after corticosteroid therapy 28%.

vol) (at 100 μM corticosteroid concentrations). Control experiments indicated that leukocyte cyclic AMP concentrations were not altered in the presence of 1% ethanol. In short incubation experiments (up to 2 h) no adverse effects of 1–100 μM hydrocortisone on lymphocyte viability (trypan blue exclusion) were observed. Cells incubated with 100 μM hydrocortisone in medium at 37° for 4 h and thoroughly washed had the expected DNA synthetic response to phytohemagglutinin P (Difco Laboratories, Detroit, Mich.).

Procedures for the purification of leukocytes and lymphocytes, enumeration of immunoglobulin containing cells and measurement of cyclic AMP have been described in detail elsewhere (8–10).

RESULTS

Possible effects of systemic corticosteroid therapy on leukocyte and lymphocyte adrenergic responsiveness. We have previously reported that when leukocytes from patients with active asthma receiving and not receiving systemic corticosteroid therapy were compared, the 10 mM isoproterenol response was somewhat greater in cells from steroid-treated patients (8). While the difference was not statistically significant, the average patient receiving corticosteroids had had more severe bronchial asthma (judging from FEV_1[1] and forced expiration time measurements) which might partially mask an improved response. It should also be pointed out that hydrocortisone dosage was equivalent to < 80 mg of hydrocortisone/day in many of these individuals.

The possible effect of systemic corticosteroid therapy on adrenergic responsiveness was also evaluated by serial leukocyte measurements at 24–48-h intervals in six asthmatic patients before and during corticosteroid therapy. Apparent improvement (in the absence of marked changes in the percentage of circulating lymphocytes) in the isoproterenol response occurred in three with increases of doubtful statistical significance in the other three (Table I).

The lymphocytes of three asthmatic patients and four normal adult controls were studied for more acute changes in catecholamine responsiveness following one or two 100-mg intravenous injections of hydrocortisone (Table II). In very instance the 10 mM isoproterenol response improved markedly within several hours, the increase ranging from 66–250%. Similar increases occurred in lymphocyte responses to 1 mM isoproterenol, 0.1 mM isoproterenol, 1 μM epinephrine–0.5 mM theophylline, and 10 mM isoproterenol–0.5 mM theophylline (Table III). An increased cyclic AMP response was also obtained with 30 μM prostaglandin E_1 (PGE_1) indicating that the alteration in the cyclic AMP response in association with corticosteroid therapy is not restricted to catecholamines. Cyclic AMP concentrations in unstimulated cells were also increased but to a lesser extent.

As expected the sizable intravenous hydrocortisone dosages used in the acute experiments produced an absolute lymphocytopenia raising the possibility of a quantitative change in lymphocyte subpopulations. The The ratio of thymus and bone marrow derived lymphocytes (T and B cells) was enumerated by indirect immunofluorescence reacting cells initially with a mixture of rabbit antibodies specific for human IgG and IgM or IgG alone. Fluorescein-tagged goat anti-rabbit IgG was used as the second antibody. Following hydrocortisone injection the percentage of immunoglobulin containing lymphocytes (presumably B cells) increased in each of three normal subjects (mean increase 88%) and in one of two asthmatic subjects (mean increase 29%) for whom data are available (Table II). In individuals

[1] *Abbreviations used in this paper:* FEV_1, 1 s forced expiration velocity; PGE_1, prostaglandin E_1.

TABLE II

Effect of Intravenous Hydrocortisone on Lymphocyte Cyclic AMP Responses to 10 mM Isoproterenol

	Cyclic AMP					% Immunoglobulin-containing cells*	
	Before hydrocortisone	After 60 min	Hydrocortisone 150 min	Hydrocortisone 210 min	Maximal change‡	Before§	After
	pmol/10⁷ cells		*pmol/10⁷ cells*		%		
Asthma M. P.	75	130	260		325	6	6
Asthma B. H.	56		108		93	24	38
Asthma A. M.‖	24			40	66		
Normal C. P.	92	118	130	180	96	24	45
Normal J. W.	62		105		70		
Normal M. H.	185		335		80	16	25
Normal D. K.	112		180		80	14	31

After drawing an initial blood specimen (before hydrocortisone) subjects were given 100 mg hydrocortisone phosphate intravenously. The 100 mg hydrocortisone injection was repeated after 100 min. Lymphocytes were purified by Ficoll-Hypaque centrifugation. 1.5 million lymphocytes in 0.5 ml Gey's solution were stimulated with 10 mM isoproterenol for 30 min at 37°.

