Alterations in Cyclic Adenosine Monophosphate Metabolism in Human Bronchial Asthma

I. LEUKOCYTE RESPONSIVENESS TO β-ADRENERGIC AGENTS

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A B S T R A C T In an effort to better define the role of β-adrenergic blockade in human bronchial asthma, peripheral blood leukocytes and lymphocytes from individuals with this condition were studied for possible alterations in cyclic AMP metabolism. Using a previously described radioimmunoassay to measure cyclic AMP, cells from asthmatic subjects were shown to have a highly significant decrease in their cyclic AMP response to β-adrenergic agents (isoproterenol, norepinephrine, and epinephrine) by comparison with normal control cells. The alteration in responsiveness was most marked at the time of severe active asthma and returned toward normal during periods of clinical remission. Evidence was presented to indicate that the reduced response in cells from asthmatic individuals was not due to marked alterations in the proportion of T and B lymphocytes. Five normal volunteers were treated with an oral bronchodilator preparation containing theophylline and ephedrine over a 2 wk period without a significant change in the lymphocyte cyclic AMP response.

These results provide unambiguous evidence for altered adrenergic responsiveness in bronchial asthma and indicate that purified peripheral blood lymphocytes should be a suitable in vitro system for further elucidation of the abnormality.

Despite the reduction in catecholamine responsiveness in the asthmatic population as a whole, major altera-

tions were largely restricted to individuals with severe, chronic asthma. Conclusive evidence for β-adrenergic blockade in individuals who have not had recent asthmatic symptoms was not obtained, casting some doubt on the theory that bronchial asthma is due to a congenital derangement of cyclic AMP metabolism. Moreover, transient episodes of bronchospasm were often accompanied by a normal cyclic AMP response indicating that episodes of asthma frequently occur in the absence of easily demonstrable adrenergic blockade.

INTRODUCTION

For about a decade it has been recognized that many individuals with bronchial asthma exhibit decreased biochemical and physiological responses to β-adrenergic drugs (1-7) but with the inherent limitations of in vivo studies involving complex hormonal responses the basis for the abnormality has remained obscure. From the elegant studies of Robison, Sutherland, and Butcher (8) it seems highly probable that most or all of the β-adrenergic effects of catecholamines are exerted through cyclic AMP. It would be important to learn whether the reduced β-adrenergic response in asthmatic individuals is due to a failure of catecholamines to produce the expected increase in cyclic AMP or whether it involves reduced cellular responsiveness to cyclic AMP. Reasoning that the reduction in responsiveness must be generalized (since it involves changes in metabolic responses that are largely extrabronchial) we recently suggested that peripheral blood leukocytes or platelets might be suitable for a detailed biochemical analysis of the phenomenon (9). When the cyclic AMP responses of asthma and normal control leukocytes to isoprotere-

nol were compared a marked decrease in the asthma cell response was observed (9). In the present paper, the results of subsequent, much more extensive studies of cyclic AMP metabolism in leukocytes of asthmatic and normal control subjects are described.

METHODS

Human subjects. Human adult volunteers were obtained from the patient population and employees of Barnes Hospital and Washington University School of Medicine. The groups studied included individuals with active asthma, inactive asthma (previous asthma with little if any clinical activity or medication within the preceding 3 months), allergic rhinitis without asthma, healthy normal controls (without known allergy and with a similar age, race, and sex distribution to patients with active asthma), control subjects with acute upper or lower respiratory infections, and individuals with chronic pulmonary fibrosis without bronchial obstruction. Results in postmenopausal women and those receiving chronic therapy with birth control pills are not included in the present paper but will be discussed elsewhere. Individuals being treated with tranquilizer, analgesic, antihypertensive, diuretic, and cardiac glycoside medications were excluded from the study. The medications being taken by the subjects with asthma are discussed in the legend to Table I. Data are available from 52 individuals with asthma (leukocytes, lymphocytes or both). All of the 52 were examined by one of the authors, 45 being examined on at least three occasions and 30 on more than four occasions. 36 were observed at least once during active asthmatic symptoms. Most of the repeatedly studied individuals were private or clinic patients of Dr. Parker with an average period of observation of 17 months during the study. The diagnosis and classification of asthma were based on routine clinical criteria including complete history, physical examination, routine blood and urine analysis, sputum and nasal smears for eosinophilic leukocytes, chest roentgenograms, pulmonary function studies before and after inhalation of isoproterenol, and skin testing with inhalent allergen extracts. As recommended by Scadding (10) all had evidence of variable bronchial obstruction. The asthma was classified as allergic in 17, idiopathic in 9, and "mixed" in 26. The term mixed asthma refers to individuals with well-defined allergies to inhalent antigens who also experience acute attacks of asthma in association with acute respiratory infections (in the absence of an identifiable exposure to a respiratory allergen). All had had at least one episode of wheezing in the absence of known respiratory infection. Individuals with chronic bronchitis and emphysema without prominent, recurrent, reversible bronchospasm were specifically excluded. The mean age of the asthmatic subjects was 34 (range 16–70) with an average interval from the time of the initial symptoms of asthma of 7 yr. Assessment of clinical activity was based on intensity of symptoms and quantity of medication. In addition, respiratory function was routinely monitored by auscultation measuring the duration of forced expiration as described by Rosenblatt and Stein (11). According to these authors there is a very good correlation between this measurement and the 1 s forced expiratory velocity (FEV_1),[1] and we are in complete agreement. FEV_1 measurements, given as percent of predicted

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[1] *Abbreviations used in this paper:* FEV_1, 1 s forced expiration velocity; Krebs' buffer, Krebs-Ringer bicarbonate buffer; VC, predicted vital capacity.

vital capacity (VC) using 83% as normal, were made frequently but not routinely. Estimation of the level of clinical activity was always completed before the results of metabolic studies were available. As used in Table I, the term "inactive asthma" refers to nine individuals with a history of asthma who did not have active asthma at any time during the period of observation. In seven of the nine, asthmatic symptoms had been absent for more than a year prior to the first cyclic AMP determination. In the remaining two the interval had been greater than 3 months. Including results both in Gey's and Krebs' buffers leukocyte data are available from 165 individual cyclic AMP experiments (each at multiple catecholamine concentrations) involving asthmatic donors. The number of experiments with purified lymphocytes from asthmatic donors in 73.

