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INHIBITION OF SPERMINE BIOSYNTHESIS AND THYMIDINE INCORPORATION IN CONCANAVALIN A TRANSFORMED LYMPHOCYTES BY S-ADENOSYL-(±)-2-METHYLMETHIONINE

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SUMMARY: S-Adenosyl-(±)-2-methylmethionine, an inhibitor of mammalian S-adenosyl-L-methionine decarboxylase, inhibited the increase in [^{3}H]-thymidine incorporation but did not interfere with the rise in [^{3}H]-uridine and [^{3}H]-leucine incorporation induced by concanavalin A in cultured mouse lymphocytes. Polyamine synthesis which increases during lymphocyte proliferation, was also selectively inhibited. Cellular levels of putrescine and spermidine increased despite the presence of the inhibitor but the rise in spermine concentration was blocked. Moreover, 24 hours after the addition of S-adenosyl-(±)-2-methylmethionine and concanavalin A to lymphocyte cultures, a concentration-dependent decrease in both [^{3}H]-thymidine incorporation and cellular spermine concentration was observed with dose-dependent increases in the other two polyamines. This is the first study in which selective inhibition of spermine synthesis also blocked DNA synthesis suggesting that this polyamine may participate in the regulation of proliferation in lymphocytes.

INTRODUCTION: The polyamines appear to play an important role in metabolism and have been shown to increase sharply during cellular proliferation (1). Selective inhibition of polyamine biosynthesis may help define the part polyamines play in cellular division and the inhibitors may in turn be useful as antiproliferative agents in cancer chemotherapy.

Inhibitors of ornithine decarboxylase, the first enzyme in the biosynthetic pathway of the polyamines, have been found to produce antiproliferative effects in some cultured cells and *in vivo* (2,3). Furthermore, MGBG**, a potent inhibitor of AdoMet decarboxylase, a key enzyme for the synthesis of Spd and Spm, also exhibited inhibitory effects on cell proliferation and is

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** Abbreviations: AdoMet, S-adenosyl-L-methionine; conA, concanavalin A; 2-methyl-AdoMet, S-adenosyl-(±)-2-methylmethionine; MGBG, methyl glyoxal bix (guanyl hydrazone); Put, putrescine; Spd, spermidine; and Spm, spermine.

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used clinically in treating acute myelocytic leukemia (4,5). The effects of MGBG on cell growth, however, even at micromolar concentrations, may not be related solely to its effect on AdoMet decarboxylase activity and polyamine biosynthesis (6,7,8).

Recently, we found that 2-methyl-AdoMet is an inhibitor of mammalian AdoMet decarboxylase (9). Because 2-methyl-AdoMet has been shown to be a substrate for some transmethylase enzymes (10), it would not be expected to interfere with AdoMet-dependent transmethylation reactions. Therefore, 2-methyl-AdoMet should be relatively specific as an inhibitor of Spd and Spm biosynthesis *in vivo*. In this paper, we describe the inhibitory effects of 2-methyl-AdoMet on the biosynthesis of polyamines, especially Spm, and on DNA synthesis in conA stimulated proliferating lymphocytes.

MATERIALS AND METHODS: Chemicals: 2-methyl AdoMet was synthesized by us as described in reference 9. Other chemicals were obtained from commercial sources. Standards used in polyamine determinations were recrystallized before use.

Lymphocyte Culture: Lymphocytes from mouse spleens were isolated and cultured as described by Wang et al. (11). DNA, RNA or protein synthesis in lymphocytes were determined by the incorporation of [^{3}H]-dThd, [^{3}H]-Urd or [^{3}H]-Leu into trichloroacetic acid precipitable material during a one hour pulse period (11).

Determination of DNA Content: Approximately 2-3 x 10^7 lymphocytes (4 ml of a culture suspension containing 4-6 x 10^6 cells/ml) were collected by centrifugation, extracted with 1.0 ml of 5% trichloroacetic acid and stored as -20°C until assayed. The DNA content of trichloroacetic acid precipitable material was determined using the method of Schneider (12) with calf thymus DNA as a standard.

Determination of Intracellular Polyamines: Approximately 1-3 x 10^7 cells (3-5 ml of a culture suspension containing 4-6 x 10^6 cells/ml) were collected by centrifugation, extracted with 0.5 ml of 0.3 N perchloric acid and the extract was stored at -20° C until assayed. The concentration of polyamines in the acid extract was determined by high pressure liquid chromatography of the dansyl derivatives of the amines as described by Newton et al. (13). For each experimental group, duplicate samples were analyzed twice for polyamine content. The polyamine concentrations are expressed as picomol per microgram of DNA.

