Pediat. Res. *12:* 703–707 (1978)

β-Adrenergic receptor
cyclic adenosine 3′:5′-monophosphate
cystic fibrosis
granulocytes
isoproterenol
leukocytes
mononuclear cells

Decreased Adenosine 3′:5′-Monophosphate Response to Isoproterenol in Cystic Fibrosis Leukocytes

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Summary

Leukocyte preparations from cystic fibrosis (CF) patients produced markedly less adenosine 3′:5′-monophosphate (cAMP) in response to isoproterenol than normal cells. Both stimulation ratio and net isoproterenol-stimulated accumulation of cAMP were significantly ($P < 0.001$) lower in mixed leukocyte preparations from 20 CF patients (29 trials) compared to 21 normal subjects (49 trials). There was no significant correlation of results with clinical score, and no differences in the CF group attributable to medications or presence of pancreatic insufficiency. CF heterozygotes had mean stimulation ratio and mean net isoproterenol-stimulated cAMP intermediate between normal and CF. There were no statistically significant differences among normal subjects, CF patients, and CF heterozygotes in basal cAMP or prostaglandin E_1-stimulated cAMP. Purified mononuclear cells from five normal persons and seven CF homozygotes had the same basal and prostaglandin E_1-stimulated cAMP, but isoproterenol-stimulated cAMP was markedly depressed ($P < 0.01$) in the CF cells. Granulocytes from six normal persons and nine CF patients also had the same basal and prostaglandin E_1-stimulated cAMP, but isoproterenol-stimulated cAMP was decreased ($P < 0.05$) in the CF samples. These results clearly demonstrate a difference between normal and CF leukocytes in cAMP response to β-adrenergic stimulation.

Speculation

The diminished cAMP response to β-adrenergic stimulation in CF is probably related to the presence of a CF gene, and may interact with other genetic abnormalities to produce the clinical syndrome of CF.

Several lines of evidence suggest that abnormalities in the autonomic nervous system (ANS) may be associated with CF, an heritable disease affecting primarily exocrine glands. Although the ANS is morphologically normal in CF patients (2), several clinical studies suggest subtle functional derangements (11, 12, 30). Also, pharmacologic manipulation of the ANS in animal model systems with reserpine (23, 24, 37), isoproterenol (22, 34), pilocarpine (19, 34), or atropine (4) can produce morphologic and physiologic changes resembling those seen in CF patients.

Most β-adrenergic receptors, including those of the ANS, are coupled to the enzyme adenylate cyclase (EC 4.6.1.1) and its product cAMP is an intracellular mediator of adrenergic signals (21). CF patients have been shown to have abnormal levels of urinary cAMP (27, 32) and CF fibroblasts have been reported to have abnormal cAMP response to isoproterenol (IPE) stimulation (8). We studied the cAMP response to β-adrenergic stimulation in CF in leukocyte preparations (15), which have been used for this purpose in asthma and other pulmonary disorders (13, 28, 29). We found that mixed leukocyte, mononuclear cell, and granulocyte preparations from CF patients produced markedly less cAMP in response to IPE than normal cells.

MATERIALS AND METHODS

SUBJECTS

Blood was obtained by venipuncture after informed consent from 21 healthy normal volunteers, ages 18–32 (23.4 ± 4.2 years) without family history of CF or pulmonary disorders, from 21 CF heterozygotes (parents of CF patients), ages 25–53 (37.5 ± 8.5 years) and from 20 patients with CF, ages 14–33 (22.4 ± 5.7 years). None was taking β-agonists, antagonists, methylxanthines, or aspirin. CF was diagnosed by sweat Cl > 80 mEq/liter and compatible clinical syndrome. Clinical Score (36), a measure of severity of illness, ranged from 38–96 on a scale of 100, *i.e.*, from severely ill to almost entirely well. All patients were taking vitamin supplements, 15 took pancreatic enzyme supplements, three took digoxin, eight took oral antibiotics (tetracycline, cloxacillin, or chloramphenicol), and two were studied both on and off antibiotics.