* The percentages are for IgG-containing cells (M. P., C. P., and M. H.) or IgG- and IgM-containing cells (B. H., D. K.).

‡ Percent increase in 10 mM isoproterenol response above "before hydrocortisone" (buffer control cell background not subtracted). Buffer control backgrounds were also increased (mean increase, 45%). Serial measurements in three normal control subjects (no hydrocortisone given) over a similar time period did not reveal significant fluctuation in the cyclic AMP response.

§ Before and after refer to before and after hydrocortisone infusion.

‖ A. M. received only a single 100 mg injection of hydrocortisone.

with a marked change in the percentage of circulating B cells we cannot exclude the possibility that the increase in catecholamine and PGE_1 responsiveness was due at least in part to the change in lymphocyte subpopulations. It seems doubtful that this is the only explanation since in three individuals (M. P., B. H., and M. H.) the increase in PGE_1 and catecholamine responsiveness considerably exceeded the increase in percentage of B cells. For example, in M. P. (Table III) pre- and post-corticosteroid lymphocyte preparations both had 6% IgG containing cells (an unusually low value) and a large increase in catecholamine and prostaglandin responsiveness nonetheless occurred.

TABLE III

Effect of Intravenous Hydrocortisone on Lymphocyte Cyclic AMP Responses to 1 and 0.1 mM Isoproterenol, Epinephrine, and PGE_1

Condition	Cyclic AMP: Before hydrocortisone	Cyclic AMP: 150 min after hydrocortisone
	pmol/10⁷ cells	
Control	22(±4)*	32(±6)
1 mM isoproterenol	65(±14)	110(±15)
0.1 mM isoproterenol	43(±10)	77(±6)
10 mM isoproterenol +0.5 mM theophylline	139(±20)	290(±26)
1 μM epinephrine +0.5 mM theophylline	70(±12)	160(±21)
30 μM PGE_1	150(±16)	375(±31)

Pooled lymphocyte cyclic AMP data from six of the seven subjects in Table II (A. M. is omitted). The cell preparations and incubation conditions are the same as those in Table II. Cells were obtained before and 150 min after the first of two injections of hydrocortisone (see Table II).

* ±SEM.

Responses of normal control lymphocytes to corticosteroids in vitro. In an attempt to elucidate how corticosteroid therapy might alter lymphocyte responsiveness to isoproterenol the effect of adding corticosteroids to normal lymphocytes in vitro was studied. In 30-min incubation experiments an increase in cyclic AMP concentration was regularly obtained at 10 μM hydrocortisone and frequently at 1 μM hydrocortisone (Table IV). Increases were also observable at earlier times (2, 5, and 10 min) and with other glucocorticoids (prednisolone and two water-soluble hydrocortisone derivatives). A greater cyclic AMP response was obtained at 30 than at 5 min in two experiments but no difference in two others (not shown). With an incubation period of 2 h the corticosteroid response was similar to or less than that obtained at 30 min (three experiments). The glucocorticoid response was not blocked by propranolol (at

TABLE IV

Response of Ficoll-Hypaque Purified Lymphocytes to Hydrocortisone

Type cell	No.	10 μM Hydrocortisone: No. with increase	10 μM Hydrocortisone: Mean % increase	No.	1 μM Hydrocortisone: No. with increase	1 μM Hydrocortisone: Mean % increase
1. Normal control	12	11	220(±40)*	10	6	100(±20)
2. Asthma	10	7	120(±35)	17	9	80(±30)

1.5 × 10^6 lymphocytes (obtained by Ficoll-Hypaque centrifugation) were incubated in 0.5 ml Gey's solution for 30 min at 37° in the presence and absence of hydrocortisone.
* ±SEM.

propranolol concentrations that markedly inhibited catecholamine effects on cyclic AMP concentrations in the same cells), indicating that beta adrenergic receptors are not involved. In the presence of 0.5 mM theophylline, the increase in cyclic AMP concentration was accentuated and responses were sometimes demonstrable at 0.1 and even 0.01 μM hydrocortisone. In both the presence and absence of theophylline there was considerable variation in individual responsiveness which was especially evident at 1 μM hydrocortisone. Elucidation of the basis for this variation will require further study. It is not likely to be due to diurnal fluctuation in endogenous glucocorticoid concentrations since samples were drawn at the same time of day.

