

Stimulation of leukocyte adenyl cyclase by hydrocortisone and isoproterenol in asthmatic and nonasthmatic subjects

Patricia J. Logsdon, B.A., Elliott Middleton, Jr., M.D., and
Ronald G. Coffey, Ph.D. *Denver, Colo.*

To evaluate the theory of beta-adrenergic blockade in asthma, we measured the effects of hydrocortisone and isoproterenol on adenyl cyclase in peripheral blood leukocytes from asthmatic and nonasthmatic children and nonasthmatic adults. An intact cell method was used, allowing cells to incorporate ^3H -adenine and convert it to ^3H -adenosine triphosphate (ATP) during a 2 hour incubation. Experimental compounds were then added for a pulse exposure of 5 minutes. Adenyl cyclase catalyzes the transformation of ^3H -ATP to ^3H -cyclic adenosine monophosphate (AMP), which was measured after chromatographic separation. To rule out effects on rate of cyclic AMP destruction, aminophylline was routinely added at 25 mM., a concentration which completely inhibited cyclic AMP phosphodiesterase. Hydrocortisone at 10^{-6}M stimulated adenyl cyclase in leukocytes from all subjects by 24 to 35 per cent. Isoproterenol at 10^{-6}M stimulated adenyl cyclase by 16 to 19 per cent in control subjects, but not in cells from asthmatic subjects, a finding which supports the beta-adrenergic blockade hypothesis. Children receiving glucocorticosteroid therapy showed a restoration of leukocyte adenyl cyclase responsiveness to isoproterenol. No synergistic effects between the steroid and catecholamine were observed in vitro. The data suggest at least 2 mechanisms by which glucocorticosteroids benefit asthmatic patients: (1) Increased activity of adenyl cyclase, and (2) Restoration of adenyl cyclase responsiveness to catecholamines.

Scott¹ first demonstrated stimulation of human leukocyte adenyl cyclase by epinephrine with broken cell preparations. Bourne and Melmon² and Lichtenstein and Bourne³ extended these studies with intact leukocytes, showing a spectrum of stimulation from phenylephrine (none) < norepinephrine < epinephrine < isoproterenol. These data are in accord with Robison, Butcher, and Sutherland's⁴ contention that stimulation of adenyl cyclase by catecholamines is a beta-adrenergic receptor function. In addition to the catecholamines, adenyl cyclase is stimulated by a large number of hormones, the specific responses depending upon the tissue. The leukocyte enzyme is also stimulated by prostaglandins^{1-3, 5} and by histamine,³ but not by glucagon or adrenocorticotrophic hormone (ACTH).²

Our interest in leukocyte adenyl cyclase receptors for hormone stimulation was prompted by Szentivanyi's⁶ theory of beta-adrenergic blockade in asthma, which implies a decreased beta-adrenergic responsiveness and possibly an in-

From the Children's Asthma Research Institute & Hospital.

Supported by United States Public Health Service Grants Nos. AI09619-02 and AI10400-01. Received for publication Jan. 14, 1972.

Reprint requests to: Ronald G. Coffey, Ph.D., 3401 West 19th Ave., Denver, Colo. 80204.

Vol. 50, No. 1, pp. 45-56

crease in alpha-adrenergic activity. Falliers and associates^{7a, 7b} and Smith and Parker^{8a, 8b} have found a diminished response of adenylyl cyclase to isoproterenol stimulation in leukocytes of patients with asthma. The effects of corticosteroids on leukocyte adenylyl cyclase have not been well characterized. Townley, Daley, and Selenke⁹ demonstrated that administration of corticosteroids restores beta-adrenergic responses in adrenalectomized or pharmacologically beta-blocked animals.

We therefore endeavored to achieve a normal beta-adrenergic response in leukocytes of asthmatic patients in vitro by the addition of hydrocortisone. In the course of these experiments the stimulation of adenylyl cyclase by hydrocortisone alone was noted. In this article we report the degree of this stimulation in leukocytes from asthmatic and nonasthmatic children and normal adults, as well as the capacity for steroid therapy in asthma to restore the responsiveness of adenylyl cyclase to isoproterenol.

MATERIALS AND METHODS

Human Subjects

Subjects included 25 asthmatic children from age 8 to 15, 7 nonasthmatic children from age 8 to 13, and 12 nonasthmatic adults from age 23 to 50. Venous blood was collected between 8:00 A.M. and 9:00 A.M. from all patients in order to eliminate any circadian bias. Some asthmatic patients were receiving prednisone therapy. In all but one instance blood was collected 24 or 48 hours after the last dose.

Chemicals

The following were obtained from Sigma Chemical Co., St. Louis, Mo.: high molecular weight dextran, SigmaCel microcrystalline cellulose, hydrocortisone, hydrocortisone-21-sodium succinate, isoproterenol hydrochloride, prednisone, aminophylline (ethylene diamine salt of theophylline) dihydrate, ATP, adenosine diphosphate (ADP), AMP, adenosine, adenine, cyclic AMP, 5'-nucleotidase (*Crotalus adamanteus* venom), and cyclic nucleotide phosphodiesterase (beef heart). Human serum albumin (4 times crystallized) was obtained from ICN Nutritional Biochemicals Corp., Cleveland, Ohio, ³H-adenine (23.4 mCi per millimole) and theophylline from Schwarz BioResearch, Orangeburg, N. Y., NCS* solubilizer from Amersham-Searle Corp., Arlington Heights, Ill., and 2,5-diphenyl oxazole (PPO) and 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP) from Packard Instruments, Inc., Downer's Grove, Ill.

