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Inhibition of Dimethyl Sulfoxide-induced Differentiation of Friend Erythroleukemic Cells by 5′-Methylthioadenosine[1]

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ABSTRACT

5′-Methylthioadenosine is a sulfur-containing nucleoside derived from the metabolism of polyamines which is known to exert an antiproliferative effect on several cell systems *in vitro*, including the Friend leukemia cell system. We have investigated the role of 5′-methylthioadenosine on the dimethyl sulfoxide-induced differentiation of this system. At a concentration of 400 μM, the drug strongly inhibited (80%) the induced differentiation of Friend cells, and this effect was already observable at a concentration as low as 10 μM (36% inhibition), as evidenced by the benzidine staining procedure and by the dot-blot hybridization of globin mRNA with a human β-globin probe. Similar results have been obtained by using 5′-*S*-isobutylthioadenosine, which is a synthetic structural analogue of 5′-methylthioadenosine. The block of differentiation produced by these nucleosides was not mediated by adenine (a catabolite of both molecules) and was not reverted by spermine or spermidine, the two polyamines whose synthesis is inhibited by 5′-methylthioadenosine.

We report a decrease of the aminopropyltransferases activities (the enzymes responsible for 5′-methylthioadenosine biosynthesis) in dimethyl sulfoxide-treated Friend cells, which could lead to a decrease of the intracellular content of 5′-methylthioadenosine during the erythroid maturation of Friend cells. The results obtained are consistent with the hypothesis that 5′-methylthioadenosine may act as an endogenous regulator of Friend cell differentiation.

INTRODUCTION

The sulfur-containing nucleoside MTA[3] derives from the metabolism of polyamines and is ubiquitously distributed in mammalian tissues and prokaryotic cells (15, 52). In the eukaryotes, MTA is synthesized by propylamine transferase reactions between decarboxylated *S*-adenosylmethionine and putrescine or spermidine (44). The nucleoside, however, does not accumulate in normal tissue but is rapidly cleaved to adenine and 5′-methylthioribose 1-phosphate by MTA phosphorylase (12, 48).

It is well documented that MTA significantly affects cell growth (48). In fact, an antimitotic effect is exerted by the thioether in several *in vitro* cultured cell systems, such as stimulated human lymphocytes (15), murine lymphoid cells (49), BHK21 cells (31), SV40-transformed murine fibroblasts (29), and FLC (7).

No effects of MTA on cell differentiation have been described so far. Recently, an involvement of polyamines in the differentiation of mammalian cells has been proposed. It has been reported that they are needed for erythroid differentiation of Friend cells (20), for adipose conversion of 3T3-L1 fibroblasts (2), and for differentiation of human normal myeloid precursors (granulocyte-macrophage colony-forming units) (46), neuroblastoma cells (9), and rabbit costal chondrocytes (45). In addition, specific inhibitors of polyamine biosynthesis are able to affect the state of cell differentiation (2, 14, 20, 45).

Since MTA is a product of the polyamine-biosynthetic pathway, it is of interest to study whether this molecule, regarded in the past as a simple by-product, might exert any significant effect on cellular differentiation.

In order to study the effect of MTA on cell differentiation, Friend erythroleukemic cells have been selected as a model system. These cells are murine proerythroblasts, infected by, and continuously producing, the Friend leukemia virus complex. They are arrested at an early stage of differentiation and can be induced to undergo erythroid differentiation by Me_2SO (17) or by a variety of other agents (27).

There are other compounds which, in turn, inhibit the induced differentiation of FLC, such as dexamethasone (25, 38), the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (35, 51), interferon (33, 34), bromodeoxyuridine (3, 37), and drugs interfering with polyamine biosynthetic pathway (20), *e.g.*, the ornithine analogs, α-methyl ornithine and α-hydrazinoornithine (29), and the inhibitor of polyamine biosynthesis, methylglyoxal bis(guanylhydrazone) (53).

The results presented here demonstrate that MTA and its synthetic structural analogue SIBA, another powerful antiproliferative drug (6, 48), are able to strongly inhibit the Me_2SO-induced differentiation of FLC to hemoglobin-containing erythroblasts. The block of hemoglobin production, exerted by MTA, resides, most probably, at the level of globin mRNA transcription, since the nucleoside strongly decreases the level of β-globin mRNA in treated FLC after 3 days of treatment. The block in Me_2SO-induced differentiation is not reversible by the addition of polyamines to the culture medium.