Preparation of leukocytes and lymphocytes. Mixed leukocytes were obtained as described previously from heparinized human blood by dextran sedimentation followed by two room temperature washes in phosphate-saline (0.15 M NaCl, 0.01 M phosphate, pH 7.4) and two low speed centrifugations to remove platelets (12). The quantity of blood used varied from 10 to 150 ml (usually 50 ml). Blood was drawn between 9 and 10:30 a.m. and processed without delay. Preparations usually contained 25–40% lymphocytes, the great majority of the remaining cells being polymorphonuclear leukocytes. Cells from individuals with asthma often contained increased numbers of eosinophilic leukocytes (usually in the 5–10% range) but were otherwise similar to control cells.

Purified lymphocytes were obtained from dextran-sedimented mixed leukocytes by nylon fiber chromatography or Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N. J.) density gradient centrifugation. Nylon fiber chromatography was carried out as described previously (12). The purified preparations contained 98–100% lymphocytes (500 nucleated cells counted) and variable numbers of erythrocytes. The average lymphocyte recovery was 25%. Ficoll-Hypaque purification was carried out according to a modification of the procedure of Mendelsohn, Skinner, and Kornfeld (13) yielding 94–99% lymphocytes and few if any erythrocytes. The remaining cells were granulocytes and monocytes. The average lymphocyte recovery was 70%. Ficoll-Hypaque purified granulocytic leukocytes were obtained as described previously (12) and were at least 99% pure. All purified cell preparations were centrifuged at low speed to remove platelets.

Reagents and solutions. Reagents and their sources were described previously (12). Solutions of catecholamines (*d,l*-isoproterenol·HCl, *l*-norepinephrine·HCl, and *l*-epinephrine ·HCl) and theophylline were prepared in 0.15 NaCl just before use.

Cyclic AMP determinations. Mixed leukocytes were suspended in Gey's solution, pH 7.8 (14), or Krebs-Ringer bicarbonate buffer (Krebs' buffer), pH 7.5 (15), at a concentration of 4–8 × 10⁶ mixed leukocytes/ml. Purified lymphocytes were used at a concentration of 3–5 × 10⁶ cells/ml. Volumes of 0.5 ml of the cell suspension were added to each tube. Additions were usually made at room temperature. Catecholamines, buffer and other solutions were added in a volume of 0.05 ml and the cells incubated at 37° for 1–60 min at 37°. In prolonged incubations cells were gently resuspended every 5 min. The pHs were stable except at the highest isoproterenol and norepinephrine concentrations used (10 mM) where a fall of about 0.3 pH units occurred in Gey's solution. Experiments with pH adjusted, 10 mM isoproterenol and norepinephrine solutions gave slightly lower cyclic AMP values. After incubation the cells were

TABLE I

The Effect of Isoproterenol on Cyclic AMP Concentrations in Mixed Peripheral Blood Leukocytes

	Number of subjects	Number of determinations	Control	10 mM Isoproterenol
			picomoles cyclic AMP/10⁷ cells	
Asthma, active (on Rx)*	26	45	5.5 (±0.5)	15.8 (±2.5)‡
On corticosteroids	12	16	5.8 (±0.7)	19.0 (±2.4)
No corticosteroids	20	29	5.3 (±0.5)	14.0 (±2.8)
Recent asthma (<14 days)				
No Rx for > 48 h	10	15	6.5 (±0.7)	19.4 (±3.8)
No Rx for > 7 days	11	16	6.2 (±0.6)	21.8 (±4.6)
Inactive asthma§ (>3 months)	9	15	6.7 (±0.5)	26.6 (±3.2)
Allergic rhinitis	21	29	7.3 (±0.7)	31.2 (±3.0)
Controls				
No illness‖	30	40	8.1 (±0.7)	34.6 (±5.0)
Acute upper respiratory infection	3	3	8.2 (±0.9)	38.2 (±5.0)
Bronchitis, acute exacerbation	3	3	7.8 (±0.9)	33.2 (±5.0)
Pulmonary fibrosis	4	4	7.3 (±1.0)	34.0 (±3.0)

2-4 × 10⁶ leukocytes were incubated in 0.5 ml Gey's solution at 37° for 30 min in the presence and absence of 10 mM d,l-isoproterenol. Means±SEM are shown. The maximal number of determinations used from a single individual is two. Postmenopausal and pregnant women as well as women taking oral contraceptives are excluded from the table. Cell preparations containing <25% and 40% lymphocytes are not included in the table.

* Medications used by individuals in the active asthma group included oral bronchodilators (36 determinations, most frequently Tedral [22 determinations], Marax, or Quadrinal [Knoll Pharmaceutical Co., Orange, N. J.] with mean daily doses of 560 mg of theophylline base, 105 mg of ephedrine·HCl and 32 mg phenobarbital), rectal aminophylline (10 determinations with mean daily doses of 420 mg theophylline base), corticosteroids (16 determinations with a mean daily dose equivalent to 80 mg of hydrocortisone/day), systemic or aerosolized catecholamines (20 determinations); and antihistamines (5 determinations). None of the individuals studied was taking analgesic medications.

‡ The difference between the isoproterenol response in active asthma and matched normal control cells is highly significant ($P < 0.001$. Student's paired t test).

§ Inactive refers to individuals with a history of asthma who were free of asthma throughout the study. Serial data from individuals with active asthma in whom observations were also made during periods of relative clinical inactivity are given in Table II.

‖ This population is matched with the active asthma group.

centrifuged for 2 min at room temperature at 2500 rpm. The supernatant solutions were decanted and the pellets frozen in liquid nitrogen or ethanol-dry ice. Specimens were stored at − 70°C until assay.

Cyclic AMP was determined by radioimmunoassay using antibody to 2′-O-succinyl-cyclic AMP and [¹²⁵I]2′-O-succinyl-cyclic AMP-tyrosine methyl ester. Details in regard to the sensitivity and specificity of the cyclic AMP immunoassay have been presented in detail previously (16).

Immunofluorescent staining. Ficoll-Hypaque purified cells were stained for surface immunoglobulins by a fluorescence double antibody technique using rabbit antibody specific for human IgG, IgM, or Fab as the first antibody and fluoresceinated goat antirabbit IgG as the second antibody. Antisera were shown to be monospecific by immunoelectrophoresis. Positive cells gave the typical speckled surface immunoglobulin fluorescence described by Papamichail, Brown, and Holborrow (17). Percentages of stained cells were corrected for the number of monocytes in the preparation. Positive responses were blocked almost completely by the appropriate human Ig at a concentration of 1 mg/ml.