Preparation of leukocytes

Blood (40 ml.) was drawn into a 50 ml. plastic syringe containing 1 ml. (5,000 units) of heparin and 6 ml. of a solution containing (per liter) 60 Gm. of high molecular weight dextran, 22 Gm. of trisodium citrate, 8 Gm. of citric acid, and 24.5 Gm. of glucose; pH 6.5. Blood was allowed to settle in the inverted syringe for 30 minutes at room temperature, after which the leukocyte-rich plasma layer was collected from the top of the syringe. Leukocyte suspensions were centrifuged at 150 g for 15 minutes at room temperature and resuspended in 20 ml. of a hypotonic solution (13.7 mM. NaCl, 5.4 mM. KCl, 1.0 mM. MgCl₂, 0.13 mM. CaCl₂, 0.44 mM. KH₂PO₄, 1.34 mM. Na₂HPO₄, and 5 mM. glucose, adjusted to pH 6.5) to lyse red cells. The suspensions were immediately centrifuged as above and suspended in a buffered solution containing 0.12 M NaCl, 5 mM. KCl, 0.6 mM. CaCl₂, 1 mM. MgCl₂, 10 mM. glucose, 1 Gm. per 100 ml. human serum albumin, and 25 mM. Tris·HCl, adjusted to pH 7.4. These suspensions typically possessed fewer than 1 platelet or red blood cell per leukocyte; the percentage of lymphocytes ranged from 25 to 35.

*Trademark of Nuclear Chicago Corporation, Des Plaines, Ill.

Adenylyl cyclase assay

Leukocyte adenylyl cyclase activity was estimated by a modification of the method described for adipocytes by Humes, Roundbehrer, and Kuehl¹⁰ and by Kuo and DeRenzo.¹¹ A cell suspension (1 ml.) containing 10^7 leukocytes was pipetted into each tube and incubated for 2 hours at 37° C. with 2.5 μ c (107 picomoles) of ^{3} H-Adenine. Freshly prepared aqueous solutions of hydrocortisone or isoproterenol (10 μ L) were then added with 50 μ L of 0.5M aminophylline, and the incubation was continued for another 5 minutes with gentle shaking. The cells were then chilled in an ice bath, centrifuged at 0° C. and washed 3 times with 2 ml. of ice-cold saline to remove traces of ^{3} H-Adenine. The cells were lysed in 0.1 ml. of distilled water, and 0.05 ml. of nonradioactive carrier solution containing 5 mM. each of cyclic AMP, ATP, ADP, AMP, and adenosine was added. Protein was denatured by boiling for 3 minutes and removed by centrifugation.

Forty μ L aliquots of the protein-free supernatant solution were spotted on thin-layer plates of SigmaCel microcrystalline cellulose. Chromatograms were developed in t-butanol : concentrated NH_4OH : ethyl acetate : H_2O (40 : 20 : 28 : 12, v/v) for 5 hours at room temperature. Cyclic AMP (R_f 0.35), adenine plus adenosine (R_f 0.5), and ATP plus ADP plus AMP (R_f 0 to 0.1) areas were visualized under short-wave ultraviolet light, removed with a razor, and placed in a vial. The nucleotides were dissolved with 0.2 ml. of H_2O , 1 ml. of NCS, and 9 ml. of toluene containing 4 Gm. per liter of PPO and 50 mg. per liter of POPOP and counted in a Picker Liqumat Scintillation Counter. Counting efficiency for ^{3} H was 30 per cent; no interference from the sedimented cellulose was observed.

Under the incubation conditions described, about 80 per cent of the ^{3} H-Adenine was incorporated into leukocytes. Control levels of adenylyl cyclase activity are expressed as per cent of protein-free supernatant counts per minute found in cyclic AMP. This was considered preferable to an alternate calculation based on per cent of adenine converted to cyclic AMP, as the aggregation of cells may result in a variable degree of adenine uptake. Aggregation may be prevented by adding autologous serum, but this alternative was not selected because of unknown effects of steroids present in serum. Effects of hydrocortisone and other experimental compounds are expressed as per cent of control adenylyl cyclase values. All incubations were performed in duplicate, and individual data are expressed as the mean $\pm$ range. Group means are indicated $\pm$ S.E.M.

Cyclic AMP phosphodiesterase assay

Leukocytes were suspended in saline, homogenized at 0° C. with a tight-fitting Teflon pestle for 3 minutes (100 strokes), and centrifuged at 10,000 g for 20 minutes at 0° C. The sediment, which contains most of the cellular ATPase and the adenylyl cyclase activities but less than 5 per cent of the phosphodiesterase activity, was discarded. The supernatant fluid (representing 5×10^6 leukocytes) was incubated for 30 minutes at 37° C. in 0.5 ml. of a solution containing at final concentrations, 0.1M of Tris-HCl, pH 8.5, 10 mM. MgCl_2 , 2 mM. cyclic AMP, and 50 μ g of 5'nucleotidase. These conditions were established as optimal in our laboratory. The 5'nucleotidase was sufficient to catalyze the conversion of 0.6 μ mole AMP to adenosine per minute, a rate 100-fold greater than our most active phosphodiesterase preparation. Hydrocortisone (10^{-6} to 10^{-2} M) had no effect on 5'nucleotidase activity, assayed at pH 8.5 with 20 mM. AMP substrate. The phosphodiesterase reaction was ended by the addition of 0.05 ml. of 50 per cent (w/v) trichloroacetic acid. Protein was removed by centrifugation at 4,000 g for 10 minutes at 0° C. and was determined according to the method of Lowry, Rosebrough, Farr, and Randall.¹² Supernatant phosphate was determined colorimetrically with molybdic acid according to the method of Ames and Dubin¹³ and equated to cyclic AMP hydrolyzed. Phosphodiesterase units were calculated as nanomoles phosphate formed in 30 minutes at 37° C. per milligram of protein, and ranged from 150 to 500 units for adult subjects.

