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The Inhibition of the Mixed Lymphocyte Culture Reaction by Cyclic Adenosine 3′,5′-Monophosphate[1] (40253)

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Various aspects of humoral and cellular immune responses appear to be modulated by cyclic adenosine 3′,5′-monophosphate (cAMP). These include the antigen-induced, IgE mediated basophil histamine release (1), lymphocyte-mediated cytolysis (2), human T-lymphocyte E-rosette function (3), hemolytic plaque formation (4, 5), complement-mediated cytolysis (6) and lymphocyte mitogenesis (7, 8). In view of the similarity of lymphocyte mitogenesis to the *in vitro* mixed leucocyte culture (MLC) reaction it was anticipated that cyclic AMP would also play an important role in the latter process. This reaction is associated with antigen recognition in homograft rejection and its dysfunction has been related to a variety of human diseases including Hodgkin's disease, sarcoidosis and systemic Lupus erythematosus (9). In the case of Hodgkin's disease particularly a deficiency of T-lymphocyte activity is evident (10). We have, therefore, in the present study sought to ascertain a modulating role for cyclic AMP in the response of human T-lymphocytes to antigenic stimulation in the MLC reaction. This has been done with agents that elevate the level of cyclic AMP in lymphocytes (11). Their effect has been evaluated by the measurement of thymidine incorporation in DNA and the stimulation of protein synthesis which occurs relatively early in the MLC reaction.

Materials and methods. Dibutyryl cyclic AMP (Bt_2-cAMP) and theophylline were purchased from Sigma Chemical Company. Prostaglandin E_1 (PGE_1) was a gift from Dr. Pike of Upjohn Company. ^{3}H-Thymidine (specific activity 6.7 Ci/mmole) and ^{3}H-leucine (specific activity 40–60 Ci/mmole) were from New England Nuclear.

Lymphocytes from peripheral blood were prepared according to the method described by Böyum (12). Heparinized blood from normal individuals free of any medication was diluted 1:1 with Hanks balanced salt solution (HBSS). Diluted blood (20–30 ml) was placed in a 50-ml conical glass bottom centrifuge tube. Ten milliliters of Ficoll Hypaque (Pharmacia) solution was added slowly to the bottom of the tube through the cell suspension. The tubes were spun for 35–40 min at 20° at a speed of 1400 rpm to give 400*g* at the interface. The cells at the interface were transferred to sterile plastic centrifuge tubes and washed three times with HBSS. To study the [^{3}H]thymidine incorporation in the 'one-way' MLC reaction, cells from one individual were suspended in medium 199 containing 5% human AB serum (heat inactivated at 56° for 30 min) supplemented with fresh glutamine (2 m*M*), streptomycin (100 μg/ml) and penicillin (100 units/ml) all obtained from Grand Island Biologicals, Grand Island, NY. Mitomycin c at a final concentration of 25 μg/ml was added to the cell suspension and the mixture was incubated at 37°. The cell pellet, after centrifugation, was washed three times with HBSS. The mitomycin c-treated cells (stimulating cells) and the cells from the other individual not treated with mitomycin (responding cells) were finally suspended in complete medium 199 buffered with 40 m*M* Na-Hepes and containing 20% human AB serum, fresh glutamine (2 m*M*), streptomycin (100 μg/ml) and penicillin (100 units/ml). Responding cells (2×10^5) and stimulating cells (4×10^5) were taken in 0.2 ml vol in a microtiter plate and incubated at 37° in a humidified atmosphere for a period of 1 to 6 days depending on the experiment, followed by the addition of 0.5 μCi of [^{3}H]thymidine 18–20 hr before terminating the culture. The cells were transferred and washed on a glass filter using a mass cell harvester. This was dried and counted in a liquid scintillation counter using 10 ml of Scintisol Complete

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(Isolab, Akron, OH).

For the measurement of the stimulation of protein synthesis (SPS), the cells (10^6/ml) were suspended in Earle's MEM without leucine (prepared from Amino Acid Kit # M11-9050, GIBCO) supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), nonessential amino acids (1 mM) and 5% human AB serum, the medium being buffered with 40 mM Na Hepes. The cell suspensions (0.5 ml) from each of two donors (X and Y) were mixed in triplicate and control cultures contained 1 ml of the individual cells in duplicate. The tubes were incubated at 37° in a humidified atmosphere for 20–45 hr and 2 hr before terminating the culture 4 μCi of [^{3}H]leucine were added to the culture. The final concentration of leucine in the medium was 0.001 μmole/ml. After the 2-hr pulse 5 ml of cold 5% TCA were added to each tube. Following an incubation in the cold (4–5°) for about half an hour, the TCA insoluble precipitate was centrifuged. The pellet was washed four times with 5% cold TCA. The washed pellet was dissolved in Protosol (New England Nuclear), transferred to a scintillation vial, mixed with 10 ml of formula 949 (New England Nuclear) and counted in a liquid scintillation counter.