RESULTS

Isoproterenol stimulation of mixed leukocytes; selection of a standardized condition. We have previously shown that isoproterenol raises cyclic AMP concentrations in purified human lymphocytes (12) and mixed peripheral blood leukocytes (9) and that the response is inhibited by d,l-propanolol, indicating that it is a β-adrenergic effect. To select an isoproterenol concentration for routine screening of adrenergic responsiveness in peripheral blood leukocytes, cells from 15 normal control subjects were stimulated with 0.0001-10 mM concentrations of isoproterenol at 37° in Gey's solution for various time periods. As a result of these preliminary studies stimulation with 10 mM isoproterenol for 30 min was selected as providing a regular and easily measurable increase in cyclic AMP concentration in normal leukocytes. Using this condition the cyclic AMP response of cells from the different experimental groups

was evaluated (Table I). In cells from healthy normal controls as well as from individuals with chronic pulmonary fibrosis and acute upper and lower tract respiratory infections, the mean increase in cyclic AMP concentration in response to 10 mM isoproterenol was about fivefold. In the group with active asthma there was a highly significant decrease ($P < 0.001$) in the cyclic AMP response as compared with normal controls. With cells from patients with recently active or inactive asthma, a less striking decrease in the isoproterenol response was observed. The response of individuals with allergic rhinitis to isoproterenol was not clearly separable from that of normal controls. In addition to an altered isoproterenol response, unstimulated (30 min at 37° without isoproterenol) cells from individuals with active asthma had lower cyclic AMP concentrations than cells from control donors (Table I).

16 of the determinations on cells from individuals with active asthma were made during systemic corticosteroid therapy. As indicated in Table I corticosteroid

FIGURE 2 The cyclic AMP response of mixed leukocytes to various concentrations of isoproterenol. Krebs' buffer, 30 min at 37°. The results given are the experimental means from four experiments with asthma (open bars) and five with normal control leukocytes (closed bars) expressed as the percent increase above 30-min buffer control values (±SEM). No theophylline present.

therapy appeared to increase catecholamine responsiveness improving the response of the group as a whole. Coffey, Logsdon, and Middleton have also found that leukocytes from asthmatic individuals receiving corticosteroids have an improved cyclic AMP response to isoproterenol (18). However, we have evidence to indicate that the corticosteroid effect is due at least in part to changes in the proportions of T and B lymphocytes (see below). This subject will be discussed in detail elsewhere.

Effect of buffer, isoproterenol concentration, and time. The leukocyte cyclic AMP response to isoproterenol was also examined in a second buffer system (Krebs' buffer), at lower isoproterenol concentrations and with different incubation periods. Representative data on the effect of buffer and incubation time are shown in Fig. 1. The asthma leukocyte cyclic AMP response in Krebs' buffer was reduced to about the same extent as in Gey's solution. Preincubation of isolated leukocytes at 37° in Krebs' buffer for periods of up to 90 min (so as to provide cells with glucose over an extended period) did not alter this result. Marked differences in the isoproterenol response in asthmatic and control subjects
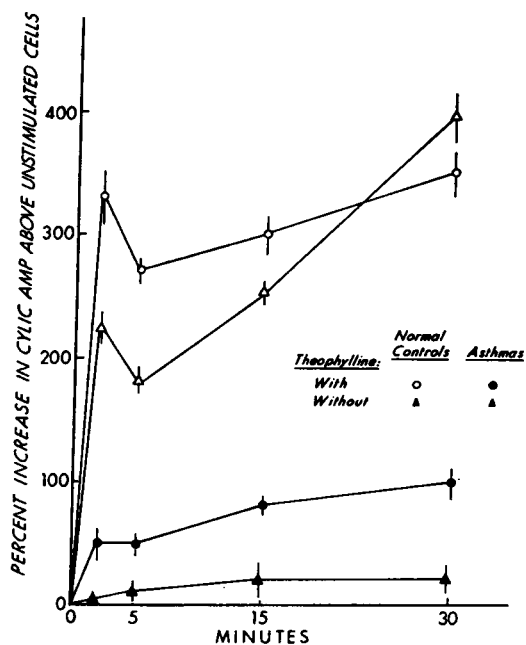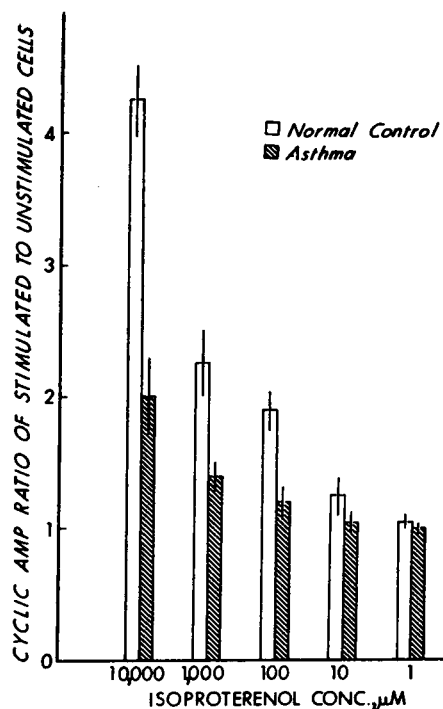

FIGURE 1 Isoproterenol stimulation of mixed leukocytes as a function of time. Cells suspended in Krebs' buffer were stimulated with 10 mM isoproterenol for the indicated time periods in the presence and absence of 0.5 mM theophylline. The results shown are mean of five experiments with normal control cells and four experiments with active asthma cells, expressed as the percent increase above the 0 time control value (±SEM). Cyclic AMP concentrations in cells not receiving isoproterenol or theophylline were essentially stable throughout the 30 min time period. In the presence of theophylline control cells had a 30% increase in cyclic AMP at 30 min as compared with a 15% increase in asthma cells.
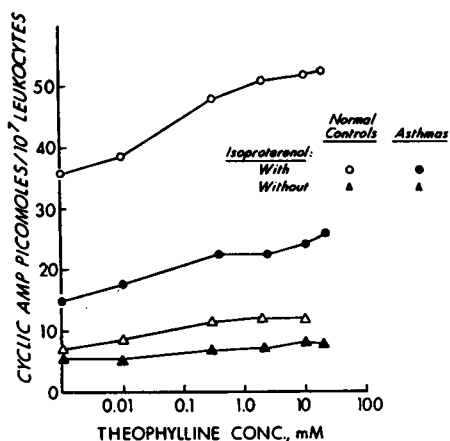
FIGURE 3 Representative data on the effect of theophylline on cyclic AMP levels in isoproterenol-stimulated cells. The response of mixed leukocytes from a subject with asthma is compared with the response of cells from a normal control. Cells in Krebs' buffer. Incubation for 30 min at 37°. The isoproterenol concentration was 10 mM.

were observed at 2 and 5 min indicating that prolonged incubation times are not required in order to see differences between the two groups (Fig. 1). The unexpected fall in cyclic AMP concentration in isoproterenol-stimulated control cells during the last 3 min of a 5 min incubation (Fig. 1) has been observed in five separate experiments but there is no information on the mechanism.