Validation of leukocyte cyclic AMP

Samples of chromatographically purified cyclic AMP, prepared from leukocytes as described, were incubated with 10 μ g of beef heart cyclic nucleotide phosphodiesterase in 0.2

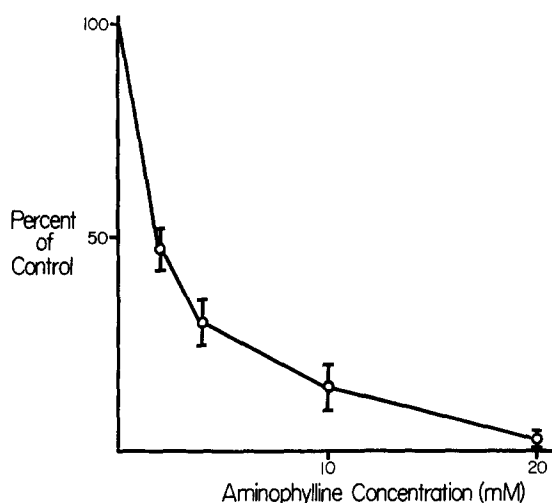


FIG. 1. Inhibition of leukocyte phosphodiesterase by aminophylline. Activity was determined as described in "Methods" for 20 nonasthmatic individuals, and ranged from 150 to 500 nmoles phosphate per milligram of protein. Error bars represent the S.E.M.

ml. of 0.1M Tris-HCl, pH 8.0, and 10 mM. $MgCl_2$ for 30 minutes at 37° C. The resultant nucleotide was chromatographed and radioactivity was recovered in the same area (R_f 0.10) as authentic AMP. This was eluted and treated with 10 μ g of 5'nucleotidase in 0.2 ml. of the above buffer for 30 minutes at 37° C. The resultant nucleotide was chromatographed and travelled identically with authentic adenosine (R_f 0.50). In addition, samples of chromatographically purified leukocyte cyclic AMP were purified by the Dowex-50 (H^+) column method of Krishna, Weiss, and Brodie.¹⁴ Between 95 and 100 per cent of the radioactivity and the ultraviolet light-absorbing material (259 $m\mu$) was recovered in the second 4 ml. of aqueous eluate, as was authentic cyclic AMP; neither sample nor standard cyclic AMP was sedimented with $BaSO_4$.

RESULTS

Cyclic AMP phosphodiesterase inhibition

The addition of an inhibitor of phosphodiesterase was required to preserve the increased cyclic AMP formed during the 5 minute incubation with hydrocortisone or isoproterenol. As shown in Fig. 1, 20 mM. aminophylline was sufficient to inhibit virtually all phosphodiesterase activity in cell-free preparations. It was still desirable to rule out the possibility that hydrocortisone might also increase cyclic AMP levels by inhibiting phosphodiesterase. In Fig. 2, the results with leukocyte phosphodiesterase indicate no effect by hydrocortisone at $10^{-6}M$, in the absence of aminophylline, when added directly to the cell-free supernatant enzyme. Similar results were obtained by incubating hydrocortisone with intact cells for one hour prior to washing, homogenization, and subsequent assay. However, significant inhibition of phosphodiesterase occurred at $5 \times 10^{-4}M$ to $5 \times 10^{-2}M$ hydrocortisone (with the sodium succinate form because of its greater solubility), a finding which ruled out the use of these concentrations in adenylyl cyclase experiments. Since the latter were performed in the presence of 25 mM. aminophylline, it is unlikely that $10^{-6}M$ hydrocortisone could stimulate cyclic AMP accumulation by any mechanism involving phosphodiesterase inhibi-

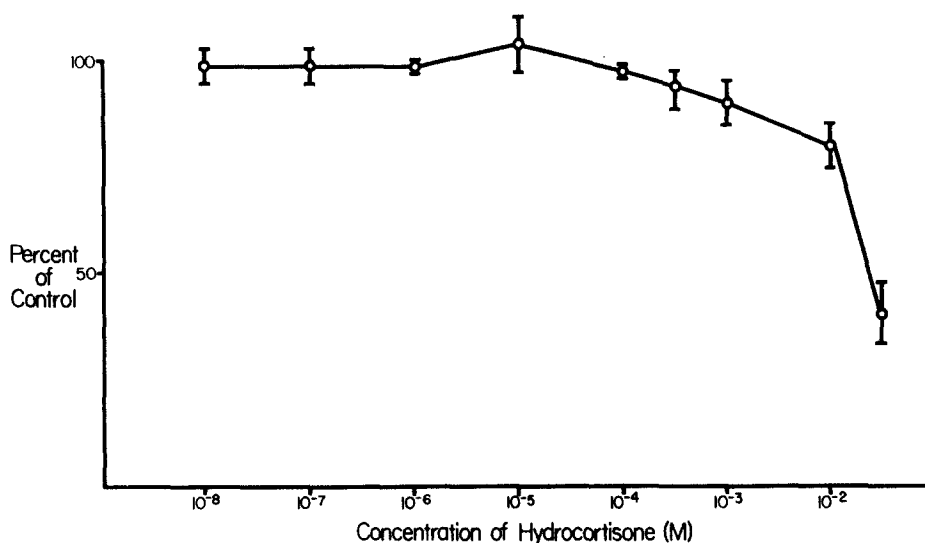


FIG. 2. Inhibition of leukocyte phosphodiesterase by hydrocortisone. Activity was determined as described in "Methods." Error bars represent the range of values for 2 dose response experiments with cells from the same individual.

tion. Sodium succinate (5×10^{-2} M) had no effect on leukocyte phosphodiesterase.

