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THE EFFECT OF THEOPHYLLINE ON THE PRODUCTION OF COLONY STIMULATING FACTOR (CSF) BY THE LUNG

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ABSTRACT

The effect of theophylline on colony stimulating factor production by the lung tissue was investigated. Addition of various concentrations of theophylline to the lung conditioned medium enhanced CSF production at 10 µg/ml and at higher concentrations showed an inhibitory effect. Determination of the phosphate content of the medium showed a decrease at 1-3 hours of incubation. Addition of AMP to the cultures in the presence of theophylline had no effect on the CSF production but compensated phosphate content of the lung cultures. Exogenous cAMP or its dibutyryl derivative also stimulated CSF production. On the other hand IBMX, another inhibitor of phosphodiesterases, exerted similar effect as theophylline. The results suggest that cAMP regulates CSF production by the lung.
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INTRODUCTION

Colony-stimulating-factors (CSFs) are a group of glycoprotein hormones necessary for the survival, proliferation and differentiation of hematopoietic progenitor cells.[1] Four different classes of murine CSFs have been identified that affect macrophage, granulocyte/macrophage, granulocyte and multipotential progenitors.[1-]
isolated and characterized in several tissues and cellular types, among which lung tissue has been reported as a very rich source of CSF[3,4] Although CSF proteins

have been studied in detail, the mechanism of their action and production remains to be elucidated.

Theophylline (1,3 dimethylxanthine) is one of the naturally occurring methylxanthines that is frequently used therapeutically in the treatment of respiratory difficulties such as controlling asthmatic manifestations, to alleviate neonatal apnoea and relieve bronchial spasms.[5,6]

Butcher and Sutherland have described the inhibition of cAMP phosphodiesterases by methylxanthines.[7] This inhibition has been further characterized by several authors [8] In addition the physiological role of methylxanthines has been extensively investigated in terms of their inhibitory action upon cAMP phosphodiesterases in various hormonal systems [9] Stimulation of neural activity in the central nervous system by theophylline has been reported.[10] Schooley and Mahlmann have demonstrated that intraperitoneal injection of theophylline potentiates the

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production of erythropoietin in rats.[11] On the other hand Butler and colleagues have reported the inhibitory effect of theophylline on the production of CSF by injecting into mice.[12]

In this paper we have used theophylline and IBMX to investigate the mechanism of CSF production by the lung tissue and explore the regulatory function of cAMP on this process.

MATERIALS AND METHODS

Animals:

Balb/c mice weighing about 40g of either sex and 20-25g were used for the lung conditioned medium and biological assay respectively.

Chemicals and reagents:

DMEM was obtained from GIBCO. It was subsequently supplemented with 30 mg/lit of asparagine and antibiotics (200 u/ml penicillin and 200mg/ml streptomycin)pH7.4. Fetal calf serum was prepared in our laboratory. Serum was heated for 30 min at 56°C and after sterilization by 0.45 μm millipore filters kept at −20°C until use. Theophylline was freshly dissolved in sterile distilled water as a stock solution (2.5 mg/ml) and then diluted to desired concentrations with DMEM befor use. cAMP, dibutyryl cAMP, AMP and IBMX (Sigma) were also freshly prepared in DMEM. Bovine serum albumine, vitamin C, ammonium molybdate and other chemicals were from Sigma.

Normal lung conditioned medium:

Mice were anesthetized by ether and the lungs removed from the chest cavity in sterile conditions. The lungs were washed with normal saline, minced and incubated in 5 ml of DMEM for 48 hours at 37°C in a fully humidified atmosphere of 5% CO_2 in the air. The supernatants obtained were heated at 56°C for 30 minutes, centrifuged at 2000g and dialysed against two changes of distilled water for 48 hours. To supernatants after centrifugation as above, polyethylene glycol was added at a final concentration of 1% before they were sterilized by filtration through 0.45 μm membrane filters.