Fig. 2 contains composite isoproterenol dose response curves from four asthma and five normal control cell preparations in Krebs' buffer. There was a difference in isoproterenol responsiveness between asthma and control cells which was present throughout the 100–10,000 μM dose range. At the 10 μM isoproterenol level there was a relatively poor response in control cells and the absolute difference between the two groups was not statistically significant. Both groups of cells had a maximal response at 10 mM isoproterenol. This suggested that the reduced 10 mM isoproterenol response in individuals with asthma was not due to a bell-shaped curve with marked inhibition at high isoproterenol concentrations. To evaluate this possibility further, dose response curves of cells from 15 additional subjects with asthma and 10 additional normal controls were analyzed. These data were obtained in Gey's buffer or using different stimulation periods and are therefore not included in Fig. 2. A modest (10–30%) decrease in the cyclic AMP response at 10 mM isoproterenol as compared with 1 and 0.1 mM isoproterenol was observed on seven occasions (four times with asthma cells and three times with normal cells) but it was similar in magnitude in the two groups. It was concluded that cells from asthmatic individuals have a

decreased cyclic AMP response over a broad range of isoproterenol concentrations.

Effect of theophylline on isoproterenol stimulation. With normal control cells in Krebs' buffer the presence of 0.5 mM theophylline in the incubation mixture increased the early (2–10 min) 10 mM isoproterenol response but there was little potentiation of the response at 15 and 30 min; in Gey's solution the combination of theophylline with isoproterenol produced an increase in the isoproterenol response over the entire 2–30 min period. In cells from asthmatic subjects 0.5 mM theophylline potentiated the isoproterenol response in both buffers throughout the 30 min period but the absolute increase in cyclic AMP (over isoproterenol alone) was usually small and the marked difference between asthma and control cells was not abolished (Fig. 1). Similarly at higher theophylline concentrations (2, 10, and 20 mM), leukocytes from asthmatic individuals continued to exhibit a substantial decrease in their cyclic AMP response to 0.01–10 mM isoproterenol as compared with control cells incubated under the same conditions (Fig. 3). Preliminary results indicate that differences may also be demonstrable at 0.001 mM isoproterenol in the presence of high concentrations of theophylline but the cyclic AMP response in control cells is relatively small and additional studies are needed.

Stimulation by other catecholamines. In previous studies we have demonstrated that 10–10,000 μM norepinephrine raises human lymphocyte cyclic AMP concentrations and that the effect is blocked by propanolol, indicating that β-receptors are involved (12). In 14 experiments with cells from asthmatic donors (Krebs' buffer), 10 mM norepinephrine produced a twofold increase in cyclic AMP concentration after 30 min as compared with a fourfold increase in cells from control subjects under the same conditions. Thus individuals with asthma also had a decrease in their leukocyte cyclic AMP response to norepinephrine. As with isoproterenol differences were obtained when norepinephrine stimulation was carried out in the presence of 0.5–20 mM theophylline.

The leukocyte cyclic AMP response to *L*-epinephrine also was examined. In normal control cells high concentrations of epinephrine, norepinephrine, and isoproterenol (100–1000 μM) produced similar cyclic AMP responses. At lower catecholamine concentrations (1–10 μM) epinephrine produced greater rises in cyclic AMP than the other two agents; in the presence of 500 μM theophylline leukocytes regularly responded quite well to 1.0 μM epinephrine. An evaluation of leukocyte responsiveness to epinephrine in individuals with asthma has been carried out only at the lower epinephrine concentrations, generally in the presence of 0.5 mM theophylline. The response of asthma leukocytes was reduced at 1, 10, and 100 μM epinephrine, and probably at 0.1 μM

epinephrine as well. (These data are not presented in tabular form because similar data for purified lymphocytes are given in Fig. 5.)

Studies with purified cell populations. To ascertain the cellular distribution of cyclic AMP in isoproterenol-stimulated, mixed leukocytes, purified cell populations were studied. Ficoll-Hypaque purified granulocytes, nylon fiber purified lymphocytes and mixed leukocytes were obtained from the same sample of normal control blood. When the cyclic AMP response of purified lymphocytes and granulocytes was compared and correlated with the differential count and cyclic AMP response of the mixed leukocyte population, 60–90% of the 10 mM isoproterenol response in mixed cells could be ascribed to the lymphocyte component. Similar results were obtained at other isoproterenol concentrations. Representative data from one of four such experiments are shown in Fig. 4 (lower portion). Purified granulocytes had substantial cyclic AMP levels but underwent a relatively modest increase in cyclic AMP concentration in response to isoproterenol. Thus the lymphocyte could be implicated as the major source of cyclic AMP in isoproterenol-stimulated leukocytes. Results with Ficoll-Hypaque purified lymphocytes substantiated this conclusion. In 21 experiments with Ficoll-Hypaque purified normal control lymphocytes (on average 95% pure), stimulation with 10 mM isoproterenol for 30 min at 37° produced a mean cyclic AMP concentration of 140 (±14 SEM) pmol/10^7 cells. If it is assumed that lymphocytes in mixed leukocyte preparations respond similarly they can account for most or all of the mixed leukocyte response.

In keeping with the apparent major role of lymphocytes in the cyclic AMP response of normal control leukocytes to catecholamines, when mixed leukocytes and nylon fiber purified lymphocytes from the blood of asthmatic donors were compared (four experiments with purified polymorphonuclear leukocytes, lymphocytes and mixed leukocytes and four experiments with lymphocytes and mixed leukocytes) catecholamine responsiveness in purified lymphocyte populations was reduced, correlating well with alterations in mixed leukocyte responses. Representative data are shown in Fig. 4 (upper portion).