In 2 experiments in which an assay for the low K_m phosphodiesterase was used, similar inhibition data were obtained. Leukocyte supernatant enzyme was incubated for 10 minutes at 37° C. with 25 nmoles ^3H -cyclic AMP in a total volume of 0.5 ml. of 50 mM. Tris, pH 7.4, and 10 mM. MgCl_2 . The unhydrolyzed ^3H -cyclic AMP was then purified by Dowex 50 (H^+) column chromatography.¹⁴ Inhibition of phosphodiesterase, calculated from the ^3H -cyclic AMP recovered, was 20 per cent and 80 per cent due to 2 mM. and 20 mM. aminophylline, respectively. The inhibition due to 10^{-6} and 5×10^{-4} M hydrocortisone was 0 per cent and 49 per cent, respectively.

Further comparisons of the effects of aminophylline, theophylline, and hydrocortisone on cyclic AMP phosphodiesterase were obtained with intact leukocytes which were allowed to accumulate ^3H -cyclic AMP as shown in Table I. During the 30 minute incubation about 5 per cent of the ^3H -cyclic AMP was incorporated. The destruction of this cyclic AMP was not affected by 10^{-6} M hydrocortisone, while progressive inhibition of cyclic AMP destruction was observed with aminophylline or theophylline from 5 to 25 mM.

Effect of corticosteroids on adenylyl cyclase

In preliminary experiments we found that 10^{-6} M hydrocortisone stimulated adenylyl cyclase in leukocytes from nearly all subjects, but the degree of stimulation varied considerably from individual to individual. Several experiments were performed in order to relate this variability to the concentration of steroid. Dose-response curves were plotted for 10 experiments involving 7 adult control subjects, in which hydrocortisone or prednisone concentrations ranged from 10^{-11} to 10^{-6} M. The concentration which provided maximum stimulation of

TABLE I. Effects of hydrocortisone and methylxanthines on intracellular cyclic AMP phosphodiesterase*

Variables	Per cent of incorporated ³ H-cyclic AMP		Per cent inhibition of phosphodiesterase
	Recovered	Destroyed	
Control	17.6 ± 0.8	82.4	0
Hydrocortisone 10 ⁻⁶ M	18.0 ± 2.6	82.0	0.3
Aminophylline 5 mM.	45.6 ± 1.5	54.4	34.0
Theophylline 10 mM.	61.6 ± 11.4	38.4	53.4
Aminophylline 25 mM.	76.0 ± 6.3	24.0	70.9

*6 × 10⁶ leukocytes from a nonasthmatic adult were added to each tube containing 6.6 μc of ³H-cyclic AMP and incubated at 37° C. for 30 minutes with or without methylxanthines at the concentrations indicated. Hydrocortisone was then added and incubations continued for 5 minutes. Cells were then centrifuged, washed, boiled, and the intracellular nucleotides purified as described in "Methods". The per cent of incorporated ³H recovered as cyclic AMP is expressed as the mean of 6 determinations ± standard deviation. The per cent inhibition was calculated as:

$$\frac{\% \text{ } ^3\text{H-cyclic AMP destroyed (control — test)} \times 100}{\% \text{ } ^3\text{H-cyclic AMP destroyed (control)}}$$

TABLE II. Dose-response of leukocyte adenyl cyclase to glucocorticosteroids in nonasthmatic adults

Subject	Age	Sex	Steroid†	Adenyl cyclase units*		Per cent of control with steroid	Optimum steroid concentration
				Control	Steroid		
T. V.	37	M	H	9.7 ± 0.1	16.8 ± 0.2	173	10 ⁻⁸ M
D. S.	33	F	H	16.0 ± 0.7	23.6 ± 0.7	148	10 ⁻⁶ M
N. S.	27	F	H	20.5 ± 0.6	26.5 ± 1.8	129	10 ⁻⁹ M
D. C.	24	F	H	14.7 ± 0.9	23.1 ± 0.6	157	10 ⁻⁸ M
P. L.	23	F	H	14.6 ± 0.1	22.8 ± 0.1	156	10 ⁻⁶ M
J. H.	27	M	H	6.3 ± 0.1	7.6 ± 0.2	121	10 ⁻⁶ M
E. M.	45	M	H	8.1 ± 0.2	11.9 ± 0.8	147	10 ⁻¹¹ M
D. C.	24	F	P	14.7 ± 0.9	19.3 ± 1.2	131	10 ⁻⁹ M
P. L.	23	F	P	13.6 ± 0.6	16.6 ± 0.8	122	10 ⁻¹⁰ M
N. S.	27	F	P	9.5 ± 1.2	11.7 ± 0.1	123	10 ⁻¹⁰ M

*Units are per cent of soluble incorporated ³H, derived from ³H-adenine, found in cyclic AMP under the conditions described in "Methods."

†Hydrocortisone (H) or prednisone (P).

adenyl cyclase is indicated for each subject in Table II. The data show that for either steroid there is no optimal concentration for every subject. It was not feasible to perform dose-response experiments with children because of limitations on the volume of blood that may be drawn. In all subsequent experiments hydrocortisone was routinely added at a final concentration of 10⁻⁶M.