Moreover, the levels of 2 enzymes involved in MTA synthesis (spermine synthase, EC 2.5.1.— and spermidine synthase, EC 2.5.1.16) increase highly during FLC growth in the absence of differentiation, whereas they increase only slightly during the course of Me_2SO induction. These results, together with the observed inhibitory activity of MTA on FLC differentiation, suggest that the nucleoside levels may physiologically regulate the cellular differentiation process.

MATERIALS AND METHODS

Cells and Culture Conditions. The F 4-6 clone of FLC was a generous gift of Dr. W. Ostertag (Hamburg, West Germany); it was cultured in

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[3] The abbreviations used are: MTA, 5′-methylthioadenosine; SIBA, 5′-*S*-isobutylthioadenosine; FLC, Friend erythroleukemic cells; Me_2SO, dimethyl sulfoxide.

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suspension in 75-sq cm flasks in minimum essential medium (Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum (Grand Island Biological Co.) in 5% CO_2 in air (11). In some experiments, where indicated, cells were cultured in 10% horse serum instead of fetal calf serum. Similar results were obtained with both types of sera. K 562 cells were obtained from Dr. R. Mertelsmann (NY) and cultured in RPMI 1640 (Flow Laboratories Inc., McLean, VA) medium containing 10% fetal calf serum in 5% CO_2 in air.

Chemicals. *S*-Adenosylmethionine was prepared by biosynthesis with yeast (39) and isolated by ion-exchange chromatography (55); *S*-adenosyl-L-[*methyl*-^{14}C]methionine (50 mCi/mmol) was purchased from Amersham, Radiochemical Centre (Amersham, Buckinghamshire, United Kingdom); MTA and 5′-[*methyl*-^{14}C]MTA were prepared according to the method of Schlenk and Ehninger (40). Adenine, putrescine, spermidine, spermine, Me_2SO, hemin, and SIBA were obtained from Sigma Chemical Co. (St. Louis, MO). *S*-adenosyl(5′)-3-methylthiopropylamine and *S*-[*methyl*-^{14}C]adenosyl(5′)-3-methylthiopropylamine were obtained from their precursors by enzymatic decarboxylation using *S*-adenosylmethionine decarboxylase from *Escherichia coli* (47). The products were purified by preparative high-performance liquid chromatography. Chemical and radiochemical purity of the above-mentioned compounds were checked by paper and thin-layer chromatography (56) and high-performance liquid chromatography (12, 54). All other chemicals were of the purest grade available from standard commercial sources.

Treatment of Cells with Drugs. All the drugs (MTA, SIBA, adenine, spermine, and spermidine) used in these experiments were dissolved in phosphate-buffered saline (137 mM NaCl-2.7 mM KCl-1.47 mM KH_2PO_4-8.1 mM Na_2HPO_4) and added to the culture medium at the time of cell seeding. Initial cell concentration was between 1.0 and 2.5 × 10^5/ml. No medium changes were performed during the experiments. Me_2SO was added at a final concentration of 1.5% (v/v) (0.21 M); the other drugs were added at the concentrations indicated in the legends to the charts.

Assay of Hemoglobin-producing Cells. The number of differentiating cells was determined by scoring benzidine-positive cells as described by Singer *et al.* (42). Five to 10 min after the addition of the benzidine reagent, an average of 300 cells was scored by 2 observers for benzidine positivity.

Cytoplasmic RNA Extraction. FLC cells were processed for the RNA extraction according to a previously described method (4). In brief, cells were washed twice with cold phosphate-buffered saline and resuspended in cold lysis buffer (10 mM Tris-HCl, pH 7.4-140 mM NaCl-1.4 mM magnesium acetate) at concentrations ranging from 10 to 40 × 10^6 cells/ml in the presence of 0.5% Nonidet P40. After 10 min, nuclei were pelleted at 3000 × *g* for 5 min, and the supernatant fluid was treated at 37° with proteinase K (500 μg/ml) plus 1% sodium dodecyl sulfate and then extracted 3 times with phenol-chloroform-isoamyl alcohol. The aqueous phase was precipitated at −20° with 2.5 volumes of ethanol in the presence of 0.3 M NaCl.

