Vol. 113, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 15, 1983 Pages 425-432

5'-DEOXY-5'-METHYLTHIOADENOSINE INHIBITION OF RAT T LYMPHOCYTE PHOSPHODIESTERASE : CORRELATION WITH INHIBITION OF Con A INDUCED PROLIFERATION

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Received April 27, 1983

5'-Deoxy-5'-methylthioadenosine inhibits Concanavalin A induced rat T lymphocyte proliferation in a dose dependent manner (50 µM to 1000 µM). The extent of inhibition by MTA of lymphocyte proliferation was greatest when MTA was added to the cells at the same time as Concanavalin A. The determination of cyclic AMP level from 30 min to the 6th hour shows that 5'-Deoxy-5'-methylthioadenosine inhibition is correlated with an elevation of cyclic AMP at this mitogen recognition phase. 5'-Deoxy-5'-methylthioadenosine concentrations that inhibit rat T lymphocyte proliferation also inhibit phosphodiesterase activity. This biochemical mechanism could be specific to 5'-Deoxy-5'-methylthioadenosine inhibition since in an another model of inhibition of rat T lymphocyte proliferation (2'-Deoxyadenosine 10 µM, in adenosine deaminase deficiency conditions : 2'-Deoxycoformycin 10 µM), no significant modification of cyclic AMP level can be demonstrated.

Lymphocyte proliferation and/or function appear to be particularly sensitive to inhibition by purine nucleosides. This is exemplified by inherited deficiencies of two enzymes of purine nucleoside metabolism, adenosine deaminase and purine nucleoside phosphorylase, which produce an immunodeficiency in humans (1, 2). A purine nucleoside, 5'-Deoxy-5'-methylthioadenosine (MTA), a by-product of polyamine synthesis, has been recently reported to produce a non toxic and reversible inhibition of mitogen stimulated blastogenesis of human peripheral lymphocytes (3) and of murine lymphoid cell proliferation (4). But the mechanism of this inhibition has not been elucidated. Recently however, Wolberg et al. have shown that MTA inhibits lymphocyte mediated cytolysis and that this inhibition can be correlated with an increase of cyclic AMP (5). Since cyclic AMP has been extensively implicated in the regulation of cell growth and differentiation in many cell types and particularly in mitogen stimulated lymphocytes (6, 7), we tried to determine whether

Abbreviations : MTA : 5'-Deoxy-5'-methylthioadenosine ; Con A : Concanavalin A ; dAdo : 2'-Deoxyadenosine ; dCf : 2'-Deoxycoformycin ; dThd : thymidine ; CFA : Bacto Complete Freund's Adjuvant ; cyclic AMP : adenosine 3',5' cyclic monophosphoric acid.

0006-291X/83 $1.50

MTA inhibition of lymphocyte proliferation could also be due to an effect on cyclic AMP metabolism. To test this hypothesis we studied the MTA effect on the proliferation of rat T lymphocytes and on the cyclic AMP level in these cells. We also compared MTA inhibition with that produced by dAdo, in adenosine deaminase deficiency conditions, a well known model of inhibition of T lymphocyte proliferation (8).

MATERIALS AND METHODS

Materials. Chemicals used in the present study included MTA, Con A grade IV, cyclic AMP, adenosine, Crotalus adamanteus Snake Venom (Sigma Chemical Co, St Louis, MO) ; dAdo (Boehringer, Mannheim RFA) ; dCf (a gift from Warner Lambert-Parke Davis, Ann Arbor, MI) ; Dulbecco's minimum essential medium (MEM Gibco, Grand Island, NY) ; fetal calf serum (Flow Labs, Irvine, Scotland), CFA (Difco Laboratories, Detroit, MI).

[6-^{3}H] dThd (1 Ci/mmol), [^{14}C-methyl] S-adenosyl-L-methionine (51 mCi/mmol) and [8-^{3}H] cyclic AMP (30 Ci/mmol) were purchased from the Commissariat à l'Energie Atomique (Saclay, France). [8-^{3}H] cyclic AMP was purified by thin layer chromatography on cellulose, developped with 2-propanol-ammonia-water (7/1/2, v/v). Radioimmunoassay for cyclic AMP determinations was from the Radiochemical Center (Amersham, England).