MATERIALS

Heparin-dextran solution was prepared with dextran (mol wt 170,000, Sigma), 5 g and heparin sodium in solid form (Sigma), 50 mg in 100 ml physiologic saline. Incubation buffer (buffer A) contained 50 mM Tris (pH 7.4) and 8 mM theophylline in physiologic saline. Solutions containing *l*-isoproterenol (IPE, Sigma), *l*-norepinephrine (Sigma), *l*-epinephrine (Sigma), and *dl*-propranolol (Sigma) and prostaglandin E_1 (PGE_1, a gift of Dr. John Pike, Upjohn Co.) were prepared immediately before use.

LEUKOCYTE PREPARATION

Leukocytes were prepared and assayed for cAMP by the method of Tallman *et al.* (35). Whole blood (5 ml) was mixed with heparin-dextran solution (1 ml), and allowed to sediment for 35 min at room temperature. The leukocyte-rich plasma was centrifuged (500 × *g*, 5 min, room temperature), and the cells were washed with physiologic saline and suspended in buffer A. Leukocyte counts were done manually. These preparations contained less than 1 platelet/50 leukocytes and were variably contaminated with erythrocytes. Osmotic lysis of erythrocytes during preparation did not affect results.

Mononuclear cells were prepared by centrifugation of leukocyte-rich plasma over a cushion of Ficoll-Hypaque solution by the method of Boyum (5). These preparations were >97% mononuclear cells. Granulocytes were recovered from the bottom of the Ficoll-Hypaque gradient and constituted >95% of the nucleated cells in this fraction. They were variably contaminated with erythrocytes.

MEASUREMENT OF LEUKOCYTE cAMP

Cells (2–4 × 10^6/tube) were preincubated for 5 min at room temperature in buffer A, and then exposed at 37° to IPE or PGE_1 in total volume 0.1 ml. The studies reported here were performed with 10^{-5}M IPE and 10^{-6}M PGE_1 for 5 min at 37°. Accumulation of cAMP was terminated by boiling for 3 min. This method permitted quantitative recovery of exogenous [^{3}H]cAMP. Samples for determination of basal cAMP were boiled immediately after the preincubation with buffer A. Incubation of cells in buffer A at 37° did not produce significant increase in cAMP. Preliminary experiments substituting Hank's balanced salt solution or Dulbecco's phosphate-buffered saline for buffer A showed no difference in cAMP accumulation. Assays for cAMP were performed by the method of Brown *et al.* (7) with appropriate internal standards.

The net IPE-stimulated accumulation of cAMP is calculated by subtracting basal cAMP from total cAMP accumulated in the presence of 10^{-5} M IPE for 5 min. The stimulation ratio is the ratio of total cAMP accumulated in the presence of 10^{-5} M IPE for 5 min to basal cAMP. Statistical comparisons were made by the *t* test (16).

RESULTS

CHARACTER OF RESPONSE

The IPE response was identified as β-adrenergic by the order of potency of agonists (isoproterenol > epinephrine > norepinephrine) and by its complete inhibition by propranolol (3, 28). The kinetics of response to IPE and the IPE dose-response curves was the same for normal and CF cells but the magnitude of response was less for the CF cells. Stimulation was maximal at 1–5 × 10^{-6} M IPE after 5 min and remained maximal at 10^{-5} IPE (Fig. 1).

MIXED LEUKOCYTE PREPARATIONS

Physiologic variations. There was an inverse relation between basal cAMP and stimulation ratio for IPE (Fig. 2) and PGE_1, but this function could not be rendered linear and the variance made uniform by log, log-log, square root, or other simple mathematical transformations.

Leukocytes from three normal drug-free women were studied every 3 days for one menstrual cycle and did not show systematic changes in their response to IPE or PGE_1. Serial studies (at least five in 2–12 months) were done in an additional five men and one woman. Maximum variation of net IPE-stimulated cAMP from the mean ranged from 13–50% except one person had one value 85% greater than his mean.