More extensive studies have been carried out with Ficoll-Hypaque purified lymphocytes stimulating with 10 mM isoproterenol, 10 mM isoproterenol-0.5 M theophylline, and 1 μM epinephrine-0.5 mM theophylline (Fig. 5). Under each of the three stimulation conditions the cyclic AMP response in active asthma cells was significantly reduced by comparison with normal control cells ($P < 0.001$). Despite the statistical difference in catecholamine responsiveness in the two groups, significant overlap was obtained. With three exceptions the response to 10 mM isoproterenol correlated well with
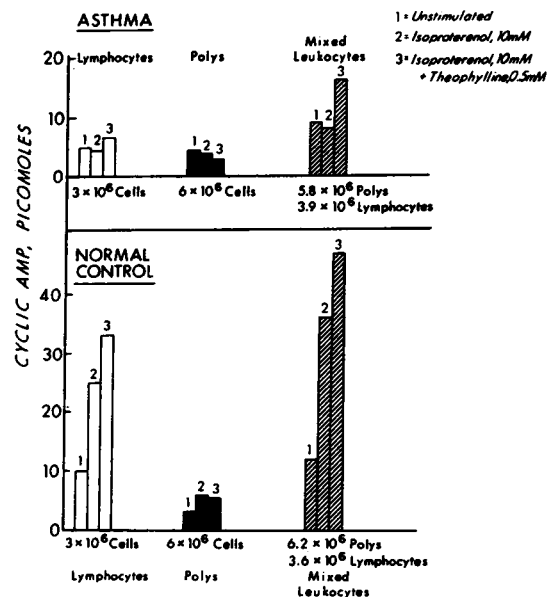

FIGURE 4 The cyclic AMP response of mixed leukocytes, polymorphonuclear leukocytes (polys), and lymphocytes from the same sample of blood. Mixed leukocytes were processed by nylon fiber chromatography and Ficoll-Hypaque density gradient centrifugation to obtain purified lymphocytes and polys, respectively. The purified cells were > 99% pure. Cells were incubated for 30 min at 37° in Gey's solution: (a) control cells; (b) 10 mM isoproterenol; (c) 10 mM isoproterenol, 0.5 mM theophylline. Results are shown for cells from an individual with active asthma (upper figure) and a normal control (lower figure). Results are expressed per 3 × 10⁶ lymphocytes, 6 × 10⁶ polys, and 10 × 10⁶ mixed leukocytes (based on total nucleated cell count). The mixed leukocyte differential is given on the abscissa.

that obtained at lower concentrations of epinephrine and isoproterenol.

The correlation between leukocyte and Ficoll-Hypaque purified lymphocyte responses in the same donor was generally quite good. However, on three occasions purified lymphocytes responded a good deal better than lymphocytes in the parent mixed leukocyte preparation. The mean leukocyte and lymphocyte cyclic AMP responses in these experiments were 17.7 and 118 pmol cyclic AMP/10^7 cells, respectively (10 mM isoproterenol for 30 min). On the basis of the proportion of lymphocytes in the leukocyte preparations (on average, 30%) the expected purified lymphocyte response would have been considerably lower. It is uncertain whether the reduction in leukocyte responsiveness is on a different basis in these individuals or whether a less readily detectible improvement in responsiveness is obtainable with most or all inhibited asthma leukocytes. Statistically, the reduction in the response of Ficoll-Hypaque purified asthma lymphocytes to 10 mM isoproterenol appears to be com-

400

CYCLIC AMP picomoles/10⁷ LYMPHOCYTES

300

200

100

○ NORMAL
▲ INACTIVE ASTHMA
● ACTIVE ASTHMA

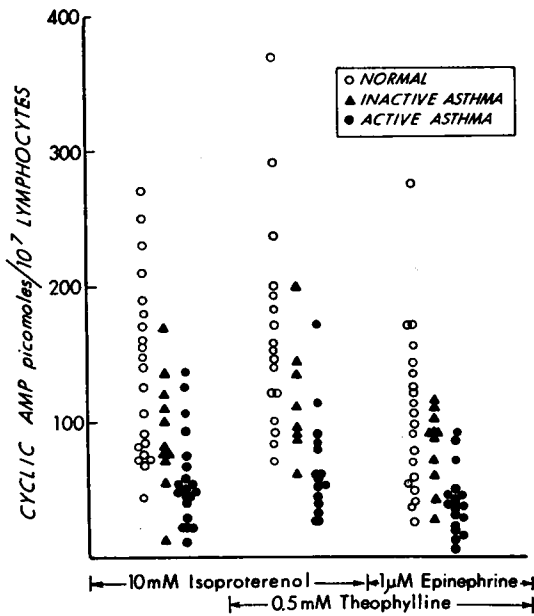|←—10mM Isoproterenol ——►|◄|1µM Epinephrine►|
|←—— 0.5mM Theophylline ———►|

FIGURE 5 Responses of Ficoll-Hypaque purified lymphocytes to isoproterenol and epinephrine; $2.5-5.0 \times 10^6$ lymphocytes were incubated in 0.5 ml Gey's solution at 37° for 30 min. Postmenopausal and pregnant women as well as women taking oral contraceptives are excluded from the figure. Under all three conditions the difference in the response in severe asthma cells and normal control cells is highly significant ($P < 0.001$, Student's paired t test).

parable to that in the mixed leukocytes which would be consistent with the former possibility. Just why certain purified lymphocyte preparations should show an improved response is uncertain. In addition to the possible effect of removing nonlymphocytic cells, dissociation of a soluble inhibitor during lymphocyte processing would be a possible explanation.

Whether purified granulocytic leukocytes from asthmatic individuals also have decreased isoproterenol responsiveness is uncertain at present. Because of the variation and restricted magnitude of the cyclic AMP response to catecholamine in these cells additional studies would be needed to answer this question.

The possible role of lymphocyte heterogeneity in catecholamine responsiveness. The next question that had to be considered was the possible role of lymphocyte heterogeneity in the altered cyclic AMP response in cells from asthmatic donors. Direct comparisons of Ficoll-Hypaque and nylon fiber purified cells from the same sample of human blood indicated that the Ficoll-Hypaque preparations are significantly more responsive to isoproterenol. Since, on average, lymphocyte recovery from nylon is only 25% a possible explanation would be the selective loss of lymphocytes that are especially responsive to isoproterenol during nylon fiber chromatog-

raphy. Recent studies in this laboratory indicate that when Ficoll-Hypaque purified lymphocytes are passed over a nylon column there is a decrease in the percentage of surface immunoglobulin containing cells (presumably B cells), apparently because of a greater nonspecific affinity of these cells for nylon (19). These observations are in accord with previous work in experimental animals indicating that lymphoid cells that contain or produce immunoglobulins selectively adhere to glass beads or nylon fibers (20, 21). Other explanations for the decreased isoproterenol response in nylon purified cells (low levels of contaminating cells in Ficoll-Hypaque preparations or toxic damage during nylon fiber purification) seem considerably less likely. The nylon had been washed exhaustively according to the procedure used by Cooper to remove toxic impurities (22). Our preparations of nylon purified lymphocytes were more than 95% viable as judged by dye exclusion. They also showed the expected increase in DNA synthesis in response to antigen and phytohemagglutinin and had a qualitatively as well as quantitatively altered cyclic AMP response pattern.