The percentage of stimulation of protein synthesis (% SPS) was calculated according to Adkinson *et al.* (13) as follows:

$$\% \text{ SPS} = \left(\frac{\text{mean cpm of stimulated culture}}{\text{mean cpm of control cultured}} - 1\right) \times 100$$

Results. The inhibitory effect of PGE_1, theophylline and Bt_2-cAMP on the [^{3}H]thymidine incorporation in MLC is shown in Table I. Except for two different concentrations of PGE_1, the degree of inhibition was directly related to the dose of the agents employed. At the concentrations of the agents studied, the cellular cyclic AMP level has been reported to be increased (7, 11) without adversely affecting the cell viability as assessed by trypan blue exclusion.

The potentiating effect of theophylline on the inhibition of [^{3}H]thymidine incorporation by PGE_1 and Bt_2-cAMP is shown in Table II. An approximately additive effect is seen when PGE_1 and theophylline or Bt_2-cAMP and theophylline are added together. On the other hand when PGE_1 and Bt_2-cAMP were used together no significant increase of inhibition was observed. No enhancement of inhibition of Bt_2-cAMP was observed when a noninhibiting dose (10^{-6} M) of theophylline was added (data not shown).

TABLE I. INHIBITION OF [^{3}H]THYMIDINE INCORPORATION IN MLC BY PGE_1, THEOPHYLLINE AND DIBUTYRYL CYCLIC AMP.[a]

Inhibitor	Concentration in molarity	[^{3}H]thymidine incorporation (cpm)	Percent inhibition
None	—	12,164 ± 211	
Prostaglandin E_1	10^{-7}	8,398 ± 63	31
	10^{-6}	8,199 ± 61	32.5
Theophylline	10^{-4}	11,420 ± 838	6
	5×10^{-4}	6,845 ± 358	43
	10^{-3}	3,764 ± 267	69
Dibutyryl cAMP	5×10^{-5}	9,448 ± 1,461	22
	5×10^{-4}	3,757 ± 1,595	69

[a] Responding (2×10^5) and stimulating cells (4×10^5) were mixed in a total volume of 0.2 ml complete medium (see text). Test agents were added in a volume of 20 μl HBSS at a final concentration shown in the table, the same volume of HBSS being added to the control culture. Results presented an average of triplicate runs ± SEM.

The kinetics of [^{3}H]thymidine incorporation in MLC in the presence or absence of theophylline and Bt_2-cAMP are shown in Fig. 1. The agents were present at the initiation of the cultures and, at the times indicated in the figure, [^{3}H]thymidine was added, *i.e.*, at 24, 48, 72 and 96 hr. The cultures were terminated 20 hr after this addition. Inhibition of thymidine incorporation in the presence of theophylline and Bt_2-cAMP is apparent as early as 24 hr. The percent inhibition was found to be higher in the early portion of the curve. The absolute degree of inhibition of [^{3}H]thymidine incorporation observed with a given agent varied among donors. Thus, for five different combinations of donors in MLC, the percent inhibition of [^{3}H]thymidine incorporation was found to vary from 39% to 69% with 5×10^{-5} M Bt_2-cAMP and from 30% to 52% with 2×10^{-4} M theophylline.

Figure 2 demonstrates the effect of delayed addition of the agents on the suppression of [^{3}H]thymidine incorporation in MLC. Maximum inhibition was observed when the agents were added at 0 hr and it gradually decreased when added at 24, 48 and 72 hr. There was no inhibition when the agents were

TABLE II. POTENTIATION OF INHIBITION OF [^{3}H]THYMIDINE INCORPORATION IN MLC BY PGE$_1$ AND Bt$_2$-cAMP IN THE PRESENCE OF THEOPHYLLINE.[a]

Agent	Concentration	[^{3}H]thymidine incorporation (cpm)	Percent inhibition
None		10,862 ± 606	
Bt$_2$-cAMP	10^{-5} *M*	6,344 ± 1,083	41.6
	10^{-4} *M*	2,580 ± 209	76
Theophylline	2×10^{-4} *M*	5,462 ± 492	49.7
PGE$_1$	10^{-6} *M*	7,895 ± 670	27.3
Bt$_2$-cAMP + theophylline	10^{-4} *M* 2×10^{-4} *M*	870 ± 197	92
Theophylline + PGE$_1$	2×10^{-4} *M* 10^{-6} *M*	3,483 ± 287	67.9
Bt$_2$-cAMP + PGE$_1$	10^{-5} *M* 10^{-6} *M*	5,447 ± 582	49.8