Comparison of adenyl cyclase in leukocytes from asthmatic and nonasthmatic subjects

The degree of stimulation of adenyl cyclase by 10⁻⁶M hydrocortisone and 10⁻⁶M isoproterenol is shown in Table III for 12 nonasthmatic adults and 7 nonasthmatic children, in Table IV for 17 asthmatic children not receiving steroid therapy, and in Table V for 8 asthmatic children on various regimens of steroid therapy as indicated. A wide variation in control levels of adenyl

TABLE III. Stimulation of leukocyte adenylyl cyclase of nonasthmatic adults and children

Adult:	Age	Sex	Adenylyl cyclase activity*			Per cent of control	
			Control	H†	I†	H†	I†
P. L.	23	F	14.6 ± 0.1	22.8 ± 0.1		156	
P. L.	23	F	8.0 ± 0.3		8.8 ± 0.8		110
F. S.	30	F	12.5 ± 0.1	18.2 ± 0.4	12.1 ± 1.0	145	97
D. C.	24	F	15.1 ± 0.4	17.9 ± 0.1	16.8 ± 1.5	119	111
N. S.	27	F	5.0 ± 0.2	7.5 ± 0.1	6.5 ± 0.4	150	130
J. H.	27	M	6.3 ± 0.2	7.6 ± 0.2	7.0 ± 0.3	121	110
T. V.	37	M	9.7 ± 0.1	13.3 ± 0.1	11.7 ± 0.2	137	121
R. S.	33	M	16.0 ± 0.7	23.6 ± 0.6	19.2 ± 0.7	148	120
R. C.	34	M	10.3 ± 1.6	14.3 ± 0.3	13.6 ± 0.2	139	132
H. C.	50	M	17.7 ± 0.1		25.2 ± 2.1		142
E. M.	45	M	8.1 ± 0.2	7.2 ± 0.5	9.4 ± 0.1	88	116
J. C.	25	M	13.8 ± 0.2		16.4 ± 0.4		122
Child:							
C. N.	13	F	5.3 ± 0.1	9.3 ± 0.6	7.2 ± 0.1	175	136
S. N.	10	F	10.1 ± 1.1	12.8 ± 1.6	11.8 ± 0.6	127	118
C. N.	12	M	5.9 ± 0.4	8.2 ± 0.5	5.3 ± 0.1	139	90
K. J.	12	M	7.8 ± 0.4	10.8 ± 1.4	8.2 ± 0.9	138	105
D. J.	10	M	13.5 ± 0.5	14.0 ± 0.4	12.8 ± 0.3	104	95
C. N.	8	M	11.0 ± 0.1	13.3 ± 0.3	13.8 ± 0.3	121	125
R. C.	10	M	11.1 ± 0.1	15.5 ± 2.9	15.6 ± 1.1	141	141

*Units are per cent of soluble incorporated ^3H , derived from ^3H -adenine, found in cyclic AMP under the conditions described in "Methods."

†Hydrocortisone (H) or isoproterenol (I) at 10^{-6}M .

TABLE IV. Stimulation of leukocyte adenylyl cyclase of non-steroid-treated asthmatic children

Child	Age	Sex	Adenylyl cyclase activity*			Per cent of control	
			Control	H†	I†	H†	I†
M. L.	13	M	11.5 ± 1.5	20.0 ± 3.0	9.5 ± 0.5	174	83
D. T.	10	M	36.0 ± 2.0	38.0 ± 4.0	35.0 ± 4.0	106	98
J. W.	13	M	17.5 ± 0.5	25.0 ± 3.0	14.5 ± 0.5	143	83
R. K.	14	M	18.0 ± 1.0	23.0 ± 0.0	22.0 ± 0.9	128	122
M. B.	10	M	9.4 ± 0.8	11.7 ± 0.9	16.0 ± 0.1	124	170
P. G.	9	M	11.5 ± 1.2	13.0 ± 1.2	10.2 ± 0.6	113	89
M. C.	12	M	6.5 ± 0.2	7.2 ± 0.7	6.2 ± 0.3	111	95
H. F.	14	M	13.4 ± 0.6	13.9 ± 0.1	13.3 ± 1.0	104	99
J. W.	9	M	6.8 ± 0.2	7.3 ± 0.2	7.1 ± 0.2	107	104
K. H.	13	M	5.2 ± 0.1	9.9 ± 0.1	6.2 ± 0.3	190	119
C. R.	15	F	14.1 ± 0.2	16.2 ± 0.1	14.0 ± 0.5	115	100
W. B.	13	M	17.6 ± 0.3	23.4 ± 3.2	22.8 ± 1.1	133	129
H. F.	13	M	4.6 ± 0.1	4.8 ± 0.0	4.4 ± 0.8	104	96
G. G.	13	M	14.5 ± 2.5	17.0 ± 2.0	12.0 ± 1.5	117	83
M. D.	15	F	4.6 ± 0.4	4.1 ± 0.1	3.5 ± 0.3	88	76
J. S.	16	M	5.3 ± 0.3	8.0 ± 0.2	5.2 ± 0.3	148	99
J. F.	16	M	5.2 ± 0.5		4.7 ± 0.5		90
J. F.	16	M	4.3 ± 0.4	4.6 ± 0.3		106	

*Units are per cent of soluble incorporated ^3H , derived from ^3H -adenine, found in cyclic AMP under the conditions described in "Methods."

†Hydrocortisone (H) or isoproterenol (I) at 10^{-6}M .

cyclase activity as well as in the per cent stimulation values is notable. Variations could not be correlated with the small differences in percentage of lymphocytes or other types of leukocytes.