Treated conditioned medium:

The experiment was essentially carried out as described for normal lung conditioned medium except that after lung tissue was minced in DMEM, theophylline at a concentration range of 0.1-250 µg/ml was added to the cultures and mixed thoroughly immediately before incubation. The volume of all samples was kept constant. cAMP, dibutyryl cAMP and IBMX were also added to the conditioned medium in separate experiments. The concentration range of 10^{-3}
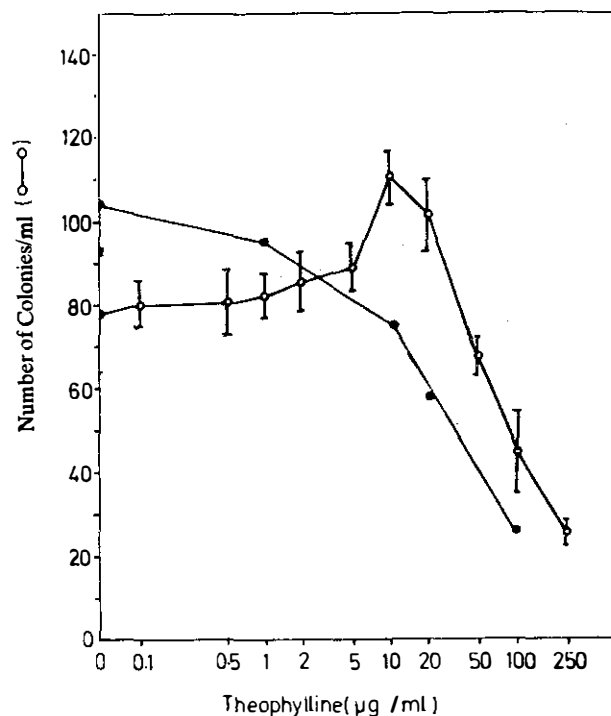

Fig.1. The effect of different concentrations of theophylline on CSF production (o——o) and the effect of CSF (0.1ml) plus theophylline on the colony formation of bone marrow cells (•——•). The values are mean ± SEM of at least 11 experiments, pair t-test was done (p< 0.05).
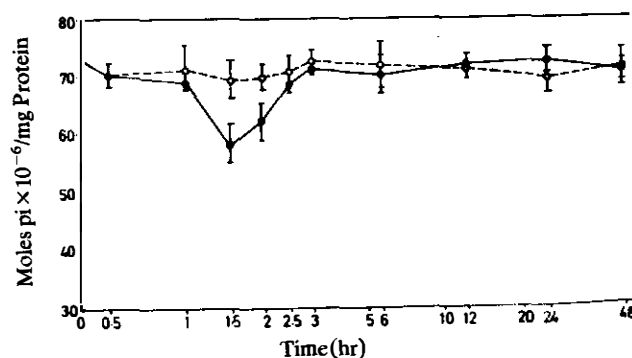

Fig.2. Phosphate content of the lung conditioned medium in the presence and absence of theophylline as a function of incubation time. o----o ; control, •——• ; plus theophylline (10µg/ml). Mean ± SD of five experiments.

10^{-8} M cAMP, 1-20 µM IBMX and 1 × 10^{-5} M and 2.25 × 10^{-5} M AMP were selected.

Biological assay:

Samples were assayed by semi-solid agar culture technique as described principally by Metcalf.[3] Bone marrow cells (10^5 cells) were plated into 35mm plastic petri-dishes (NUNC) containing 1ml of DMEM supplemented with 0.3% agar, 20% fetal calf serum and
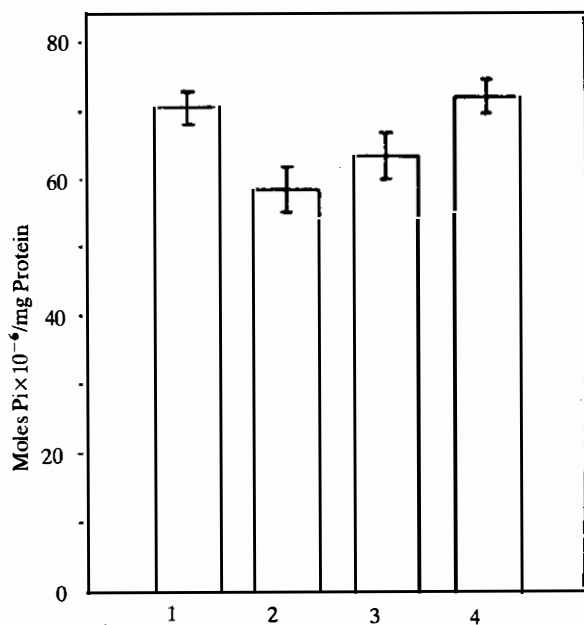
Fig.3. Combination of AMP in the experiment indicated in Fig.2. 1;control, 2;theophylline (10 μg/ml), 3;theophylline (10 μg/ml) plus AMP (1×10^{-5} M), 4; theophylline (10 μg/ml) plus AMP (2.5×10^{-5} M). Incubation time was 1.5 hours. Mean ±SD of six experiments.
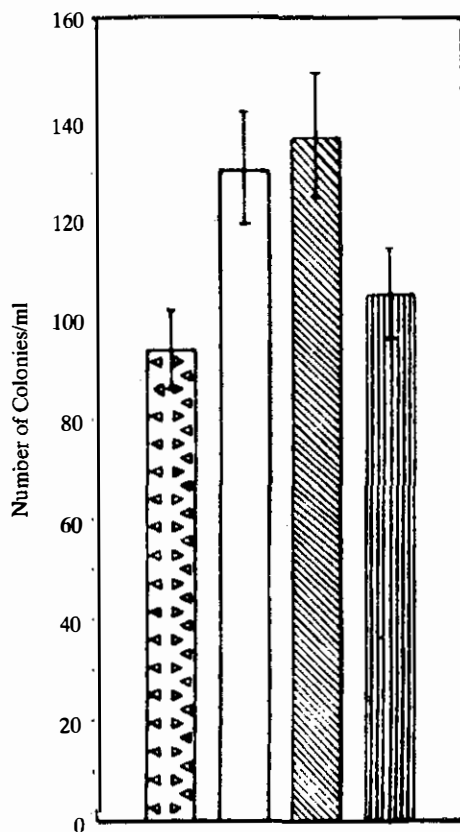
Fig.4. The effect of AMP on CSF production. ⬛; control, □; theophylline (10 μg/ml), ▧; AMP (2.5×10^{-5} M) plus theophylline (10 μg/ml), ▥; AMP (2.5×10^{-5} M). Mean ±SD of six experiments.

0.1 ml of conditioned medium. The plates were incubated as above for seven days. Colonies with greater than 50 cells were scored using a dissecting microscope.

Protein assay:

The amount of proteins were measured by Lowry method modified by Hartree.[13] Bovine serum albumin was used as a standard.

Theophylline content determination:

Conditioned media after extensive dialysis were analysed by radioimmunoassay and HPLC methods. Theophylline at 10,50 and 100 μg/ml was used as a standard.

Phosphate determination:

The amount of phosphate in the culture medium was determined using Hosono method.[14] Lung conditioned media were prepared as above in the presence or absence of theophylline (10 μg/ml). At different time intervals of incubation, the lung particles in each sample were homogenized in 0.1 M tris-HCl pH 7.5 and 0.1 ml of 55% trichloroacetic acid was added. The samples were centrifuged for 15 min at 2000g (4°C) and the clear supernatants were used for phosphate assay. To each sample, and equal volume of solution A (1% vitamin C: H$_2$O: 6N H$_2$SO$_4$: Ammonium molybdate 1:2:1:1 v/v) was added and incubated for 30 min at 37°C. The absorbance was measured at 720 nm. Phosphate monosodic was used as a standard.

cAMP measurements:

Samples at desired time of incubation were homogenized at 8000rpm for 2 min and an equal volume of 50% trichloroacetic acid was added. After centrifugation at 2000g for 10 min, to 50 μl of each 3 μl of 0.3N ZnSO$_4$, 3 μl of 0.3N barium hydroxide added and incubated for 10 min at room temperature. The samples were centrifuged as above and the supernatants were lyophilized, redissolved in D.H$_2$O and plated onto TLC plates. CAMP at known concentrations was used as a standard. TLC solvent was isopropanol: H$_2$O: N NaOH (1.5:1.5:7). The plates were scanned at 260 nm by Shimadzu TLC scanner and the amount of cyclic nucleotide determined by calculating the area under the peaks and molar concentration of standard.