Methods

- Preparation and culture of rat T lymphocytes. Rat T lymphocytes from CFA activated lymph nodes were prepared as previously described (8, 9). The cell suspensions with or without Con A (5 µg/ml) were maintained in macroculture (1 x 10^6 cells) in Dulbecco's minimum essential medium supplemented with 10 % fetal calf serum, in Nunclon flasks (Nunc, Roskilde, Denmark) for 24 h, 48 h or 72 h at 37°C in 5 % CO_2 in humidified air, and the appropriate concentrations of MTA were added at culture initiation or at different states of activation of the cells.

- [^{3}H] dThd uptake. After the designated incubation time, stimulated and unstimulated cultures were pulsed for 17 h with [^{3}H] dThd (1 µCi/well) in 96-well microtiter plates (3040 Falcon Labware Oxnard CA)(2 x 10^5 cells/well). [^{3}H] dThd incorporation in DNA was then quantified according to the technique of Mans and Novelli (10). All assays were done in triplicate. In macrocultures, the cell number was evaluated after three days of culture by optic microscopy.

- MTA phosphorylase assays. Lymphocytes were centrifuged at 145 g for 10 minutes, washed and resuspended in 0.05 M potasium phosphate buffer (pH 7.2) containing 4 mM dithiothreitol. The cells were freeze-thawed, and sonicated. [^{14}C-methyl] -MTA was prepared by the procedure of Schlenk et al. (11). MTA phosphorylase activity was determined by measuring the conversion of [^{14}C-methyl] -MTA to [^{14}C-methyl] -methylthioribose as described by Ferro et al (12). The standard reaction mixture contained 50 mM sodium Hepes, (pH 7.5), 5 mM potassium phosphate (pH 7.5) 0.130 mM [^{14}C-methyl] -MTA (30 000 cpm) and about 0.6 mg protein in a total volume of 0.25 ml. After a 30 min incubation at 37°C, the reaction was stopped by the addition of 0.05 ml of 1.8 M trichloracetic acid. The resulting precipitate was removed by centrifugation and a 0.25 ml aliquot of the supernatant fluid was applied to a Dowex 50 H^+ X4 (100-200 mesh) column (2 x 10 cm) equilibrated with 0.2 N trichloracetic acid. [^{14}C-methyl] -methylthioribose was eluted with 2 ml H_2O directly into scintillation vials. Under these chromatographic conditions, [^{14}C-methyl] -MTA was quantitatively retained on the column. Radioactivity was counted in 0.4 % PPO in toluene/Triton X-100 (2:1 v/v) with a Packard Tricarb 300 scintillation spectrometer.

- Cyclic AMP determinations. Aliquots of ten million lymphocytes from cultures run in triplicate were collected, cooled rapidly to 4°C, in NaCl 9 ‰ , EDTA 4 mM (pH 7.2), centrifuged at 145 g for 10 min. The supernatant

was immediately removed and the pellet was resuspended in 0.3 ml Tris-HCl buffer 50 mM (pH 7.5), EDTA 4 mM, and heated at 100°C for 10 min. After centrifugation, the supernatant was frozen and lyophilized. Cyclic AMP content was measured by using a radioimmunoassay kit.

- Cyclic AMP phosphodiesterase assay. Rat T lymphocytes were disrupted for enzyme assays as described above for MTA phosphorylase except that the cells were resuspended in 50 mM Tris-HCl buffer (pH 7.5). Cyclic AMP phosphodiesterase was assayed by the two-step procedure of Thompson et al (13) with the following modifications : the assay mixture contained 50 mM, Tris-HCl buffer pH 7.5, 2 mM $MgSO_4$, the cell extract (0.4×10^6 cells), MTA at various concentrations and [8-^{3}H] cyclic AMP 10 µM (50 000 cpm) in a final volume of 0.2 ml. After various incubation times, the reaction was stopped by heating at 96°C for 2 min. The tubes were cooled to 37°C and 0.01 ml of Crotalus adamanteus snake venom (10 mg/ml) (devoid of cyclic AMP phosphodiesterase activity) was added as a source of 5'-nucleotidase.(This activity was not affected by MTA 500 µM). After another 15 min incubation at 37°C, the reaction was terminated by heating at 96°C for 2 min. Nucleosides (adenosine and inosine), nucleotides (AMP, IMP) and cyclic AMP were separated by descending chromatography on DEAE 81 paper in Paladini-Leloir solvant mixture (ammonium acetate 1 M : ethanol, 30/75 v/v). Cyclic AMP phosphodiesterase activity was calculated from the percentage of radioactivity appearing in adenosine plus inosine.