Group means and statistics for all subjects are presented in Table VI. There are no significant differences between mean control values of adenylyl cyclase

TABLE V. Stimulation of leukocyte adenyl cyclase of steroid-treated asthmatic children

Child	Age	Sex	Adenyl cyclase activity*			Per cent of control		Steroid treatment	
			Control	H†	I†	H†	I†	Mg.	hr. Before test
T. C.	16	M	11.1 ± 0.9	16.2 ± 0.4	16.2 ± 0.4	146	146	10	24
E. F.	9	M	16.4 ± 0.5	23.4 ± 1.3	19.6 ± 3.2	143	119	40	48
G. O.	10	M	12.8 ± 1.1	24.2 ± 2.7	16.1 ± 1.7	189	126	30	24
M. M.	14	M	21.4 ± 1.1	25.3 ± 0.2	24.3 ± 0.3	118	113	40	24
B. M.	14	M	21.0 ± 1.0	23.6 ± 0.9	22.1 ± 0.4	112	105	60	24
J. T.	12	M	9.8 ± 0.7	12.5 ± 1.2	11.6 ± 0.5	128	118	15	10
M. R.	15	F	12.1 ± 0.7	15.1 ± 0.3	12.5 ± 0.0	125	103	9	48
S. C.	13	F	10.6 ± 1.4	12.4 ± 0.3	12.2 ± 0.3	117	115	40	25

*Units are per cent of soluble incorporated ^3H , derived from ^3H -adenine, found in cyclic AMP under the conditions described in "Methods."

†Hydrocortisone (H) or isoproterenol (I) at 10^{-6}M .

activity for any of the groups. It is remarkable that the means for per cent stimulation by hydrocortisone (24 to 35 per cent) are similar for all groups. This stimulation, with few exceptions, was greater than the stimulation with isoproterenol (16 to 19 per cent), which was absent in most asthmatic subjects not receiving steroids. However, steroid therapy was associated with a response to isoproterenol (18 per cent stimulation) similar to that of the nonasthmatic control subjects. The mechanism by which systemic treatment with glucocorticosteroids restores this beta-adrenergic response to isoproterenol is not understood. In several experiments (not shown), no synergistic effects of hydrocortisone and isoproterenol upon leukocyte adenyl cyclase activity in vitro were found.

DISCUSSION

The present studies constitute the first observation, to our knowledge, of the stimulation of adenyl cyclase activity by physiologic concentrations of glucocorticosteroids. The degree of stimulation is similar among several groups of subjects: asthmatic children, nonasthmatic children, and nonasthmatic adults. No sex differences were noted. The degree of stimulation did not differ significantly between asthmatic subjects receiving steroid therapy and those not treated. The absence of stimulation by isoproterenol in most non-steroid-treated asthmatic subjects provides additional evidence for the theory of beta-adrenergic blockade.^{6, 15, 16} The stimulation by isoproterenol (18 per cent) was significant in most steroid-treated asthmatic subjects, indicating that this treatment has partially restored the beta-adrenergic response in leukocytes. Comparable restoration was not demonstrable simply by incubating hydrocortisone and isoproterenol with leukocytes in vitro. Further work is in progress to evaluate the effect of prolonged incubation of leukocytes with hydrocortisone in vitro on the degree of stimulation which can be obtained by catecholamines in leukocytes of normal and asthmatic subjects. It should be noted that our choice of 10^{-6}M isoproterenol, although higher than physiologic concentrations of epinephrine, is yet 100-fold less than the optimal concentration found by Bourne and Melmon² and Lichtenstein and Bourne,³ and is 4 orders of magnitude less than that

TABLE VI. Summary of adenylyl cyclase data

Subjects	No.	Adenylyl cyclase units*			Per cent increase	
		Control	Hydrocortisone	Isoproterenol	Hydrocortisone	Isoproterenol
Adult controls	12	11.4 ± 1.2	14.7 ± 1.9	13.4 ± 1.7	34 ± 7	19 ± 4
Children controls	7	9.2 ± 1.1	12.0 ± 0.9	10.7 ± 1.3	35 ± 8	16 ± 7
Asthmatic subjects not on steroids	17	11.8 ± 1.9	14.5 ± 2.2	12.1 ± 2.0	24 ± 6	2 ± 6 (NS)
Asthmatic subjects on steroids	8	14.4 ± 1.6	19.1 ± 1.9	16.8 ± 1.7	35 ± 9	18 ± 5

*Means ± S.E.M. are expressed for each group. The paired Student's *t* test showed no significant differences among control levels. *P* values for stimulation by hydrocortisone and isoproterenol were derived from *t* tests by comparing control means, defined as 100 per cent, to the means for the hormones, expressed as per cent of control

$$\left(\frac{100 \times \text{enzyme units with hormone}}{\text{enzyme units without hormone}} \right).$$

All *P* values for stimulation by either hormone were less than 0.01 except that indicated by NS.

used by Smith and Parker^{8a, 8b} for stimulation of leukocyte adenylyl cyclase. Lichtenstein and Bourne observed a 30 per cent stimulation by 10^{-6} M isoproterenol, compared to the 16 to 19 per cent stimulation noted by us. At present it appears reasonable to perform experiments with the lower concentrations of catecholamine in order to gain insight into the possible interactions between catecholamines and steroids.

One difference between our procedure and that of others is our use of aminophylline in place of theophylline. We recently discovered that theophylline permits a greater stimulation of adenylyl cyclase by isoproterenol, while providing lower basal levels of cyclic AMP. In preliminary experiments with theophylline, isoproterenol at 10^{-6} M stimulated adenylyl cyclase by 42.7 per cent (± 6.6 , 9 subjects) in leukocytes from nonasthmatic adults, but only 2.5 per cent (± 4.9 , 4 subjects) in cells from asthmatic children not receiving steroid therapy. Further work is now planned to find whether the variable degree of isoproterenol response in asthmatic subjects is related to severity of asthmatic or other symptoms.