Dot-Blot Hybridization. RNA resuspended in 6× SSC (1× SSC: 0.15 M NaCl-0.015 M sodium citrate) at the concentrations indicated in Fig. 3, in a final volume of 5 μl, was spotted onto a nitrocellulose filter (Schleicher and Schüll, Dassel, West Germany) that had been washed first in water and then in 10× SSC for at least 4 hr, and the filter dried in a vacuum oven at 80° for 2 hr. Hybridization was carried out with a ^{32}P-labeled human β-globin probe (courtesy of Dr. E. V. Avvedimento, Naples, Italy) prepared by nick translation (32), in 1 × Denhardt's solution containing 50% formamide, 10% dextrane sulfate, 5 × SSC, and calf thymus DNA (100 μg/ml), at 42° for 24 hr. Following hybridization, the filters were washed in 0.1% sodium dodecyl sulfate-0.1× SSC at 58° and then exposed to Kodak X AR-5 film with intensifying screens.

Enzyme Assays. Cell-free extracts were obtained by hypotonic lysis of 250 μl of cells resuspended in 2 ml of H_2O and 3 cycles of freeze-thawing. After addition of 250 μl of 1 M potassium phosphate buffer, pH 7.4, and 200 μl of 40 mM dithiothreitol, the mixture was centrifuged at 1500 × *g* for 30 min. The supernatant was used to assay the enzyme activity. MTA phosphorylase was assayed by following the production of 5-[*methyl*-^{14}C]thioribose 1-phosphate (5). The assay mixture contained 15 to 60 μg of protein-100 mM sodium phosphate buffer, pH 7.4-1 mM dithiothreitol-50 μM [*methyl*-^{14}C]MTA (25 μCi/μmol). Spermidine synthase activity was assayed according to previously reported methods (21) following the production of 5′-[*methyl*-^{14}C]MTA from [*methyl*-^{14}C]*S*-adenosyl(5′)-3-methylthiopropylamine in the presence of putrescine. The radioactive product was isolated on a phosphocellulose column (Cellex-P; Bio-Rad, Richmond, CA). Spermine synthase was assayed in the same way by replacing putrescine with spermidine. Assay mixtures contained 10 μM *S*-[*methyl*-^{14}C]adenosyl-(5′)-3-methylthiopropylamine (25 μCi/μmol), 0.5 mM putrescine or spermidine as the acceptor polyamines, 50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, and 30 to 300 μg of proteins. All enzyme assays were carried out under conditions in which their activity was proportional to the amount of protein added and to the time of incubation.

RESULTS

No Induced Differentiation in FLC by MTA and Its Analogue, SIBA. In order to investigate whether MTA could act as an inducer of FLC erythroid differentiation, we treated the cells with 1, 10, 100, and 400 μM of MTA for 5 days; we used the benzidine staining procedure to score the hemoglobin-producing cells during the differentiation period. As shown in Chart 1*A*, at the tested concentrations, FLC are not induced to differentiate by MTA, whereas they can differentiate up to 70% when treated with 1.5% Me_2SO. Chart 1*B* depicts the growth curve of the MTA-treated cells, showing the typical, and previously described antiproliferative effect of MTA on FLC (7). Identical results were obtained with SIBA, the synthetic analogue of MTA, as measured at the fifth day of treatment and at the same concentrations as for MTA.

Inhibition of Me_2SO-induced Differentiation of FLC by MTA and Its Analogue, SIBA. We observed a dose-dependent inhibition of Me_2SO-induced differentiation at concentrations ranging from 1 to 400 μM of MTA and SIBA; these results are reported in Chart 2. Both drugs acted in a similar way, reducing the percentage of benzidine-positive cells to 9% for MTA, 400 μM (Chart 2, *left*) and 16% for SIBA, 400 μM (Chart 2, *right*), hence obtaining, at the fifth day of treatment, about 80% of inhibition of the Me_2SO-induced differentiation with either MTA or SIBA. It is interesting to note that this action on the maturative process was already appreciable at one of the lowest concentrations tested, reaching 37 and 34% of inhibition for a 10 μM concentration, and 13% of inhibition for a 1 μM concentration of MTA and SIBA, respectively.