RESULTS AND DISCUSSION: In initial studies, we found that 2-methyl-AdoMet preferentially inhibited the increase in [^{3}H]-dThd incorporation in lymphocytes stimulated with conA. The concentration of 2-methyl-AdoMet which reduced the increase in [^{3}H]-dThd incorporation by 60%, decreased [^{3}H]-Urd or [^{3}H]-Leu incorporation by 8%. Therefore, in subsequent experiments, only

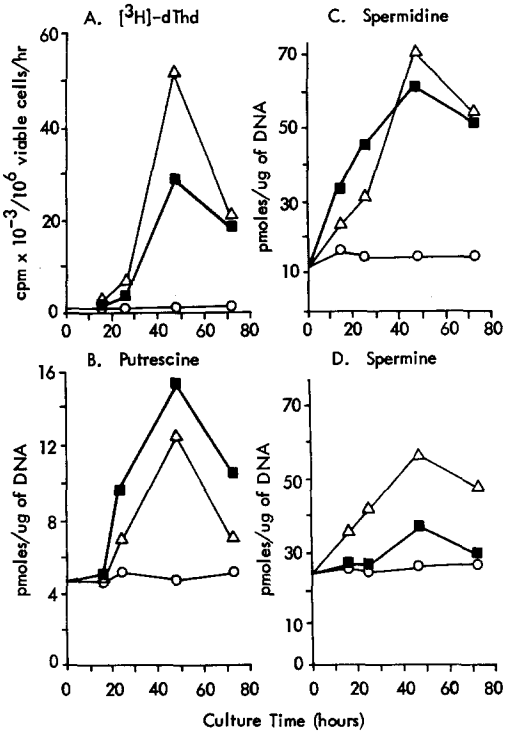

Fig. 1 Time course of effects of 2-methyl-AdoMet on [^{3}H]-dThd incorporation and intracellular levels of polyamines in conA stimulated lymphocytes. Mouse spleen lymphocytes (5 x 10^6 cells/ml) were cultured without conA (○), with 1 ug/ml conA (△), or with 1 ug/ml conA and 1 mM 2-methyl-AdoMet (■) in RPMI 1640 medium supplemented with 1% heat inactivated human serum, 2 mM L-glutamine, 50U/ml penicillin, and 50 mg/ml streptomycin. At time points indicated, the rate of [^{3}H]-dThd incorporation and the levels of polyamines in lymphocytes were determined as described in Materials and Methods.

[^{3}H]-dThd incorporation was determined in assessing the inhibitory effects of 2-methyl-AdoMet.

The time course of inhibition of polyamine biosynthesis and [^{3}H]-dThd incorporation in conA transformed lymphocytes by 2-methyl-AdoMet is shown in Fig. 1. In the presence of 1 mM 2-methyl-AdoMet, the increase in [^{3}H]-dThd incorporation was reduced by 60% but the peak was not delayed in its occurrence. Thus, the inhibition caused by 2-methyl-AdoMet was not due to a delay in the onset of DNA synthesis.

ConA stimulation also produced increases in cellular polyamine levels which paralleled the rise in the rate of [^{3}H]-dThd incorporation (Fig. 1). Treatment with 2-methyl-AdoMet, however, selectively altered the pattern of

change in polyamine levels. The increase in Spm was inhibited by 60% while the accumulation of Put and Spd continued or was enhanced. The correlation between the inhibition of [^{3}H]-dThd incorporation and the prevention of Spm rise at 24 hr suggests that intracellular Spm content and DNA synthesis may be interrelated.

To further study this relationship, parallel conA stimulated lymphocyte cultures were incubated with different doses of 2-methyl-AdoMet for 24 hr and the effects of the compound on polyamine levels and [^{3}H]-dThd incorporation were measured (Table 1). The inhibitor produced a dose-dependent decrease in both the cellular Spm content and the incorporation of [^{3}H]-dThd. Furthermore, the decrease in Spm level showed a good linear correlation with the decrease in [^{3}H]-dThd incorporation ($r = 0.971$, $r^2 = 0.901$). Over the concentration ranges tested, cell viability was not decreased by more than 10% suggesting that the inhibitory effect of 2-methyl-AdoMet was not due to general cytotoxicity.

In other experiments, lymphocytes treated with 2-methyl-AdoMet were washed free of the inhibitor after 24 hr of incubation with the compound. The proliferative response to conA recovered completely one day after reversal, indicating 2-methyl-AdoMet did not cause permanent damage to the inhibited cells.

2-methyl-AdoMet also produced a dose-dependent increase in Put and Spd levels at 24 hr (Table 1). The increases in Put and Spd concentrations showed a good linear correlation with the decrease in [^{3}H]-dThd incorporation suggesting that the decrease in DNA synthesis may be a result of the increases in Put and/or Spd concentration and not the decrease in Spm concentration. However, the time-course studies (Fig. 1) do not support a causal relationship between the increase in Spd levels and the inhibition of [^{3}H]-dThd incorporation. At 48 and 72 hr after the addition of the inhibitor, the incorporation of [^{3}H]-dThd was inhibited in spite of a slight decrease in Spd levels.