Hydrocortisone is indirectly involved with beta-adrenergic responses in many tissues, lending a "permissive" effect to the stimulation by catecholamines.^{9, 17, 18} A similar "permissive" effect on the stimulation of adenylyl cyclase by ACTH was found by Braun and Hechter.¹⁹ Such studies have been performed with adrenalectomized, hydrocortisone-treated animals, and it is difficult to rule out the possibility that the steroid acted over a period of time to alter synthesis of proteins related to hormone receptor sites. Moskowitz and Fain²⁰ found that dexamethasone plus growth hormone caused an increase in adenylyl cyclase activity in adipocytes, but this increase required a time lag of one hour and was blocked by inhibitors of protein synthesis. Smith and Parker^{8a, 8b} reported in a preliminary communication that 5×10^{-4} M hydrocortisone increased the level of cyclic AMP 2-fold in human leukocytes after incubation for one hour. The increase was greatly reduced in cells from asthmatic subjects. However, the

concentration of steroid was sufficient, according to the present results and also according to Senft, and associates,²¹ to inhibit phosphodiesterase, and the time period was sufficient to allow altered rates of enzyme synthesis to affect the results. Since we often found that much lower concentrations of hydrocortisone provided optimal stimulation of adenylyl cyclase, it is possible that higher levels of steroid increase cyclic AMP by another mechanism. We have noted that the direct inhibition of phosphodiesterase by hydrocortisone occurs only at concentrations exceeding 10^{-6} M. Our complete observations on the effects of high concentrations of hydrocortisone on leukocyte phosphodiesterase will be presented elsewhere.

In the present studies leukocytes were exposed to hydrocortisone for only 5 minutes in order to rule out the possibility of significant changes in protein synthesis. Smith, and colleagues²² found that a short pulse with phytohemagglutinin (PHA) is optimal for demonstrating stimulation of lymphocyte adenylyl cyclase. Bourne and associates^{2, 5} and Lichtenstein and Bourne³ obtained similar results by using prostaglandin E_1 , catecholamines, and histamine to stimulate leukocyte adenylyl cyclase. With the use of the adenine uptake technique, a short exposure to hydrocortisone is desirable for 2 additional reasons: (1) hydrocortisone may cause a reduction in the level of intracellular ATP,²³ and (2) hydrocortisone inhibits uptake of glucose into thymus²³ and other peripheral tissues,²⁴ a process that, according to Humes and associates¹⁰ is required for uptake of adenine and subsequent conversion to ATP. The short pulse is also convenient for catecholamines, obviating the need for reducing agents to prevent oxidation to chromophores.

Additional problems of interpretation arise when one considers the contributions of various types of leukocyte to the enzyme being measured. For example, Smith and associates²² found that phytohemagglutinin stimulates adenylyl cyclase in purified human lymphocytes but not in granulocytes. Our cell suspensions usually contained mixtures of leukocytes consisting of 25 to 35 per cent lymphocytes. Bourne and associates⁵ estimated that in such mixtures lymphocytes contribute less than 5 per cent of the total adenylyl cyclase. Furthermore, it is likely that platelets contribute less than one per cent of the measurable adenylyl cyclase activity.⁵ We therefore suggest that the present data primarily reflect the stimulation of the neutrophil enzyme by hydrocortisone.

The use of intact cells to demonstrate interactions between hormones and receptors is often preferable to the use of broken cells or membrane fragments. The incorporation of radioactive adenine into intact cells results in a pool of labeled ATP which is more accessible to adenylyl cyclase than the unlabeled pool.^{10, 11} Thus one can detect a small effect of a hormone on adenylyl cyclase activity which may be difficult to demonstrate by other methods. The optimal concentration of isoproterenol to stimulate leukocyte adenylyl cyclase is lower in intact cells than in homogenates.⁵ In brain tissue Krishna and associates²⁵ found reproducible stimulation of adenylyl cyclase by catecholamines and histamine only with intact cells; the hormone receptors seem to be labile to homogenization. It is probable that the receptors for hydrocortisone are also extremely labile. In preliminary experiments we have been unable to demonstrate

stimulation of adenylyl cyclase by hydrocortisone in leukocyte homogenates comparable to the degree observed with intact cells. One disadvantage of the use of intact cells is that additional proof is required that the agent in question is not acting by inhibition of cyclic nucleotide phosphodiesterase. The present report describes effects by hydrocortisone at a concentration proved to be ineffective in altering phosphodiesterase activity.

While we have demonstrated the stimulation of adenylyl cyclase by hydrocortisone at low concentrations, several other possible effects of the use of glucocorticosteroids to increase cellular cyclic AMP *in vivo* might be considered: (1) increased synthesis of new adenylyl cyclase molecules, (2) decreased synthesis of new phosphodiesterase molecules, and (3) direct inhibition of phosphodiesterase.

The technical assistance of Mrs. Diane Carnright is gratefully acknowledged. The cooperation of all children and staff members at Children's Asthma Research Institute and Hospital, who provided blood in copious quantities, is greatly appreciated.