[a] Responding cells (2×10^5) and stimulating cells (4×10^5) were mixed in a total volume of 0.2 ml, the agents being added at the initiation of the culture in a volume of 20 μl HBSS at a final concentration shown in the table, the same volume of HBSS being added in the control culture. 0.5 μCi of [^{3}H]thymidine added to each culture at 96 hr and the culture was terminated 20 hr later (see text). Results presented are the average of six replicates ± SEM.

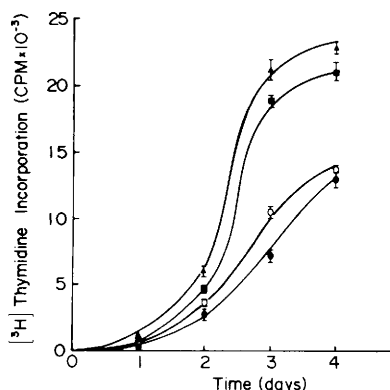

FIG. 1. Kinetics [^{3}H]thymidine incorporation in MLC in the presence of theophylline and Bt$_2$-cAMP. Responding cells (2×10^5) and stimulating cells (4×10^5) were mixed in 0.2 ml of complete medium and incubated at 37°C (for details see text). The agents were added at the time of initiation of the cultures. [^{3}H]Thymidine (0.5 μCi) was added at the time indicated in the figure, the cultures being terminated 20 hr after addition of [^{3}H]-thymidine. Control ▲——▲; theophylline (10^{-4} *M*) ■——■; Bt$_2$-cAMP (10^{-4} *M*) ●——● and Bt$_2$-cAMP (5×10^{-5} *M*) ○——○. Results represent mean of three experiments ± SEM.

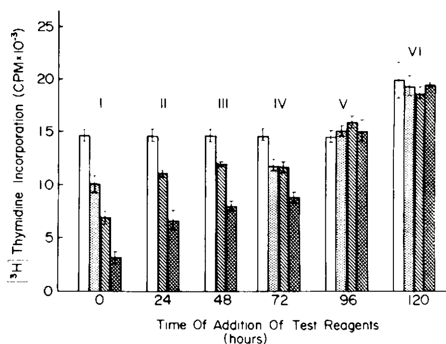

FIG. 2. Effects of adding PGE$_1$, theophylline or Bt$_2$-cAMP at different periods of time during incubation on [^{3}H]thymidine incorporation in MLC. Responding cells (2×10^5) and stimulating cells (4×10^5) were mixed in 0.2 ml of complete medium and incubated at 37° in a humidified atmosphere. Test agents were added at the times indicated. In experiments I through V, [^{3}H]thymidine (0.5 μCi) was added at 96 hr and the cultures were terminated 20 hr later. In experiment VI [^{3}H]thymidine (0.5 μCi) was added at 120 hr and the cultures were terminated 20 hr later. PGE$_1$ (10^{-6} *M*) ▧; theophylline (2×10^{-4} *M*) ▨, Bt$_2$-cAMP (10^{-4} *M*) ▩ and control □. Results presented are the average of three experiments ± SEM.

added at 96 hr even though the addition preceded the period of peak [^{3}H]thymidine incorporation. Viability of the cells in the presence of the agents was found to be within ±2–3% of the control culture.

The stimulation of protein synthesis in the absence and presence of Bt$_2$-cAMP (5×10^{-5} *M*) is presented in Table III. Although the addition of Bt$_2$-cAMP to individual cultures produced variable effects, the % SPS in MLC

TABLE III. EFFECT OF DIBUTYRYL CYCLIC AMP ON THE STIMULATION OF PROTEIN SYNTHESIS IN MLC.[a]

	[^{3}H]leucine incorporation in absence of Bt_2-cAMP				[^{3}H]leucine incorporation in presence of 0.05 m*M* Bt_2-cAMP			
Expt. number	10^6 cell (X) (cpm)	10^6 cell (Y) (cpm)	0.5×10^6 cell (X) + 0.5×10^6 cell (Y) (cpm)	%SPS	10^6 cell (X) (cpm)	10^6 cell (Y) (cpm)	0.5×10^6 cell (X) + 0.5×10^6 cell (Y) (cpm)	%SPS
1	14,331 ± 238	12,604 ± 478	17,392 ± 983	29	12, 194 ± 730	8,170 ± 450	10,620 ± 729	4
2	12,289 ± 204	10,983 ± 420	15,214 ± 544	32	10,503 ± 242	13,311 ± 739	12,807 ± 528	8
3	12,842 ± 1,032	12,638 ± 1,125	16,464 ± 1,009	24.3	14,209 ± 527	13,263 ± 739	15,295 ± 1,251	7.6