To further explore this inhibitory effect, cytoplasmic RNAs from Me_2SO-induced FLC treated with MTA concentrations from 1 to 400 μM were extracted and hybridized against a ^{32}P-labeled human β-globin-specific cloned probe. RNAs were obtained from cells treated for 3 days. As shown in Fig. 1, we observed a pronounced dose-dependent reduction of the β-globin-specific mRNA content, a definite effect being very clear for a dose of 1 μM MTA (*Lane 3*). A total disappearance of β-globin-specific mRNA was observed with 100 and 400 μM MTA (*Lanes 5* and *6*, respectively). The inhibiting action of MTA on FLC-induced differentiation, as measured by β-globin mRNA decrease, seemed to be even more pronounced when compared to the benzidine staining procedure; this might be due to differences in the sensitivity and/or specificity of the 2 techniques used.

MTA Action not via Conversion to Adenine. It has been suggested that the MTA antiproliferative effects could be mediated via its catabolism into adenine by MTA phosphorylase

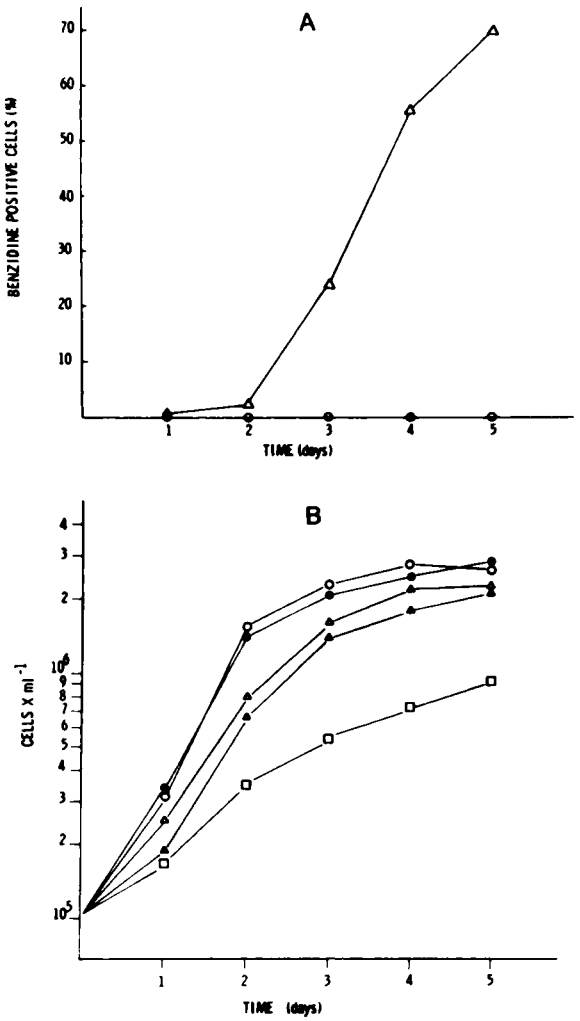

Chart 1. Effect of MTA on cell growth and erythroid differentiation of FLC. In *A*, erythroid differentiation was measured, during 5 days of continuous treatment of FLC with either Me_2SO or MTA, by counting benzidine-positive cells (see "Materials and Methods"). △, Me_2SO, 1.5% (v/v); ○, control FLC; ●, MTA 1, 10, 100, and 400 μM. In *B*, cells were seeded at a concentration of 1×10^5/ml and treated with MTA throughout the experiment. ○, control FLC; ●, MTA, 1 μM; △, MTA, 10 μM; ▲, MTA, 100 μM; □, MTA, 400 μM. The results are the mean of at least 3 experiments.

(48). In a previous report, we concluded that adenine was unable to mimic the antiproliferative effect of MTA on FLC. These results, however, do not exclude the hypothesis that the effect of MTA on the induced differentiation was mediated by its catabolite adenine. To rule out this possibility, we treated FLC with adenine in the same range of concentrations used for MTA. The results of this experiment are shown in Chart 3. No effect of adenine on Me_2SO-induced erythroid maturation was noticed, even at an adenine concentration as high as 400 μM.