RESULTS

- MTA effect on lymphocyte proliferation. MTA inhibited Con A stimulated rat T lymphocyte proliferation as measured by [^{3}H] dThd uptake (Table I). MTA was added to the medium at the same time as the mitogen at concentrations

Table I 5'-Deoxy-5'-methylthioadenosine inhibition of [^{3}H] dThd incorporation in rat T-lymphocytes

Addition of MTA		cpm [^{3}H] dThd			Total cell number 10^6/ml
	at 0 h	24 h	48 h	72 h	72 h
NONE		16 000 ± 5 000	138 085 ± 9 719	225 747 ± 23 057	1.25*
MTA (µM)	50	9 946 ± 2 219	75 306 ± 3 933	133 039 ± 14 963	1.03*
	100	15 167 ± 5 000	59 381 ± 3 963	124 075 ± 10 219	0.72*
	250	7 290 ± 1 530	25 775 ± 5 128	75 694 ± 10 809	0.50*
	500	1 797 ± 197	6 178 ± 713	10 286 ± 3 582	0.33*
	1 000	173 ± 62	337 ± 66	585 ± 129	0.37*

Lymphocytes (1 x 10^6/ml) were stimulated with Con A (5 µg/ml) in the presence of different concentrations of MTA. After addition of lectin and MTA, aliquots of 2 x 10^5 cells were incubated in Falcon microtest II plates. [^{3}H] dThd (1 µCi/well) was added for 17 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The addition of [^{3}H] dThd occurred at the time 0, 24, and 48h of the culture period. Results were expressed in cpm as the mean ± SD of triplicate determinations of a single experiment (similar data were obtained in three additional experiments). Cell numbers were determined 72 h after Con A and MTA additions.

*In the presence of Con A typical blast cell transformation was demonstrated in up to 90% of cells. With concentration of MTA 500 µM and 1000 µM there is no more any blast cell transformation.

Viability of the unstimulated cells was the same in the presence or in the absence of MTA (1000 µM).

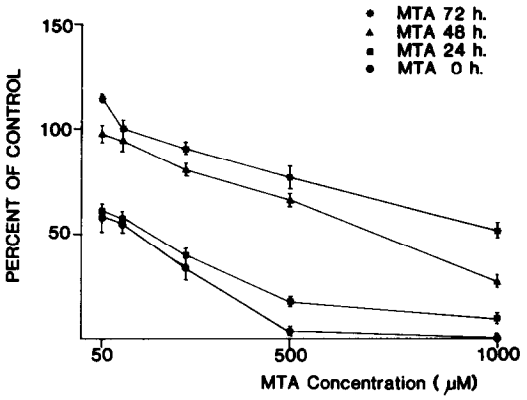

Fig. 1 Effect of addition of 5'-Deoxy-5'-methylthioadenosine at various times after Con A stimulation.

Lymphocytes (1 x 10^6/ml) were stimulated with Con A (5 μg/ml) for 72 h. Aliquots of 2 x 10^5 cells were dispensed in Falcon microtest II plates at time 0 of the stimulation period. MTA at different concentrations was added at times 0 h (●) ; 24 h (■) ; 48 h (▲) and 72 h (✱). At 72 h of the stimulation period [^{3}H] dThd (1 μCi/well) was added for 17 h at 37°C in a humidified atmosphere of 5 % CO_2 in air.
Bars represented the standard deviation of triplicate determinations of a single experiment (similar results were obtained in three additional experiments). The percent of control was calculated from the values in cpm of [^{3}H] dThd incorporated in the acid-insoluble fraction for 2 x 10^5 cells incubated in the presence of Con A (5 μg/ml) alone.

between 50 and 1000 μM. After 24 h, 48 h, 72 h of the incubation time, stimulated and unstimulated cultures were pulsed for 17 h with [^{3}H] dThd. In our culture conditions, the maximum blast number and [^{3}H] dThd uptake occurred 72 h after the stimulation with Con A (5 μg/ml).

MTA inhibition of [^{3}H] dThd uptake was dose dependent between 50 μM and 1000 μM. The number of blasts decreased with increasing MTA concentrations between 50 and 250 μM. With MTA concentrations of 500 μM and 1000 μM there was no longer blastogenesis. Even with the higher MTA concentration used, 1000 μM, the viability of cells was the same as that of unstimulated cells.