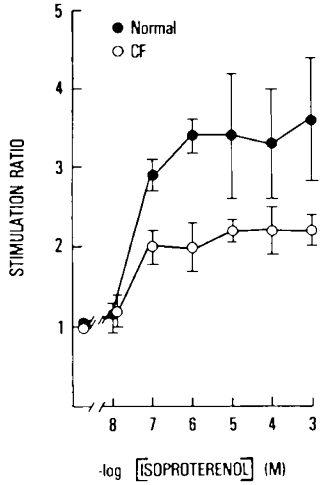

Fig. 1. Isoproterenol dose-response curves for normal (●) and CF (○) mixed leukocyte preparations. Incubations were performed in buffer A for 5 min at 37° with the indicated concentrations of isoproterenol as described in the text. Symbols represent mean and standard error of the mean for five normal and five CF subjects.

Leukocytes from six persons, four normal volunteers and two CF patients, were studied while the subjects were fasting and at intervals after they consumed a 400-cal breakfast. The optimal time for stimulation by IPE was 30 min after eating. At this time there was a mean 25% increase in stimulation ratio over the fasting state. At 120 min following a meal, response to IPE was significantly decreased compared to either 30 min after eating (40% less, $P < 0.01$) or the fasting state (25% less, $P < 0.01$). Blood for the studies reported here was obtained 30–45 min after breakfast.

Response of cAMP to IPE. Both stimulation ratio and net IPE-stimulated accumulation of cAMP were significantly ($P < 0.001$) lower in mixed leukocytes from 20 CF homozygotes (29 trials) compared to 21 normal subjects (49 trials) (Fig. 2, Table 1). Separating the data by sex did not alter this result.

There was no significant difference in basal cAMP among normal, CF heterozygote, and CF homozygote cells (Table 1).

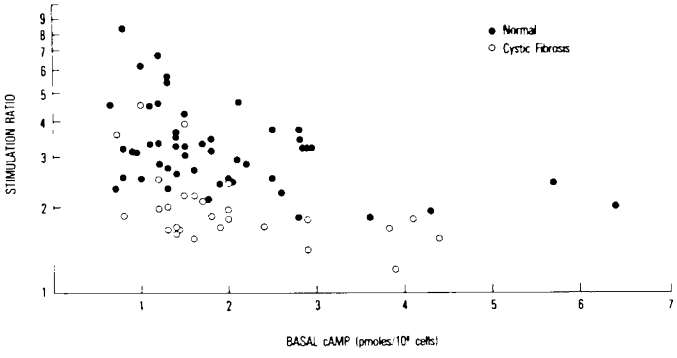

Fig. 2. Correlation of stimulation ratio of cAMP production in response to isoproterenol with basal cAMP in normal and CF homozygote mixed leukocyte preparations. Experiments were performed in buffer A at 37° with 10^{-5} M isoproterenol as described in the text. Stimulation ratio is significantly ($P < 0.001$) reduced in CF cells.

Table 1. *Production of cAMP by leukocytes in response to agonists (picomoles per 10^6 cells ± SD)*[1]

	Normal	CF	CF heterozygotes
Mixed leukocytes	(n = 49)	(n = 29)	(n = 21)
Basal	1.9 ± 0.2	1.9 ± 0.2	2.6 ± 0.6
Net IPE-stimulated	4.1 ± 0.3	1.8 ± 0.2[2]	3.3 ± 0.5
Net PGE_1-stimulated	15.7 ± 2.8	10.9 ± 2.3	12.3 ± 2.6
Mononuclear cells	(n = 5)	(n = 7)	
Basal	14.6 ± 2.9	14.3 ± 3.0	
Net IPE-stimulated	15.4 ± 1.8	5.0 ± 3.0[3]	
Net PGE_1-stimulated	22.2 ± 10.3	22.2 ± 6.3	
Granulocytes	(n = 6)	(n = 9)	
Basal	5.5 ± 1.3	4.3 ± 1.6	
Net IPE-stimulated	9.9 ± 2.8	4.6 ± 3.0[4]	
Net PGE_1-stimulated	11.2 ± 1.5	10.4 ± 4.5	

[1] n represents number of trials. Net IPE-stimulated cAMP is calculated by subtracting basal cAMP from total cAMP generated with 10^{-5} M IPE, in 5 min, at 37°. Net PGE_1-stimulated cAMP is calculated by subtracting basal cAMP from total cAMP generated with 10^{-6} M PGE_1, in 5 min, at 37° (see text).