RESULTS

Varying concentrations of theophylline (0.1-250 μg/ml) were added to the mouse lung conditioned medium at the start of incubation and after 48 hours the excess of theophylline removed by extensive dialysis. Fig. 1 illustrates the results obtained from the biological assay of conditioned media on Balb/c bone marrow

151

Table I. Amount of cAMP in the conditioned media in the presence and absence of theophylline (10μg/ml)

Incubation time (hours)	[M]cAMP×10⁻⁶	
	Normal	+theophylline
0	1.18	1.06
1.5	1.45	2.94
3	1.63	1.48

culture. As it is shown theophylline influenced CSF production in a dose dependent manner. It had no detectable effect up to a concentration of 1 μg/ml but the number of colonies increase gradually and reached a maximum at 10 μg/ml. Higher concentrations of theophylline progressively diminished CSFlevels, thus giving a few colonies at 250 μg/ml possibly through its toxicity effect. It is desirable to note that the enhancing effect was not due to the effect of theophylline on bone marrow cells. On one hand the amount of theophylline remained in the conditioned medium after dialysis was measured by radioimmunoassay and HPLC. In both cases no theophylline was detected in the conditioned medium before biological assay. On the other hand addition of theophylline at different concentrations to the bone marrow cultures in the presence and absence of 0.1 ml conditioned medium (Fig.1) showed an inhibitory effect, even at 10 μg/ml.

Theophylline is a potent inhibitor of cAMP phosphodiesterases.[9,15] In this process cAMP degradation is inhibited followed by a decrease in the AMP or inorganic phosphate content of the medium. Therefore it was proposed that the enhancing effect of theophylline might have occurred as a regulatory function of the end products of cAMP itself. Determination of the phosphate content of the lung conditioned medium at different time intervals (0-48 hours) in the presence (10 μg/ml) or absence of theophylline is given in Fig.2. A reduction is observed at 1-33 hours of incubation and after that reached normal level and remained constant until 48 hours. Designing the experiment in combination with AMP is shown in Fig.3. It is apparent that AMP compensated phosphate content and brought it to normal level at 2.5×10⁻

this process was further investigated by addition of AMP to the lung cultures. The result is presented in Fig.4. It is obvious that AMP had no considerable effect on the CSF production either on normal lung conditioned medium or treated with theophylline (10 μg/ml), indicating that most probably the enhancing effect did not correspond to the reduction of AMP concentration in the medium.

In order to confirm the second possibility (cAMP accumulation), the following experiments were conducted. Addition of cAMP or its dibutyryl derivative to the lung conditioned medium was carried out at a
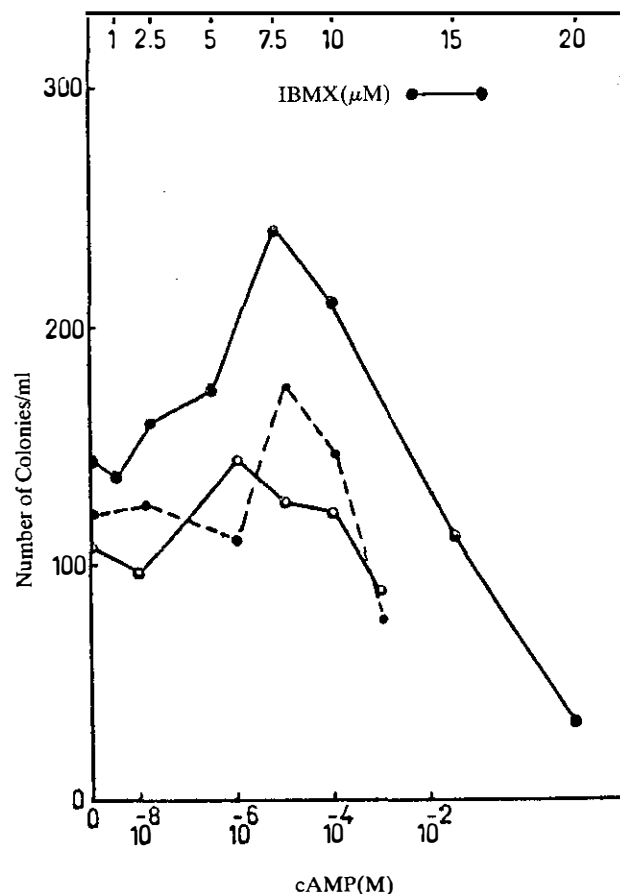
Fig 5: The effect of various concentrations of cAMP, dibutyryl cAMP and IBMX separately on the production of CSF by the lung. Mean ±SD of eight experiments. o——o ; cAMP, ●----● ; dibutyryl cAMP.