Table 1. Effects of Varied Concentrations of 2-methyl-AdoMet on Polyamine Accumulation and [^{3}H]-Thymidine Incorporation in Concanavalin A-Stimulated Lymphocytes

Culture Conditions[a]	Polyamine Levels (pmoles/ug DNA)[b]			[^{3}H]-Thymidine Incorporation
	Putrescine	Spermidine	Spermine	(cpm/10^6 viable cells/hr.)[b]
Unstimulated (- conA)	5.4	11.1	18.0	662
Stimulated (+ conA)	10.7	27.9	37.1	7144
+ 0.2 mM 2-methyl AdoMet	12.3(130)	38.2(160)	37.0(100)	6568(91)
+ 0.5 mM 2-methyl AdoMet	17.6(230)	45.1(200)	35.6(92)	5048(68)
+ 1.0 mM 2-methyl AdoMet	17.9(240)	51.5(240)	32.7(77)	3625(46)
+ 2.0 mM 2-methyl AdoMet	19.2(260)	59.6(290)	25.0(37)	2085(22)

a) Cultures were harvested 24 hours after addition of conA and 2-methyl-AdoMet.

b) Numbers in parentheses are % of control (+ conA); measured values are means of at least two separate determinations; the coefficient of variation of these measurements is not more than $\pm$ 5%.

To explore a possible causal relationship between the increase in Put concentration and the inhibition of [^{3}H]-dThd incorporation, the effects of combining α-methyl-(±)-ornithine, a potent inhibitor of Put accumulation (14), with 2-methyl-AdoMet on the cellular polyamines concentration and [^{3}H]-dThd incorporation were studied (Table 2). The combination of the two inhibitors produced a decrease in the concentrations of Put and Spm and an inhibition of [^{3}H]-dThd incorporation greater than that observed with either inhibitor alone. These results suggested that the inhibition of [^{3}H]-dThd incorporation produced by 2-methyl-AdoMet could not be attributed to the increase in Put concentration.

The changes in the intracellular concentrations of the polyamines in 2-methyl-AdoMet treated lymphocytes were, in part, consistent with an inhibition of AdoMet decarboxylase by this compound. Inhibition of this enzyme by 2-methyl-AdoMet should result in a decrease in the intracellular concentration of decarboxylated AdoMet which is needed for the sequential conversion of Put to Spd and then Spd to Spm. Consequently, an increase in the concentration of Put and a decrease in the concentrations of Spd and Spm were predicted. We found, however, that Spd levels increased normally and that synthesis of only the final product of the polyamine pathway, Spm, was inhibited. Thus, a rise in Spm levels is clearly linked to DNA synthesis.

The unexpected elevation of Spd concentration in inhibited cells, measured by high pressure liquid chromatography, may have been due to the presence of some structurally similar compound which cochromatographed with Spd. It is possible that 2-methyl-AdoMet is a substrate for AdoMet decarboxylase and was transformed to 2-methyl decarboxylated AdoMet. The product 2-methyl-decarboxylated AdoMet could then become a substrate for Spd synthase and be converted to 2-methyl Spd. To test this possibility, the chromatographic peak corresponding to the tridansyl Spd was collected and subjected to chemical ionization mass spectrometric analysis. The mass spectrum of the material obtained from cells treated with the inhibitor was identical to that of authentic tridansyl Spd. Furthermore, the spectrum did not show the presence

Table 2. Effects of 2-methyl-AdoMet and α-Methyl-(±)-ornithine on Polyamine Accumulation and ^{3}H-Thymidine Incorporation in Concanavalin A-Transformed Lymphocytes

Conditions of Incubation[a]	Polyamine Levels (pmoles/μg DNA)[b]			^{3}H-Thymidine Incorporation
	Putrescine	Spermidine	Spermine	(cpm/10^{6} viable cells/hr.)[b]
Unstimulated (- Con A)	4.6	13.7	27.3	937
Stimulated (+ Con A)	9.5	32.9	43.4	6527
+ 2.0 mM α-Methyl-(±)-ornithine	4.7(2)	24.3(55)	36.2(55)	5020(73)
+ 1.0 mM 2-methyl-AdoMet	14.0(192)	48.7(182)	33.2(37)	2879(35)
+ 2.0 mM α-Methyl-(±)-ornithine + 1.0 mM 2-methyl-AdoMet	4.5	41.6(145)	33.0(35)	1815(16)

a) Cultures were harvested at 24 hours after addition of Con A, α-methyl-AdoMet and α-methyl-(±)-ornithine.

b) Numbers in parentheses are % of control (+ Con A); measured values are means of at least two separate determinations; the coefficient of variation of these measurements is not more than ± 5%.

of tridansyl 2-methyl Spd. These results unambiguously established that the observed increase in Spd concentration was not due to the presence of 2-methyl Spd.

Alternatively, the increase in Spd could result from 2-methyl-AdoMet acting as an inhibitor of Spm synthase. Inhibition of this enzyme would account for the differential increase in Spd and decrease in Spm concentrations Previously, 2-methyl-AdoMet has been shown not to inhibit Spm synthase from bovine brain (15), however, the enzyme in mouse spleen lymphocytes may be different from the bovine brain enzyme in its sensitivity toward the compound.

These results show that a rise in cellular Spm level is necessary for lymphocyte DNA synthesis. The exact mechanism of the differential inhibition of Spm biosynthesis by 2-methyl-AdoMet remains to be determined, nevertheless, this is the first demonstration that Spm, the final product of the polyamine synthetic pathway, is specifically involved in cell proliferation.

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