[2] $P < 0.001$ compared to normal level.

[3] $P < 0.01$ compared to normal level.

[4] $P < 0.05$ compared to normal level.

However, because of the inverse relation of basal cAMP and stimulation ratio (Fig. 2), data were analyzed in groups according to basal cAMP. In each group CF homozygote cells had lower stimulation ratio than normal cells except for basal cAMP >3.0 pmol/10^6 cells where the difference was not significant but the number of trials was small (Fig. 2). Net IPE-stimulated cAMP was not related to basal cAMP, and the difference between normal and CF cells was significant at all basal levels.

Leukocytes from CF heterozygotes had mean stimulation ratio and mean net IPE-stimulated cAMP intermediate between normal and CF values (Fig. 3, Table 1). However, the heterozygote values were not normally distributed about the mean, but showed a biphasic distribution (Fig. 3). Eight of 21 heterozygotes had stimulation ratio in or above the normal range, 11 were in or below the CF range, and 2 fell in between. Moreover, parents of the same child seemed to segregate with one partner's leukocytes normal and one in the CF range. This non-normal distribution may account for the marginal statistical significance ($0.05 < P < 0.10$) of the difference between normal subjects and CF heterozygotes. Similarly, for net IPE-stimulated cAMP, 10 heterozygote values were in or above the normal range, 7 were in or below the CF range, and 4 were intermediate. The mean was intermediate and the difference from normal again was of marginal statistical significance. However, when data were expressed in this way, two of six families had values from both parents in the normal range.

There was no significant difference in the results from 15 CF patients using pancreatic enzyme supplements and 5 who were not: between the 3 without pancreatic insufficiency and the other 17; between 3 taking digoxin and the other 17; or between 8 patients taking antibiotics and the 10 who were not. One patient was studied while on and off tetracycline and cloxacillin, and another was studied on three occasions while taking tetracycline, while taking chloramphenicol, and while taking no antimicrobial.

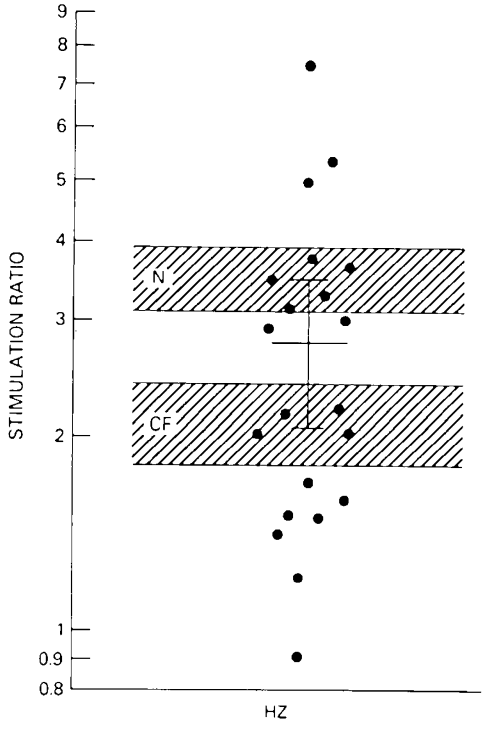

Fig. 3. Stimulation ratio of cAMP production in response to isoproterenol in mixed leukocyte preparations from 21 CF heterozygotes (●), compared to normal and CF homozygotes. Shaded areas indicate 95% confidence interval derived from 49 trials in 21 normal subjects (N) and 95% confidence interval derived from 29 trials in 20 CF homozygotes (CF). These describe a "normal range" and a "CF range." Vertical bar shows mean and 95% confidence interval for heterozygotes. Figure demonstrates biphasic grouping of heterozygotes in or above the "normal range" and in or below the "CF range." (See text.)