Responses of lymphocytes from asthmatic donors to corticosteroids in vitro. In purified lymphocytes from asthmatic donors not receiving glucocorticoid therapy a cyclic AMP response to corticosteroids was ordinarily obtained at 10 and 1 μM hydrocortisone, the response overlapping that of normal control cells (Table IV). Incubation time effects were similar to those obtained with normal cells. The response of asthma cells to 10 μM hydrocortisone was statistically decreased ($P < 0.05$).

Responses of lymphocytes from asthmatic donors to hydrocortisone and epinephrine in combination in vitro. In order to evaluate the ability of hydrocortisone to potentiate the cyclic AMP response to beta adrenergic drugs, the effect of 1 μM hydrocortisone on asthma lymphocyte cyclic AMP concentrations in the presence of 1 μM epinephrine, 0.5 mM theophylline was studied. In 9 of 17 experiments in which a 30 min incubation time was used a greater response was obtained to hydrocortisone, epinephrine, and theophylline in combination than to epinephrine and theophylline alone. In the 17 experiments as a whole a 40(±14)% greater response was obtained in the presence of hydrocortisone than in its absence. In five of nine experiments in which hydrocortisone clearly augmented the epinephrine-theophylline response, synergistic effects were obtained. In three experiments, large hydrocortisone effects were obtained with cells that had decreased responsiveness to epinephrine-theophylline (two such experiments, closed circles and triangles, are shown in Fig. 1). In other experiments hydrocortisone either did not potentiate the epinephrine-theophylline response or did so only at concentrations of 5 μM or higher (see, for example, the experiment denoted by open circles in Fig. 1). In three experiments in which potentiation of the epinephrine response by hydrocortisone was obtained, data at shorter incubation times are also available. In two of the three

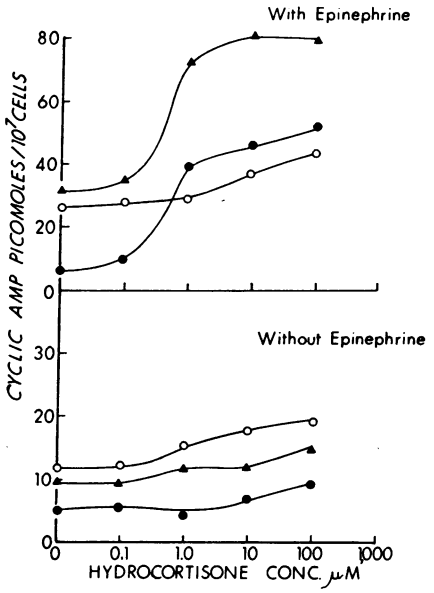

FIGURE 1 Effect of hydrocortisone on the cyclic AMP response to epinephrine in lymphocytes from asthmatic donors. ▲——▲, ○——○, and ●——● are Ficoll-Hypaque purified lymphocytes from three different donors with active bronchial asthma (none on corticosteroid therapy). Cells were incubated with various amounts of hydrocortisone in the presence and absence of 1 μM epinephrine (0.5 mM theophylline was present in each instance). Buffer control values without theophylline were 6, 11, and 5 pmol cyclic AMP/10^7 cells, respectively. Incubation for 30 min at 37° in Gey's solution.