- Cell activation state dependency of MTA inhibition (Fig. 1). In these experiments, MTA at concentrations between 50 μM and 1000 μM, was added at different times (0 h, 24 h, 48 h, 72 h) following Con A stimulation of rat T lymphocytes. At the end of the culture period, we measured the [^{3}H] dThd uptake in acid-insoluble material. The dose dependent inhibitory effect of MTA was modulated by the activation state of the cells. For example, MTA (50 μM), added simultaneously with Con A,inhibited the [^{3}H] dThd uptake by 57 percent of the control (Con A alone). The same MTA concentration, added at 72 h of the stimulation period no longer had any effect on [^{3}H] dThd uptake.

- MTA phosphorylase induction during rat T lymphocyte transformation. Preliminary experiments were designed to further characterize the kinetic parameters of stimulated rat T lymphocyte MTA phosphorylase. The enzyme de-

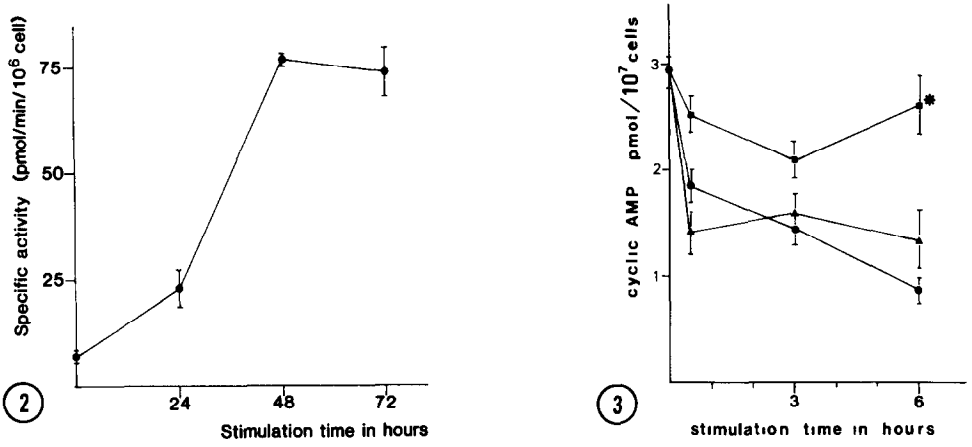

Fig. 2 Induction of 5'-deoxy-5'-methylthioadenosine phosphorylase activity during rat T lymphocyte transformation. Lymphocytes (1 x 10^6/ml) were stimulated with Con A (5 µg/ml) as described in Materials and Methods. The enzyme activity was measured at each of the designated times. Bars represented the standard error for triplicate determinations of a single experiment (similar results were obtained in four additional experiments). In the same experiment (Table I) the uptake of [^{3}H] dThd was measured at the same designated times.

Fig. 3 Time course of cyclic AMP level in rat T lymphocytes

Cultures run in triplicate (10^6 cells/ml) were preincubated 20 min either with MTA 10^{-3}M (culture I) (■), with dCf (10^{-5}M) (culture II) (▲), or without effector (culture III) (●). Con A was added in the 3 cultures, simultaneously with dAdo 10^{-5}M in culture II. The level of cyclic AMP (pmol/10^7 cells) was measured as described in Materials and Methods at each of the designated time after the addition of Con A.

Each point was the mean ± SEM for seven experiments. The statistical comparisons between levels of cyclic AMP found in cells in the presence of Con A alone and in the presence of Con A plus effectors (MTA or dAdo + dCf) were calculated by Student's t test (* $p < 0.02$).

monstrated normal Michaëlis-Menten kinetics. Apparent K_M values for MTA of 10.81 ± 1.97 µM and for phosphate of 1.52 ± 0.10 mM were calculated by the G. N. Wilkinson test (14)(mean ± SEM, n = 5). In order to determine whether increased levels of MTA phosphorylase might be responsible for the reduced effectiveness of MTA in activated lymphocytes, we assayed the enzymic activity in unstimulated and Con A activated rat T lymphocytes (Fig. 2). Rat T lymphocyte transformation was accompanied by an increase in MTA phosphorylase activity. The activity at 24 h of the stimulation time, was 250 % of the control and was 650% of the control value at 48h.

- Modification of cyclic AMP level by MTA, or by the combination of dAdo + dCf (Fig. 3). After 30 min, 3 h, and 6 h of stimulation by Con A, cells were collected for cyclic AMP determinations as described in Materials and Methods. After a 6 h Con A stimulation there was a significative accumulation of cyclic AMP in the presence of MTA (1000 µM), added 20' before the lectin : cyclic AMP concentration was 304 percent of results obtained with Con A alone.