concentration range of 10⁻
in Fig.5 and shows that cAMP and, at higher levels, its derivative, stimulated CSF production in a dose dependent manner as judged by increasing the number of colonies produced in the semi-solid agar cultures. Preliminary experiments were also performed to measure the content of cAMP directly in the medium (Table I). It is shown that an increase in the cAMP content at 1.5 hours of incubation is obviously in comparison to normal samples. In addition, when cAMP or dibutyryl cAMP and theophylline were added together and simultaneously to the lung tissue cultures at the start of incubation, the number of colonies was reduced to lower than normal (from 180 colonies per ml to 65 colonies).

On the other hand experiments were carried out in the presence of other methylxanthine such as IBMX. As shown in Fig.5 similar results were observed when lung tissue was incubated with various concentrations of IBMX (1-20 μM). Stimulatory effect of IBMX on CSF production reached a maximum at a concentration of 7.5 M.

DISCUSSION

The purpose of this study was to monitor the effect of methy lxanthines on-the granulocyte-macrophage colony stimulating factor production by the lung tissue. Clearly the apparent role of CSF in the control of myelopoiesis has added great impetus to the study of this protein. From the results obtained above it is concluded that methylxanthines such as theophylline and IBMX, potent inhibitors of cAMP phosphodiesterases,[9,16] significantly increased CSF production. In both cases the enhancing effect is dose dependent. Toxicity of theophylline at high concentration has already been published.[17]

cAMP phosphodiesterases catalyse the hydrolysis of cAMP to corresponding adenosine 5'monophosphate.[18] Therefore the results arouse two distinct questions: 1) whether this enhancement is due to AMP reduction in the conditioned medium, or 2) corresponds to the accumulation of cAMP as a function of theophylline. Enhancement was also accompanied by a reduction in the inorganic phosphate content of the medium. If the AMP reduction is responsible, then the addition of exogenous AMP might reverse the effect. In this study although AMP compensated phosphate content of the medium, it did not show any detectable effect on CSF production by the lung even in the presence of theophylline. It is suggested that the reduction of AMP does not play an important role on CSF production. This together with the stimulatory effect of cAMP or its dibutyryl derivative on CSF production confirm the second possibility that cAMP regulates CSF production in the lung conditioned medium. Also preliminay results obtained indicated that the level of cAMP increase at desired time of incubation.

It is also desirable to note that the observed enhancing effect is not due to the stimulatory effect of these compounds on the bone marrow stem cells in the agar cultures. Since they are small molecules capable of passing through dialysis tubing pores (cut off 10000D), our measurements indicated that no theophylline is being left in the medium after dialysis to be able to stimulate colony formation. On the other hand addition of theophylline to the bone marrow cultures had an inhibitory effect.

The regulation of CSF-induced myelopoiesis at the level of stem cell proliferation has shown that several factors are known to inhibit CSF-induced stem cell proliferation, among which prostaglandin E and cAMP have extensively been studied.[19-21] The inhibitory effect of theophylline and cAMP on murine erythroleukemia cell differentiation has been reported.[22]

The mechanism regulating CSF production, however is currently less clear but several lines of circumstantial evidence suggest CSF mediation.

Schooley and Mahlmann[11] have studied the effect of theophylline on the action and production of erythropoietin in the intact animal and shown that theophylline only increases the hormone production when given after hypoxic exposure. They have discussed a direct action of cAMP on this process. However opposite results have been reported by others. Butler, et al. have shown that injection of theophylline into mice lowers the production of CSF.[12] On the other hand, Moore, et al[23] using indomethacin have discussed the prostaglandins and cAMP regulation of CSF production by IPS-stimulated murine leukocytes. Our results, although in contrast to the latter two findings, is in agreement with the observations of Schooley and Mahlmann which indicate that cAMP stimulates CSF production. This increase in the production of CSF may be a direct action of cAMP, but the possibility that the nucleotide stimulates other physiological processes which indirectly influence the production of CSF can not be excluded.

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