TABLE V

Effect of Preincubation with Hydrocortisone on Lymphocyte Cyclic AMP Responses to Isoproterenol

	Preincubation condition		Final incubation condition: Cyclic AMP		
	Time	Hydrocortisone concentration	Time	Buffer	10 mM Isoproterenol
	min	*μM*	*min*	*pmol/10⁷ cells*	
1.	120	—	10	32	70
2.	120	3.0	10	28	70
3.	120	1.0	10	27	84
4.	120	0.3	10	38	72
5.	120	0.1	10	30	80
6.	30	—	10	36	100
7.	30	3.0	10	40	112
8.	30	1.0	10	36	90
9.	30	0.3	10	40	90
10.	30	0.1	10	34	108
11.	10	—	10	32	106
12.	10	3.0	10	40	120
13.	10	1.0	10	35	112
14.	10	0.3	10	30	108

The effect of preincubation with hydrocortisone on the cyclic AMP response to 10 mM isoproterenol. Ficoll-Hypaque purified lymphocytes from a normal control donor (selected because of a feeble cyclic AMP response to hydrocortisone in short incubation experiments). 1.5 million cells were preincubated at 37° in 0.5 ml Gey's for 10–120 min in the presence and absence of hydrocortisone. Isoproterenol or buffer was then added and incubation was continued for another 10 min.

experiments potentiation was observed at 2 and 5 min as well as at 30 min.

In normal control cells amplification of the epinephrine response at low hydrocortisone concentrations was also observed, although to a lesser extent than in cells from asthmatic donors. At high (100 μM), nonpharmacologic hydrocortisone concentrations a paradoxical effect was sometimes obtained in which the two agents in combination produced a smaller rise in cyclic AMP than either agent alone.

Despite the impressive potentiation of epinephrine stimulation by hydrocortisone in some experiments, more frequently marked changes in the catecholamine response were not observed. The possibility was considered that corticosteroids might exert a delayed effect on adrenergic responsiveness in addition to whatever changes were obtained in short incubation experiments. Three preparations of asthma lymphocytes and three of normal control lymphocytes in which hydroocrtisone did not augment epinephrine or isoproterenol responsiveness in 10 and 30 min incubation experiments were studied. Preincubation of these cells with pharmacologic concentrations of hydrocortisone for 30 and 120 min did not increase the catecholamine response. Representative data are given in Table V.

The ability of hydrocortisone to potentiate the catecholamine response in beta blocked (*d,l*-propranolol) normal control cells also was studied (three experiments, one of which is shown in Table VI). Essentially negative results were obtained.

DISCUSSION

The results of these studies indicate that pharmacologic concentrations of corticosteroids are capable of promoting the accumulation of cyclic AMP in human lymphocytes in vitro, confirming our earlier observations at higher corticosteroid concentrations (7). Similar observations have recently been reported by Logsdon, Middleton, and Coffey in mixed human leukocytes using a radioactive precursor method for measuring cyclic AMP formation (11). A significant difference between the two studies is the greater variability we have observed in the ability of 1 μM hydrocortisone to raise lymphocyte cyclic AMP concentrations in preparations from different individuals. As a rule the pattern of responsiveness is reproducible in a given individual raising the possibility of a genetic influence on the response. Whether in vitro corticosteroid responsivenes is in any way a reflection of corticosteroid reactivity in vivo remains to be established. It will be of interest to correlate the results of in vitro studies with corticosteroid effects on glucose tolerance, blood pressure, and intraocular pressure in vivo.

The mechanism by which corticosteroids alter lymphocyte cyclic AMP metabolism and improve catecholamine responsiveness will require further study. Effects are observable at 2–5 min and maximal within 5–30 min. The rapidity of the response would indicate that corticosteroid induction of protein synthesis is probably not involved. However, delayed effects developing over a period of many hours are not excluded. There is no indication the corticosteroid effect is a selective one on adrenergic responsiveness. Corticosteroids promote cyclic AMP accumulation in normal control cells as well as in cells from asthmatic donors and responses both to PGE_1 and catecholamines are increased. Under short incubation conditions corticosteroids do not restore catecholamine responsiveness in lymphocytes that have been preincubated with *d,l*-propranolol.

The results of prospective studies in asthmatic and normal individuals indicate that systemic corticosteroid therapy can alter cyclic AMP metabolism in human peripheral blood lymphocytes in vivo. It is uncertain whether effects of glucocorticoids in short incubation experiments in vitro fully explain their potentiating action in vivo. Corticosteroid-induced alterations in lymphocyte cyclic AMP metabolism seem to evolve

TABLE VI

Effect of Preincubation with Hydrocortisone on the Ability of d,l-Propranolol to Block Lymphocyte Beta Adrenergic Responsiveness