Inhibitory Effect of MTA not Reverted by Polyamines. Since MTA is a powerful inhibitor of polyamine biosynthesis (28), our results could be ascribed to an intracellular depletion in polyamines. If this were the case, the exogenous addition of polyamines should be able to revert the effect of MTA. In order to test this hypothesis, we added spermine or spermidine to the culture medium of FLC treated with Me_2SO and MTA. The results of these experiments are shown in Table 1. Neither spermine nor spermidine were able to revert MTA inhibition, even at a concentration of 100 μM. In some experiments, horse serum was substituted for fetal calf serum, since a certain toxic effect of polyamine by-products was reported for cells growing *in vitro* in fetal calf serum (29). This might be due to a high polyamine oxidase content of fetal calf serum compared to horse serum. We obtained similar results using both types of sera.

Enzymatic Activities Involved in MTA Metabolism in FLC Undergoing Erythroid Differentiation. In order to evaluate the physiological significance of the inhibition of the differentiation exerted by MTA, the activities of the enzymes involved in the biosynthesis and catabolism of the nucleoside have been tested during the differentiation process. In Charts 4 and 5 are depicted the patterns of the enzymatic activities of spermine and spermidine synthases over the period of growth. We have observed an increase in the aminopropyltransferase activity during the growth of both Me_2SO-treated and control populations. However, the enzymatic activities were significantly lower in cells undergoing differentiation than in control FLC. In fact, spermidine synthase activity increased about 8-fold in control cells, reaching a peak value at the fourth day of culture, while Me_2SO-treated cells displayed only a 3-fold increase at the same peak time (Chart 4). Spermine synthase also increased within 5 days of culture, although to a lesser extent. We observed a 3.5-fold elevation in

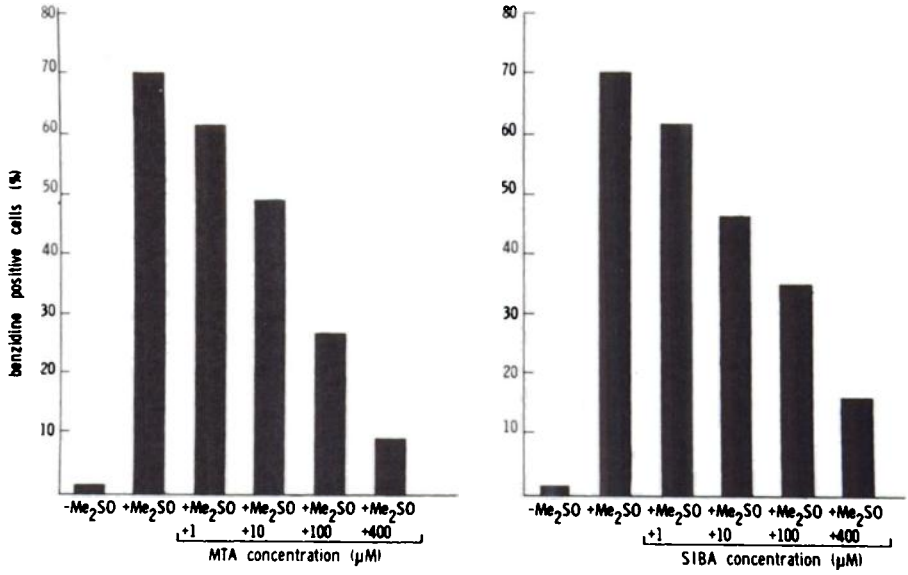

Chart 2. Effect of MTA and SIBA on the Me_2SO-induced differentiation of FLC. *Left*, cells were plated at a concentration of 1×10^5/ml and Me_2SO 1.5% (v/v) or MTA, at the concentrations indicated, were added at the time of cell seeding. Erythroid differentiation was measured by scoring benzidine positive cells; *right*, experiment performed as above, substituting SIBA, at the concentrations indicated, for MTA. The results are the mean of at least 3 experiments.