Both patients gave the same results in all trials. There was no significant correlation of results with clinical score (36) (Fig. 4).

Of the 20 CF patients studied, 18 had IPE-stimulated cAMP below the 95% confidence interval derived from the results from our normal subjects. However, 2 patients had values reproducibly in the normal range.

Response of cAMP to PGE_1. There were no statistically significant differences in PGE_1-stimulated cAMP among normal subjects, CF patients, and CF heterozygotes (Table 1).

MONONUCLEAR CELLS AND GRANULOCYTES

Purified mononuclear cells from five normal persons and seven CF homozygotes had the same basal cAMP and PGE_1-stimulated cAMP, but IPE-stimulated cAMP was markedly depressed ($P < 0.01$) in the CF cells (Table 1). One CF patient had IPE-stimulated cAMP in mononuclear cells in the normal range; his mixed leukocytes had also responded normally to IPE. Thus the findings in mononuclear cells reflect the findings in the mixed leukocyte preparations. Similarly, granulocytes from six normal subjects and nine CF patients had the same basal and PGE_1-stimulated cAMP, but IPE-stimulated cAMP was decreased ($P < 0.05$) in the CF samples (Table 1).

DISCUSSION

These results clearly demonstrate a difference between normal and CF leukocytes in cAMP response to β-adrenergic stimulation. It is likely that this difference is related to the presence of a CF gene and does not merely represent the secondary effects of disease, because of the lack of effect of severity of illness or of the drugs used by our patients, and the response of CF heterozygotes in this system. The proximity of this abnormality to the genetic lesion is uncertain, and it may be a distant consequence. However, of the 20 CF patients we studied, 2 had IPE-stimulated cAMP reproducibly in the normal range. Since it is quite likely that more than one genetic lesion can produce the clinical syndrome of CF (17), it may be that these two patients have a different inborn error from the others.

Leukocyte preparations from patients with asthma (13, 28, 29) and atopic eczema (9) have also been reported to have reduced cAMP response to β-adrenergic stimulation. Although recent evidence suggests that the defect in asthmatic patients may result from therapy with β-agonists (13), there is some question of an intrinsic defect in this system in asthmatics. But defective leukocyte cAMP response to β-adrenergic stimulation is not a general feature of lung diseases, since Parker and Smith (28) reported four patients with pulmonary fibrosis, three with acute exacerbations

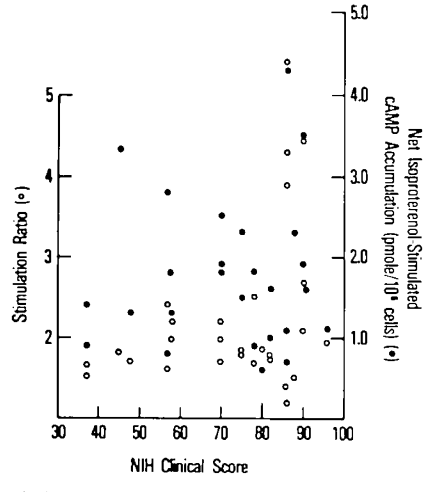

Fig. 4. Correlation of NIH Clinical Score and isoproterenol stimulation of cAMP in mixed leukocyte preparations from CF patients. There is no significant correlation for data expressed either as stimulation ratio (○) or as net isoproterenol-stimulated cAMP (●).

of chronic bronchitis, and three with acute upper respiratory infection who had normal response.

It is also unlikely that the diminished cAMP response in CF results from pancreatic insufficiency alone since three CF patients with normal pancreatic function were indistinguishable from the others.