	Initial preincubation, hydrocortisone concentration	Second preincubation, propranolol concentration	Final incubation condition (10 min)			
				Cyclic AMP		
			Buffer	0.5 mM Theophylline	1 μM Epinephrine + 0.5 mM theophylline	10 mM Isoproterenol + 0.5 mM theophylline
	μM	μM			*pmol/10⁷ cells*	
1.	—	—	16	26	52	230
2.	—	20	15	24	20	114
3.	—	4	—	27	23	150
4.	3	—	23	40	76	270
5.	3	20	22	24	17	106
6.	3	4	—	30	26	—
7.	1	—	21	27	58	225
8.	1	20	19	22	59	110
9.	1	4	—	17	20	180

The effect of preincubation with hydrocortisone on the ability of *d,l*-propranolol to block beta adrenergic responsiveness in human lymphocytes. Normal control lymphocytes purified by centrifugation in a Ficoll-Hypaque gradient were used. 1.5 million cells were incubated at 37° in 0.5 ml Gey's solution in the presence and absence of hydrocortisone for 120 min (fiirst preincubation condition). *d,l*-Propranolol or buffer was added and the incubation was continued for an additional 10 min (second preincubation condition). Buffer, theophylline, or catecholamine plus theophylline was then added and after a further 10 min the reaction was terminated by centrifugation.

rather slowly in vivo raising the possibility of an entirely different mechanism such as the induction of protein synthesis or a more complex response involving the participation of nonlymphocytic tissues or plasma itself. There may also be a discrepancy in the concentration of glucocorticoid needed to alter hormonal responsiveness in the two situations. In the absence of stress hydrocortisone concentrations in human plasma are in the 0.3–0.6 μM range and much of the steroid is bound to protein (12–13). During high dose corticosteroid therapy (which is being used with increasing frequency during severe status asthmaticus) the unbound hydrocortisone concentrations in plasma might reach 1 μM, a level at which many but by no means all asthma lymphocyte preparations show an increased cyclic AMP response. During less aggressive glucocorticoid therapy, the circulating free hydrocortisone concentration is below the concentration ordinarily required to obtain steroid effects on asthma lymphocyte cyclic AMP metabolism in vitro. While Logsdon et al. have observed large effects on leukocyte cyclic AMP responses in individuals receiving intermediate doses of corticosteroids, the changes we have observed are modest and of questionable statistical significance.

In attempting to explain the improved cyclic AMP response to catecholamines and prostaglandins in lymphocytes from individuals treated with large doses of corticosteroids the possible effect of a change in circulating lymphocyte subpopulations must be considered. It is known that corticosteroids produce marked lymphocytopenia (12, 15) and in corticosteroid treated mice there is selective trapping of thymus derived lymphocytes in the bone marrow (12, 16). The possibility that a change in the proportion of T and B lymphocytes might be involved when leukocytes from individuals receiving corticosteroid therapy showed altered hormonal responsiveness was not studied by Logsdon et al. (11) who assumed that most of the cyclic AMP response was taking place in polymorphonuclear leukocytes. Our own observations indicate the proportion of B lymphocytes does increase significantly during corticosteroid therapy and we cannot exclude the possibility that such changes partially explain the improvement in hormonal responsiveness. However, we doubt that this is the sole explanation since the increase in hormonal responsiveness sometimes exceeds the increase in percentage of circulating B cells by a considerable margin.

In view of the increased hormonal responsiveness in cells from corticosteroid treated individuals it is tempting to speculate that corticosteroids might alter catecholamine responsiveness in lung in much the same way, accounting for the beneficial effect of these agents in

bronchial asthma. Townley and his colleagues have reported that corticosteroids increase beta adrenergic responsiveness in human tracheal muscle preparations exposed to *d,l*-propanolol (17, 18). However, 100 μM corticosteroid concentrations were used and concentrations of this magnitude would not be obtained in vivo, even during massive corticosteroid therapy. It should also be kept in mind that individuals with acute asthmatic attacks do not necesarily have evidence for altered adrenergic responsiveness in the lymphocyte assay even though corticosteroids are effective in this situation. In addition to a corticosteroid action on cyclic AMP metabolism, possible corticosteroid effects on cellular responsiveness to cyclic AMP, catecholamine biosynthesis and release, and immunological mediator release in this situation also merit careful study.

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