REFERENCES

- 1 Scott, R. E.: Effects of prostaglandins, epinephrine, and sodium fluoride on human leukocyte, platelet, and liver adenylyl cyclase, *Blood* **35**: 514, 1970.
- 2 Bourne, H. R., and Melmon, K. L.: Adenylyl cyclase in human leukocytes: Evidence for activation by separate beta-adrenergic and prostaglandin receptors, *J. Pharmacol. Exp. Ther.* **178**: 1, 1971.
- 3 Lichtenstein, L. L., and Bourne, H. R.: Inhibition of allergic histamine release by histamine and other agents which stimulate adenylyl cyclase, in Austen, K. F., and Becker, L., editors: *Biochemistry of acute allergic reactions*, Philadelphia, 1972, F. A. Davis Company.
- 4 Robison, G., Butcher, R., and Sutherland, E.: Adenylyl cyclase as an adrenergic receptor, *Ann. N.Y. Acad. Sci.* **139**: 703, 1967.
- 5 Bourne, H. R., Lehrer, R. I., Cline, M. J., and Melmon, K. L.: Cyclic 3',5'-adenosine monophosphate in the human leukocyte: Synthesis, degradation, and effects on neutrophil candidacidal activity, *J. Clin. Invest.* **50**: 920, 1971.
- 6 Szentivanyi, A.: The beta-adrenergic theory of the atopic abnormality in bronchial asthma, *J. ALLERGY* **42**: 203, 1968.
- 7a Falliers, C. J., deA. Cardoso, R. R., Bane, H. N., Coffey, R. G., and Middleton, E., Jr.: Clinical, physiologic, and biochemical differences of identical twins, related to discordant allergic manifestations, American Academy of Pediatrics, San Francisco, Calif. (abst.), 1970.
- 7b Falliers, C. J., deA. Cardoso, R. R., Bane, H. N., Coffey, R. G., and Middleton, E., Jr.: Discordant allergic manifestations in monozygotic twins: Genetic identity vs. clinical, physiologic, and biochemical differences, *J. ALLERGY* **47**: 207, 1971.
- 8a Smith, J. W., and Parker, C. W.: The responsiveness of leukocyte cyclic adenosine monophosphate to adrenergic agents in patients with asthma, *J. Lab. Clin. Med.* **76**: 993, 1970.
- 8b Smith, J. W., and Parker, C. W.: The responsiveness of leukocyte cyclic AMP to adrenergic agents in patients with asthma, *Proc. Cent. Soc. for Clin. Res.* **43**: 76, 1970.
- 9 Townley, R. G., Daley, D., and Selenke, W.: The effect of agents used in the treatment of bronchial asthma on carbohydrate metabolism and histamine sensitivity after beta-adrenergic blockade, *J. ALLERGY* **45**: 71, 1970.
- 10 Humes, J. L., Roundbehrer, M., and Kuehl, F. A., Jr.: Assay for measuring adenylyl cyclase activity in intact cells, *Anal. Biochem.* **32**: 210, 1969.
- 11 Kuo, J., and DeRenzo, E. C.: Comparison of the effects of lipolytic and antilipolytic agents on adenosine 3',5'-monophosphate levels in adipose cells as determined by prior labeling with adenine-8-¹⁴C, *J. Biol. Chem.* **244**: 2252, 1969.
- 12 Lowry, O., Rosebrough, N., Farr, A., and Randall, R.: Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* **193**: 265, 1951.
- 13 Ames, B. N., and Dubin, D. T.: The role of polyamines in the neutralization of bacteriophage DNA, *J. Biol. Chem.* **235**: 769, 1960.

- 14 Krishna, G., Weiss, B., and Brodie, B. B.: A simple, sensitive method for the assay of adenylyl cyclase, *J. Pharmacol. Exp. Ther.* **163**: 379, 1968.
- 15 Reed, C. E.: The role of the autonomic nervous system in the pathogenesis of bronchial asthma, *in* Rose, B., Richter, M., Schon, A., and Frankland, A. W., editors: *Allergology, Proceedings of the Sixth International Congress of Allergology*. Amsterdam, 1968, Excerpta Medica Foundation.
- 16 Middleton, E., Jr.: Autonomic imbalance in asthma with special reference to beta adrenergic blockade, *Adv. Intern. Med.* **18**: 1972. In press.
- 17 Brodie, B. B., Davies, J. I., Hynie, S., Krishna, G., and Weiss, B.: Interrelationships of catecholamines with other endocrine systems, *Pharmacol. Rev.* **18**: 273, 1966.
- 18 Miller, T. B., Exton, J. H., and Park, C. R.: Block in epinephrine-induced glycogenolysis in hearts from adrenalectomized rats. *J. Biol. Chem.* **246**: 3672, 1971.
- 19 Braun, T., and Hechter, O.: Glucocorticoid regulation of ACTH sensitivity of adenylyl cyclase in rat fat cell membranes, *Proc. Natl. Acad. Sci. U.S.A.* **66**: 995, 1970.
- 20 Moskowitz, J., and Fain, J. N.: Stimulation by growth hormone and dexamethasone of labeled cyclic adenosine 3',5'-monophosphate accumulation by white fat cells, *J. Biol. Chem.* **245**: 1101, 1970.
- 21 Senft, G., Schultz, G., Munske, K., and Hoffman, M.: Effects of glucocorticosteroids and insulin on 3',5'-AMP phosphodiesterase activity in adrenalectomized rats, *Diabetologia* **4**: 330, 1968.
- 22 Smith, J. W., Steiner, A. L., Newberry, W. M., Jr., and Parker, C. W.: Cyclic adenosine 3',5'-monophosphate in human lymphocytes. Alterations after phytohemagglutinin stimulation, *J. Clin. Invest.* **50**: 432, 1971.
- 23 Young, D. A.: Glucocorticoid action on rat thymus cells. Interrelations between carbohydrate, protein, and adenine nucleotide metabolism and cortisol effects on these functions, *J. Biol. Chem.* **244**: 2210, 1969.
- 24 Munek, A.: Glucocorticoid inhibition of glucose uptake by peripheral tissues: Old and new evidence, molecular mechanisms, and physiological significance, *Perspect. Biol. Med.* **14**: 265, 1971.
- 25 Krishna, G., Forn, J., Voigt, K., Paul, M., and Gessa, G. L.: Dynamic aspects of editors: Role of cyclic AMP in cell function; advances in *Biochemical Psychopharmacology neurohumoral control of cyclic 3',5'-AMP synthesis in brain*, *in* Greengard, P., and Costa, E., 3, New York, 1970, Raven, p. 155.