[a] For each of the three representative experiments the donors X and Y were different. Total incubation period for experiments 1, 2 and 3 was 45 hr, 22 hr and 30 hr respectively. Bt_2-cAMP in each experiment was added at the time of initiation of the culture and [^{3}H]leucine was added 2 hr before termination of the culture.

reaction was always low in the presence of the agent when compared to control.

Discussion. The main finding of this study is the inhibition of thymidine incorporation into DNA in the MLC reaction by the elevation of intracellular levels of cAMP. This elevation was effected by PGE_1, a stimulator of adenylate cyclase, by theophylline, an inhibitor of phosphodiesterase, and by Bt_2-cAMP. Measurement of the intracellular level of cAMP by the method of Sinha and Colman (23) revealed a twofold increase with 10^{-3} *M* theophylline and a two- to threefold increase with 10^{-6} *M* PGE_1 over the control (112 ± 2 pmoles/10^8 cells). The additive responses of theophylline with Bt_2-cAMP and with PGE_1 point also to the key role of the level of intracellular cAMP. Our results are in accord with the observations of DeRubertis *et al.* (7) for mitogenesis, Chisari and Edgington (3) for E-rosette formation and Henney *et al.* (2) for lymphocyte-mediated cytolysis.

Since the assay used in this study does not directly measure thymidine incorporation into DNA, the inhibition of its uptake in the stimulated cells in the presence of the agents under study, relative to the untreated control, may be attributed to the inhibition of other processes associated with cellular uptake. However, there appears to be no evidence to date that other modes of thymidine accumulation are affected by cAMP. Furthermore, toxic effects by these agents at the concentrations used have been ruled out by previous observations (7, 11, 14). In the case of [^{3}H]-thymidine incorporation associated with PHA stimulation, the reversibility of its inhibition by PGE_1 and theophylline has been demonstrated (7).

The inhibition by elevation of the intracellular level of cAMP is time-dependent with respect to the interval between the initiation of stimulation of the responding cells and the exposure of the culture to agents causing the elevation (Fig. 2). The resistance to inhibition of responding cells which have been stimulated for 96 hr prior to the addition of these agents indicates that a cellular process required for subsequent thymidine incorporation has been completed for the entire cell population within the 96-hr period. Furthermore, the inhibition of the early stimulation of protein synthesis by Bt_2-cAMP (Table III) suggests that the intracellular cAMP level modulates cellular activity throughout the entire process of the MLC reaction.

The relevant inhibition of protein synthesis may not be restricted to the responding cells in the MLC reaction. We have observed (data not given) as has been previously reported (13, 15) that the stimulation of protein synthesis and thymidine incorporation in the responder cells are strongly inhibited if the stimulating cell population is treated with inhibitors of protein synthesis such as emetine or streptovitacin. The requirement for continued protein synthesis in the stimulating cells during the MLC reaction is also suggested by the observations that heat-killed lymphocytes with unmodified HL-A antigens are inactive in MLC (16, 17) and that lymphocytes killed by uv irradiation are also ineffective (18). Thus, the increased intracellular level of cAMP in the stimulating cells may be inhibiting required protein synthesis.

The profound influence on lymphocyte function of intracellular cAMP points to the possibility that the depressed cellular immunity observed in patients with Hodgkin's disease, sarcoidosis and rheumatoid arthritis (9) may be related to a disorder in the regulation of its concentration and distribution.

Summary. Prostaglandin E_1, theophylline and dibutyryl cyclic AMP uniformly suppressed the [^{3}H]thymidine incorporation in human mixed lymphocyte culture in a dose

dependent manner. The inhibitory effects were time dependent, with the maximum when the agents were added at the time of the initiation of the culture, decreasing gradually when added at 24 hr, 48 hr and 72 hr and becoming undetectable when added at 96 hr or later. When prostaglandin E_1 and theophylline or dibutyryl cyclic AMP and theophylline were added together an additive effect was found. Dibutyryl cyclic AMP also inhibited the stimulation of protein synthesis in mixed lymphocyte culture. It is concluded that the T-cell response in the MLC reaction is modulated by the intracellular level of cyclic AMP as observed with a variety of other immune reactivities.

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