With indirect evidence indicating that immunoglobulin-containing lymphocytes have an especially active cyclic AMP response to isoproterenol, the possibility that the proportion of T and B lymphocytes might be altered in individuals with bronchial asthma had to be seriously considered. In 12 experiments, Ficoll-Hypaque purified lymphocytes from asthmatic donors (with reduced cyclic AMP responses to isoproterenol) were analyzed for surface immunoglobulins by immunofluorescence. The mean number of asthma cells staining for IgG (eight experiments) was 28%, averaging somewhat higher than the value obtained in normal control lymphocytes (19%, 10 experiments), purified in the same way. In four experiments in which the percentage of cells staining for both IgM and IgG was determined the mean percentages in asthma and normal control cells were 33 and 28%, respectively. Thus, if anything, the B cell content of cells from asthmatic donors was slightly increased which, of the arguments outlined above are valid, would be expected to increase rather than decrease the isoproterenol response. If, instead, it were assumed that T cells are the sole source of cyclic AMP in purified lymphocytes, a decrease in the percentage of T cells from 72 to 67% (neglecting IgA-containing cells) could hardly explain the marked (> twofold) decrease in isoproterenol responsiveness in asthmatic individuals.

Serial studies in normal controls. The consistancy of the normal control leukocyte cyclic AMP response to 10 mM isoproterenol (30 min, 37°) in a given individual was examined. In serial studies of 14 young adults (seven male, seven female) examined sporadically on four or more occasions over a period of a year or

TABLE II
Serial Leukocyte Studies in 13 Individuals with Asthma of Fluctuating Severity

Asthmatic symptoms	Mean FEV₁	Mean forced expiratory time	No. determinations*	Control	10 mM Isoproterenol
	% predicted VC	s		cyclic AMP/10⁷ cells	
Severe	38%‡	7.5	19	5.3 ($\pm$0.7)	13.3 ($\pm$2.6)
Minimal (>3 wk)	65%§	3.4	42	6.3 ($\pm$0.7)	21.9 ($\pm$3.0)

Leukocytes were stimulated with 10 mM isoproterenol in Gey's solution for 30 min at 37° (see legend to Table I). Determinations made during corticosteroid therapy are not included in the table. Otherwise, medications in use in the severe asthma group are similar to those in Table I. The mean percentage of lymphocytes was 28% in the severe asthma group and 32% in the minimal asthma group. Preparations containing <25% and >40% lymphocytes were excluded from the Table. In 16 of the 19 leukocyte determinations made during periods of severe asthma symptoms had been present for at least 48 h.

* In individuals with more than two determinations during severe symptoms or four determinations during minimal symptoms data are averaged and the number of determinations taken as two and four, respectively. This is done to avoid undue statistical weighting of the sample by results in a few individuals.

‡ Mean of 14 determinations. In six individuals, the FEV₁ after inhalation of isoproterenol was also determined and averaged 50% of the predicted VC.

§ Mean of 15 determinations.

more the average maximal deviation in a single experiment from the overall experimental mean was 42 and 65%, respectively. The greater variation in females presumably is due to fluctuation in hormonal levels during the menstrual cycle as will be discussed elsewhere. Despite some variation in the cyclic AMP response it was rare to obtain less than threefold stimulation with 10 mM isoproterenol (30 min at 37°).

Serial studies in individuals with asthma. The most marked impairment of leukocyte responsiveness was in association with severe, persistent asthma. In three such individuals (mean FEV₁, 32% of predicted vital capacity, duration of forced expiration 7.6 seconds) leukocyte responsiveness was regularly reduced (18 determinations, mean cyclic AMP concentration 11.6 pmol-10⁷ cells, 10 mM isoproterenol for 30 min).

The relationship between impairment of pulmonary function and leukocyte responsiveness to catecholamine was further examined in serial studies involving 13 individuals with asthma of fluctuating severity (Table II). During periods of reduced clinical activity there was a significant increase in the cyclic AMP response. Even at such times, however, the response appeared to be less than that obtained in cells from individuals whose asthma was inactive throughout the study (Table I).

Despite the overall correlation between alterations in leukocyte responsiveness and severity of asthmatic symptoms considerable variation was noted. In eight individuals with recently developed symptoms of asthma (< 48 h) the leukocyte (or lymphocyte) cyclic AMP

response was clearly abnormal in only three (represented by L. G. in Fig. 6 and M. H. in Fig. 7, both of whom are also in Table II). Both individuals eventually had a complete return of leukocyte responsiveness to normal within several months after evidence of clinical bronchospasm had cleared. A return to normal or near normal responsiveness was observed in three other individuals included in Table II. By contrast the cells of M. S. (Fig. 7) exhibited decreased, isoproterenol responsiveness on each of 10 occasions, even at times when she was largely free of airway obstruction and receiving little or no medication. A persistent reduction in responsiveness was observed in four additional individuals in Table II. In the remaining three subjects in this table the results of serial studies were inconclusive.

The effect of bronchodilator therapy in control subjects. The effect of oral bronchodilator therapy on lymphocyte cyclic AMP metabolism in five normal volunteers was studied (Table III). After control blood samples had been obtained subjects were given four Tedral tablets/day (Warner Chilcott Laboratories, Morris Plains, N. J.) over a 2 wk period. Since 1 Tedral tablet contains 130 mg theophylline, 24 mg ephedrine·HCl, and 8 mg phenobarbital, a daily dose of 4 tablets/day corresponds closely to what was being used in patients with active asthma on oral bronchodilator therapy. Aside from a possible modest fall in catecholamine responsiveness on days 7 and 8 (days 6 and 7 of therapy) (P values of 0.2–0.4, Student's paired t test) the oral bronchodilator agent did not alter the lymphocyte cyclic AMP
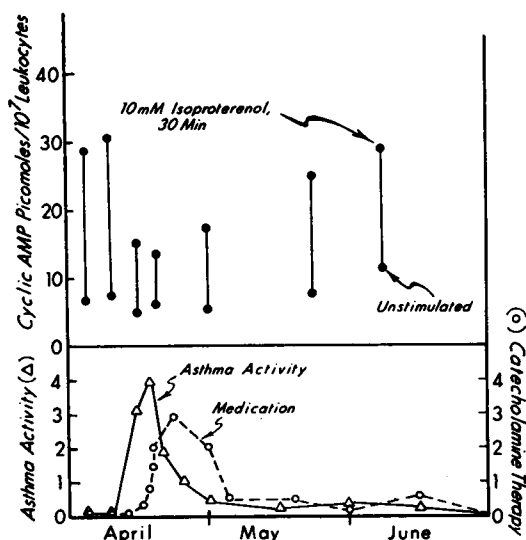
FIGURE 6 Serial mixed leukocyte studies in L. G., a 23 yr old Negro male with pollen allergy, just before, during, and after a sustained period of asthmatic symptoms. The upper closed circles are cyclic AMP values obtained with 10 mM isoproterenol, 37°, 30 min; the lower closed circles are values for unstimulated cells. The lower section of the figure gives the relative severity of asthmatic symptoms and signs (open triangles) and the relative 24 h catecholamine dosage (open circles). In order to compare these responses with the mean isoproterenol response in normal control cells see Table I. At the time of the initial three determinations he had received no therapy. Subsequently he was treated with oral bronchodilator tablets (maximal dose, 4 Tedral tablets/day). During active asthmatic symptoms (> 3 in the figure) the mean FEV_1 was 45% of the predicted vital capacity (two determinations) and the mean duration of forced expiration, 6.2 s. The corresponding values during symptom-free intervals were: FEV_1 93% (the average of single determinations in early April and late May); and mean duration of forced expiration 2.7 s.

response to either isoproterenol or epinephrine (compare with Fig. 5).