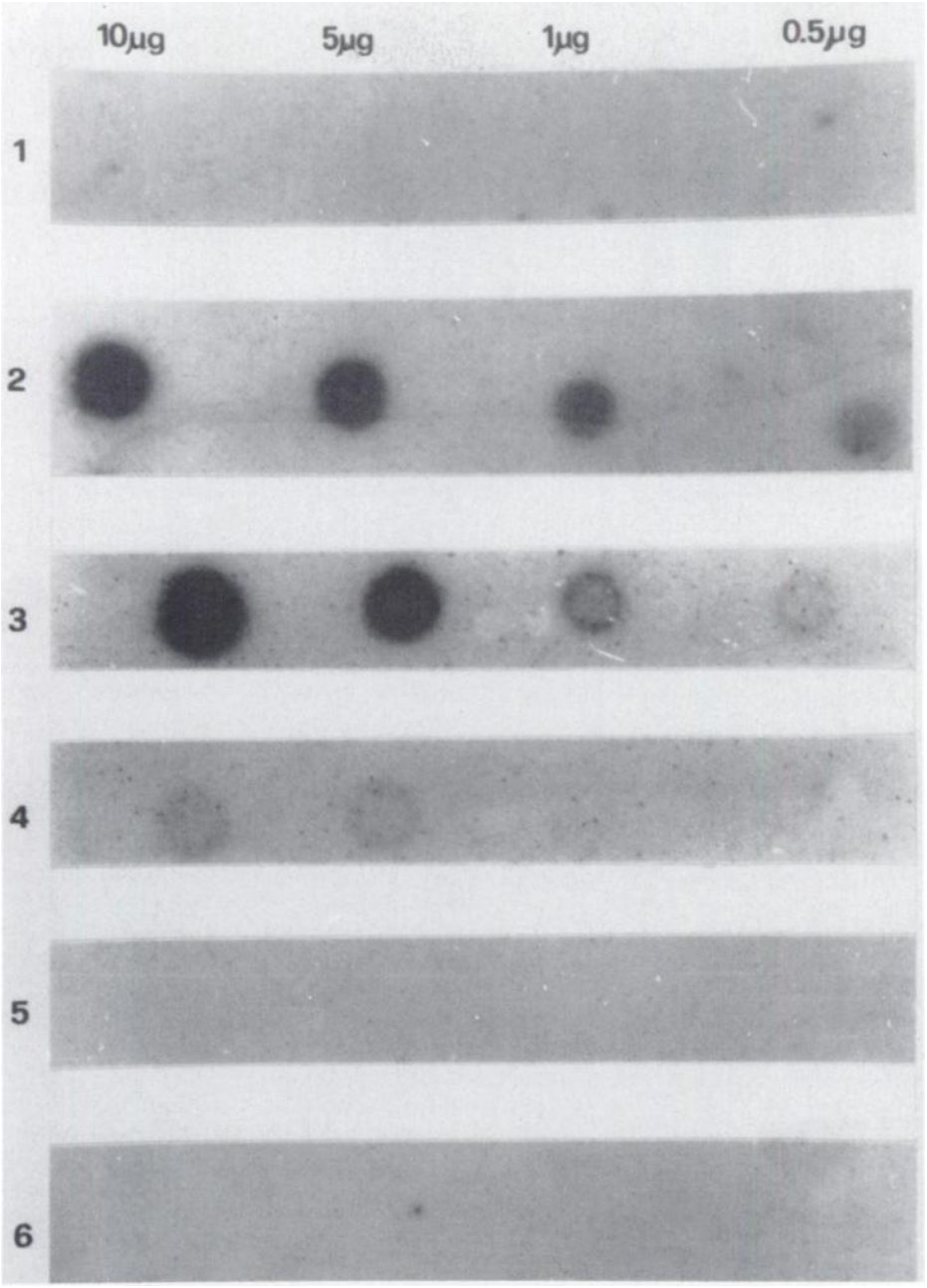

Fig. 1. Cytoplasmic β-globin-specific mRNA in FLC treated with Me_2SO alone or with Me_2SO and MTA. The analysis was performed by means of a dot-blot hybridization technique as described in "Materials and Methods." *Lane 1*, control FLC; *Lane 2*, 1.5% (v/v) Me_2SO; *Lane 3*, Me_2SO + MTA, 1 μM; *Lane 4*, Me_2SO + MTA, 10 μM; *Lane 5*, Me_2SO + MTA, 100 μM; *Lane 6*, Me_2SO + MTA, 400 μM. 10, 5, 1.0, and 0.5 μg of total cytoplasmic RNA were spotted for each lane.

the untreated cells (peak at the third day of culture) compared to a 2-fold increase (second day of culture) in differentiating cells (Chart 5).