Table II

5'-Deoxy-5'-methylthioadenosine inhibition of cyclic AMP phosphodiesterase activity in a homogenate of rat T lymphocytes

MTA (μM)	Phosphodiesterase activity (percent of control) mean ± SEM	
0	100	
50	86 ± 1.5	(3)
100	84,81	(2)
250	71 ± 2	(4)
350	64,67	(2)
500	56 ± 0.7	(6)

Phosphodiesterase activity was tested in extracts of lymphocytes after a 72 h Con A stimulation as described in Materials and Methods.

On the contrary, in adenosine deaminase deficiency conditions (dCf 10^{-5}M), dAdo, at a concentration of 10^{-5}M, which is inhibitory for lymphocyte proliferation (8) did not produce a significative accumulation of cyclic AMP.

- Inhibition of lymphocyte cyclic AMP phosphodiesterase by MTA (Table II)

An increase of cyclic AMP level may be due to an increase of adenylate cyclase activity or to a decrease of cyclic AMP breakdown. MTA has been reported to inhibit cyclic AMP phosphodiesterase of mouse normal spleen lymphocyte (5). Our results show that this is true also for rat T lymphocytes. The phosphodiesterase enzyme activity was measured with MTA between 50 μM and 500 μM. MTA inhibited cyclic AMP phosphodiesterase at concentrations which were also inhibitory for lymphocyte transformation : with MTA concentration of 500 μM, which completely inhibits lymphocyte proliferation, the residual phosphodiesterase activity was 56 % of control. The preincubation of the cell extract with MTA 500 μM at 37°C, during 30 min did not modify the percentage of this inhibition, indicating that MTA inhibition is independent from its metabolism by the cells.

DISCUSSION

MTA has been reported to inhibit the proliferation of phytohemagglutinin stimulated human peripheral lymphocytes (3, 15). In this study, MTA is found to similarly inhibit the Con A induced proliferation of rat T lymphocytes in a dose dependent manner. This inhibition depends on the activation state of the cells ; this is probably due to the 10-fold increase of MTA phosphorylase activity during blastogenesis, as suggested by Ferro et al for human lymphocytes (3).

The mechanism of MTA inhibition of mitogen stimulated lymphocyte proliferation has not been clearly elucidated. Indeed, one or more of the following effects of MTA, demonstrated in various systems, could interfere with cell proliferation : regulation of polyamine synthesis (16) ; regulation of cell division by the level of the methythiol group, produced by MTA (17) ; modulation of adenosine kinase activity (18) ; inhibition of RNA synthesis (19) ; inactivation of S-adenosyl-L-homocysteine hydrolase (20) ; inhibition of protein methylation (21) ; inhibition of phosphodiesterase activity (5).

In a similar model of mitogen stimulated lymphocyte proliferation, Ferro et al suggested that the probable mechanism of MTA inhibition was inactivation of S-adenosyl-L-homocysteine hydrolase, with a subsequent interference with methylation. This mechanism has also been proposed to explain, at least in part, the toxicity of dAdo, in adenosine deaminase deficiency conditions, since dAdo produces a suicide inactivation of S-adenosyl-L-homocysteine hydrolase (22). More recent studies however, cast a doubt on the role of this inhibition on T lymphocyte proliferation (23). Of interest to us, was the recent report of Wolberg et al (5) that MTA inhibits lymphocyte mediated cytolysis in the mouse presumably by interfering with cyclic AMP metabolism. This lymphocyte mediated cytolysis is a function which does not implicate cell proliferation ; however it should be noted that, in the model of Wolberg et al, as well as in our model of Con A stimulated rat T lymphocytes, the concentration of MTA that inhibits cell function or proliferation also inhibits phosphodiesterase and produces an elevation of cyclic AMP levels.

MTA induced inhibition of lymphocyte proliferation thus appears to be another example of the effects of phosphodiesterase inhibition in these cells. On the contrary, our results demonstrate that dAdo inhibition of adenosine deaminase deficient lymphocyte proliferation is not mediated by an interference with cyclic AMP metabolism, at least after the first 30 min of stimulation time. It should be noted too that dAdo inhibition of lymphocyte mediated cytolysis cannot be attributed to an effect on cyclic AMP metabolism (24). The inhibitory effects of the two purine nucleosides, MTA and dAdo on lymphocyte function and proliferation appeared to be mediated by different biochemical mechanisms.

ACKNOWLEDGMENT

We would like to thank Ms Annie Munier for her assistance with the cell culture experiments, Ms Danielle Bresson for secretarial assistance and Mr Thierry Hernandez for the illustrations.

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