DISCUSSION

The results of this study confirm and extend our earlier observations that peripheral blood leukocytes from subjects with symptomatic bronchial asthma exhibit a decreased cyclic AMP response to β-adrenergic agents by comparison with normal control cells. A change in the cyclic AMP response was obtained with isoproterenol, norepinephrine, and epinephrine and presumably would occur with any catecholamine that raises leukocyte cyclic AMP concentrations. A reduced catecholamine response could be demonstrated with a variety of incubation times, catecholamine concentrations, in the presence and absence of theophylline and in at least two different buffers making it clear that the phenomenon is not restricted to a single set of in vitro conditions. In addition to an altered
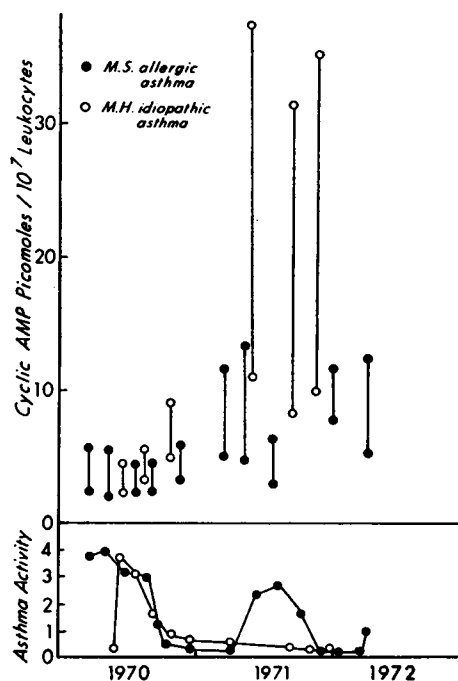
FIGURE 7 Serial measurements of the mixed leukocyte cyclic AMP response to 10 mM isoproterenol (37°, 30 min) in two subjects with asthma. M. S., a 39 yr old female with allergic asthma (closed circles). M. H., a 68 yr old male with idiopathic asthma (open circles). In the upper portion of the figure the upper and lower connected circles are cyclic AMP levels in isoproterenol stimulated and control cells, respectively. The lower section of the figure gives the severity of asthmatic symptoms and signs (same symbols, graded on a 0–4 scale). To compare with the isoproterenol response in normal control cells, see Table I.

In M. S. the mean FEV_1 (percent of VC) during periods of marked asthma activity (graded > 3) was 32% (three determinations) as compared with 63% when asthma activity was < 1 (three determinations). Mean forced expiration times were 7.3 s (asthma activity > 3) and 3.3 s (asthma activity < 1). During 3+ or > asthmatic symptoms she was receiving an average of 4.6 Tedral or Marax (J. B. Roerig & Co., New York) tablets/day as well as intermittent therapy with aerosolized isoproterenol and rectal aminophylline. During < 1+ asthma she was taking an average of 1 bronchodilator tablet every 1.5 days.

M. H. had had dramatic exacerbations of asthmatic symptoms in June 1970, at the time of an acute upper respiratory infection. The initial leukocyte cyclic AMP study was obtained within 12 h after the onset of symptoms at a time when he had received very little catecholamine or methylxanthine therapy (1 Tedral tablet). In the subsequent 2 wk he received an average of 4.2 Tedral tablets/day as well as limited treatment with aerosolized isoproterenol. On subsequent occasions he was taking an average of < 2 Tedral tablets/wk. Single FEV_1 determinations are available in June 1970 (FEV_1 34% of predicted VC, asthma activity > 3) and December 1971 (FEV_1 73% of predicted VC, asthma activity < 1). Mean forced expiration time at these times were 8.2 and 3.1 s, respectively.

TABLE III

The Effect of Oral Bronchodilator Therapy on Normal Control Lymphocyte Responsiveness to Catecholamines

	Control	During therapy		
Condition	Days 1, 2	Day 3	Days 7, 8	Days 14–16
		cyclic AMP picomoles/10⁷ cells		
10 mM isoproterenol	109 ($\pm$11)	94 ($\pm$18)	85 ($\pm$14)	119 ($\pm$20)
10 mM isoproterenol-0.5 mM theophylline	122 ($\pm$9)	107 ($\pm$10)	103 ($\pm$9)	135 ($\pm$14)
1 μM epinephrine-0.5 mM theophylline	72 ($\pm$9)	68 ($\pm$7)	59 ($\pm$11)	67 ($\pm$14)

Five normal males ages 17–32 were studied. Blood samples were obtained on days 1, 2, 3, 7, 8, 14, 15, and 16. From day 2 (after the second blood sample had been obtained) to day 16 subjects received 4 tablets of Tedral/day (at 8 a.m., 12 noon, 4 p.m., and 8 p.m.). On days 7, 14, and 15 the 8 a.m. dose was delayed until after blood had been obtained. Data are given for Ficoll-Hypaque purified lymphocytes incubated in Gey's solution for 30 min at 37°.

catecholamine response unstimulated cells from asthmatic subjects had a decreased cyclic AMP content. The change in leukocyte adrenergic responsiveness could be correlated with the severity and chronicity of asthmatic symptoms. In association with severe, persistent asthma, leukocyte responsiveness was regularly decreased. By contrast, an entirely normal leukocyte response was obtained in many individuals with inactive or recently developed asthma. Even in severe chronic asthma the reduction in responsiveness was relative rather than absolute, a limited response being obtained at at least one catecholamine concentration.