The effect of a possibly abnormal circulating milieu in CF which in turn affects the adrenergic responsiveness of the leukocytes is difficult to gauge. Desensitization of β-receptors in CF due to high levels of circulating catecholamines caused by the stress of chronic disease is unlikely for several reasons. First, plasma norepinephrine is low in CF patients (20). Second, half the heterozygotes are similarly unresponsive but clinically well. Third, our preliminary studies with [^{3}H]dihydroalprenolol binding indicates that the number of β-receptors on the CF cells is not reduced (14). One prominent mechanism of desensitization or tachyphylaxis involves decreased numbers of receptors (26), although other mechanisms are undoubtedly also operative. However, interactions with other circulating factors may contribute to the abnormality we find in the CF cells.

The segregation of CF heterozygotes as either normal or CF-like responders may imply that the trait for this biochemical abnormality is passed by only one parent, consistent with an AaBb "double heterozygote" model of CF genetics (31). Or, since generation of cAMP in response to IPE clearly requires the interaction of numerous separate components, it may be that small heterozygote defects are compensated by other parts of the system, and are not detectable in this assay, whereas large defects cannot be easily compensated, and are observed.

There is a range of basal cAMP levels and IPE and PGE_1 effects on leukocytes reported in the literature (1, 3, 9, 13, 28, 29, 33, 38). Most of these differences probably reflect technical factors such as anticoagulants (1), presence of phosphodiesterase inhibitors in the buffers, method and time of cell preparation, and methods of cAMP recovery and measurement. However, clinical variables such as aspirin ingestion (33), use of β-agonist pharmaceuticals (13), and food ingestion are important as well. As many variables as possible were controlled in this study and the data were analyzed for the effects of drugs which could not be eliminated, sex, and the effects of the menstrual cycle. Still, there is probably important individual variability (13).

The leukocyte, mononuclear cell, and granulocyte preparations are all of mixed cell type. However, the consistency of the impaired cAMP response to IPE in all these preparations (Table 1), the known normal proportions of B and T lymphocytes in CF mononuclear cells prepared in this way (6), and the normal leukocyte differential counts in the CF samples all make it unlikely that the decreased cAMP response in the CF cells is related to varying proportions of different cell types alone.

Buchwald (8) studied cAMP response to IPE and PGE_1 stimulation in cultured fibroblasts, and found a significant difference between normal and CF cells for IPE stimulation but not for PGE_1. However, he finds an increase in cAMP response in the CF cells, and we find a decrease. There are several possible reasons for this discrepancy. The technical details of cAMP extraction and measurement differ between this study and Buchwald's (8). In addition, there may be genetic differences between Buchwald's 5 CF subjects and most of our 20 subjects. However, one report in abstract form (39) did not confirm Buchwald's results, and we have studied five normal and five CF cultured fibroblast lines, and find statistically significant decrease in the ratio of stimulation by IPE in the CF lines (14). However, our culture conditions differ from Buchwald's and further studies are under way to elucidate this problem.

This study demonstrates significant abnormalities in CF leukocytes in cAMP response to β-adrenergic stimulation. Although the normal basal cAMP levels and PGE_1-stimulated cAMP production in CF cells suggest that adenylate cyclase itself is intact, further studies, including direct measurement of adenylate cyclase activity in broken cell preparations and measurement of [^{3}H] dihydroalprenolol binding to β-receptors are in progress to localize the lesion more precisely. The recent emphasis on abnormal distribution of essential fatty acids in CF plasma and erythrocyte membranes (10, 25) raises the intriguing possibility that abnormal membrane lipids may affect the function of the receptor or of adenylate cyclase, as lipids are known to do in other systems (18, 40). But the nature and etiology of the CF fatty acid abnormality are not yet established, and if they are strictly related to pancreatic insufficiency or vitamin E deficiency, they will probably not explain our results. It will be important to determine the relation of the abnormality in cAMP response to β-adrenergic stimulation to the CF gene and also to the pathogenesis of clinical disease. Because of the extensive available ANS-active pharmacopeia these investigations might have distant therapeutic implications besides providing one approach to the genetic lesion of CF.

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41. We thank Drs. John Tallman and Paul A. di Sant'Agnese for advice, and the NICHD for laboratory space.
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43. Received for publication May 16, 1977.
44. Accepted for publication August 26, 1977.

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0031-3998/78/1206-0699$02.00/0

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