The alteration in adrenergic responsiveness in asthma leukocytes is presumably on the same basis as the reduced response of asthmatic patients to catecholamines in vivo. The studies of Middleton, Finke, and Arce (6), Reed (24); Inoue (4), Kirkpatrick and Keller (3), and Lockey, Glennon, and Reed (5) have indicated that blood glucose and lactate responses to the intravenous or subcutaneous injection of epinephrine are frequently reduced in asthmatic patients. Szentivanyi has theorized that β-adrenergic blockage may be an important mechanism in the pathogenesis of human asthma (23). It is difficult to study the basis for altered catecholamine responsiveness in vivo because of the complexity of the response. Our group was the first to obtain evidence to indicate that peripheral blood leukocytes might be of value in the study of adrenergic responsiveness in this condition (9, 25). Our own subsequent, extensive observations as well as the independent studies of Coffey et al. (18) and Falliers et al. (26) now provide strong evidence that leukocytes from individuals with asthma have an abnormal cyclic AMP response validating the potential usefulness of these cells for further studies. A major advantage in using leukocytes is their ready availability. With a highly sensitive method for measuring cyclic AMP such as radioimmunoassay (16),

considerable information can be obtained on as little as 5–10 ml of blood, making serial measurements possible, even in children. While it could be argued that the catecholamine concentrations used to study leukocyte responsiveness are high, in the presence of theophylline a decrease in the asthma cell response was observable at 1 μM epinephrine and probably at 1μM isoproterenol which is the concentration of isoproterenol used by Coffey et al. in their studies (18). These concentrations may well approximate levels achieved intrabronchially following inhalation of potent bronchodilator preparations. It will now be important to determine whether in vitro alterations in the cyclic AMP response can be correlated with changes in adrenergic responsiveness in vivo.

In undertaking more extensive studies of cyclic AMP metabolism in cells from asthmatic donors it would be highly desirable to utilize as homogeneous a population of cells as possible. With this goal in mind purified cell populations were studied. On a per cell basis the cyclic AMP response to isoproterenol and epinephrine was much greater in lymphocytes than in polymorphonuclear leukocytes. When calculations based on the differential count were made it was concluded that the majority of the catecholamine response in mixed leukocyte populations could be attributed to the lymphocytic segment of the population. It was therefore not surprising to find that purified lymphocytes from asthmatic subjects exhibited the same reduction in responsiveness to isoproterenol that was seen in the unfractionated leukocytes. However, before concluding that the altered response in asthmatic subjects is a manifestation of β-adrenergic blockade, it was necessary to consider the possibility that a change in the proportion of T and B lymphocytes might be involved. This control took on added importance when parallel studies in this laboratory provided highly suggestive, although indirect evidence to indicate that immunoglobulin containing cells (presumably B cells) have

a considerably greater cyclic AMP response to isoproterenol than cells lacking immunoglobulin (presumably T cells). However, when the percentage of immunoglobulin containing cells was studied by the double fluorescent antibody technique, the difference between asthma and normal control cells was relatively small and seemingly insufficient to account for the striking reduction in isoproterenol responsiveness. Thus no evidence was obtained to indicate that quantitative alterations in lymphocyte subpopulations could explain the reduced catecholamine response.

Since lymphocytes from asthmatic individuals have a reduced β-adrenergic response and are obtainable in substantial quantities as a highly purified cell population, they would appear to be suitable for detailed metabolic studies. The use of Ficoll-Hypaque rather than nylon fiber purified cells seems indicated because the former are obtained in higher yield and are more responsive to isoproterenol. Another advantage of the Ficoll-Hypaque purification is that it removes erythrocytes which would be a source of contaminating enzymes and substrates. The major continuing problems are lymphocyte heterogeneity and the variability of the cyclic AMP response in normal control cells. Fortunately a quantitative evaluation of lymphocyte subpopulations is possible by means of immunofluorescence. Nonetheless, it would be preferable if an entirely homogeneous cell population could be used and further exploration of other readily accessible cell populations which might permit an evaluation of adrenergic responsiveness (particularly platelets) seems desirable.

From serial studies it is apparent that decreased catecholamine responsiveness is especially marked at the time of severe asthmatic symptoms. A possible relationship of reduced adrenergic responsiveness to catecholamine and methylxanthine therapy must be seriously considered since nearly all of the individuals under study were receiving or had previously received some form of therapy. Unfortunately final conclusions are not possible, but there is reason to suspect that therapy alone may not account for the abnormal leukocyte response: (a) Our in vitro data as well as that of most in vivo studies have indicated that the change in catecholamine responsiveness is correlated more closely with the severity of asthmatic symptoms than it is with catecholamine dosage per se (4). In addition, as a group, individuals who have not had recent drug therapy for asthma appear to have a modest reduction in their mean leukocyte cyclic AMP response to β-adrenergic drugs (Table I). (b) Serial lymphocyte studies in normal volunteers receiving oral bronchodilator therapy over a 15 day period have failed to reveal convincing changes in isoproterenol responsiveness (Table III). In another recent study Fireman and his colleagues reported that when normal subjects ingested 100–200 mg of ephedrine per day over a 7–30 day period there was no decrease in the blood glucose response to epinephrine (27). These two studies indicate that reduced responsiveness to catecholamines is not readily induced by oral catecholamine and methylxanthine therapy in moderate dosage. However, the possibility that changes might occur during more aggressive treatment with these agents is not yet excluded and more extensive studies are needed. (c) Recent studies in this laboratory with leukocytes from patients with atopic eczema (who have a strong genetic predisposition to asthma and allergic rhinitis but at the time of study had not had asthmatic symptoms or received catecholamine therapy) indicate a reduction in isoproterenol responsiveness of similar magnitude to that in individuals with active asthma (28). This finding is of considerable interest since patients with atopic eczema typically have increased serum IgE concentrations and many have bronchial hyper-reactivity to methacholine and histamine similar to that in patients with overt bronchial asthma (29, 30). In one study the blood glucose response to epinephrine was reduced in these individuals (27). Thus, there is evidence to support the view that some patients with bronchial asthma (or an atopic condition often associated with bronchial asthma) have a long standing alteration in catecholamine responsiveness which is not solely attributable to catecholamine therapy as such. However, there are other individuals who have an entirely normal leukocyte catecholamine response. This is more frequent during prolonged symptom free periods but can occur during active asthmatic symptoms. While it is possible that a subtle derangement of cyclic AMP metabolism is being overlooked in these individuals, the existing data must be interpreted as indicating that adrenergic responsiveness is not invariably altered in association with bronchial asthma.

While further studies are needed it is already apparent that leukocytes from asthmatic patients exhibit significant heterogeneity in their cyclic AMP response to catecholamines, prostaglandins, histamine, glucocorticoids and α- and β-blocking agents. Further elucidation of these differences may provide a better means of classification of asthma and could conceivably suggest new therapeutic approaches to this disease.

ACKNOWLEDGMENTS

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