The activity of MTA phosphorylase, EC 2.4.2.1, the enzyme responsible for MTA cleavage, has also been investigated. The enzyme activity was measured over a 5-day growth period, as for aminopropyltransferase. No differences were detected between treated and untreated cells, and only slight variations were observable during cell growth (data not shown). MTA phosphorylase activity was significantly higher when compared to that of aminopropyltransferase, ranging from 40 to 80 nmol of MTA degraded per mg of protein per hr.

No Inhibition of Hemin-induced Erythroid Differentiation in K 562 Cells by MTA. In order to verify whether the inhibitory effect of MTA on erythroid differentiation is a phenomenon restricted to the FLC system or is common also to other differentiation-inducible systems, we have tested the effect of MTA on the human cell line K 562 (26), another system which was available in our laboratory. This human leukemic cell system can be induced to differentiate *in vitro* by chemical compounds (1, 36) along the erythroid differentiation pathway, showing the production of several types of embryonic and fetal hemoglobins (10, 36). In this case, the inducer of differentiation was hemin, since this cell system is insensitive to Me_2SO (1). The results

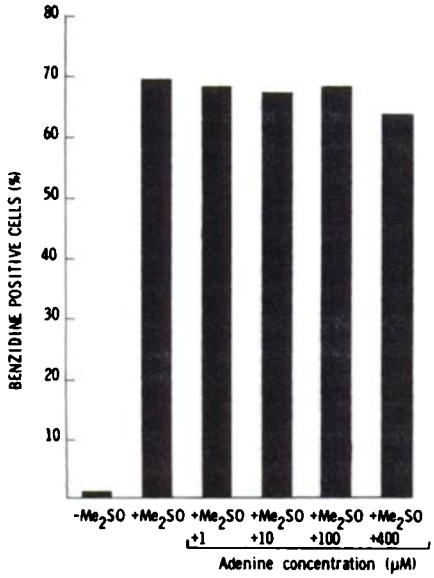

Chart 3. Effect of adenine on cell growth and Me_2SO-induced erythroid differentiation of FLC. Cells were plated at 1×10^5/ml and Me_2SO, 1.5% (v/v) or adenine, at the concentrations indicated, were added at the time of cell seeding. Differentiation was assayed by means of the benzidine method. The results are the mean of 2 experiments in duplicate.

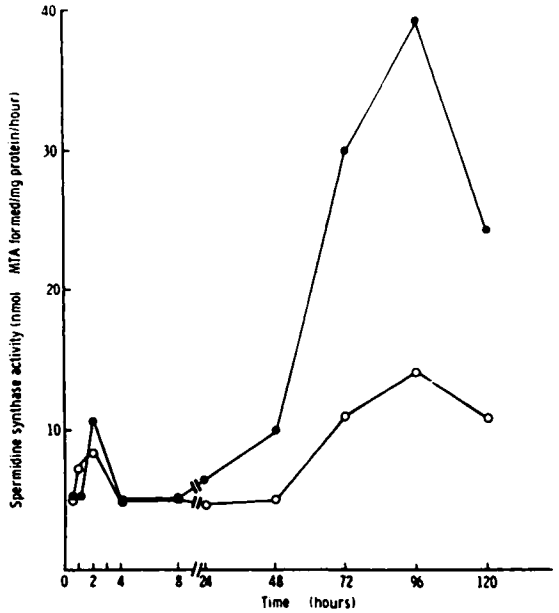

Chart 4. Effect of Me_2SO on the levels of spermidine synthase in differentiating FLC. Log-phase cells were plated as described in "Materials and Methods," and the inducer was added at the time of cell seeding. At the times indicated, cells were harvested, and cell-free extracts were prepared to assay the enzymatic activity. ●, control FLC; ○, Me_2SO, 1.5% (v/v).

Table 1

Effect of spermine and spermidine on the MTA-induced inhibition of FLC differentiation

The results are the mean of at least 3 experiments.

Treatment	Saturation density (cells × 10^{-5})/ml		Induction (% of benzidine-positive cells)[a]	
	HS[b]	FCS	HS	FCS
None	1.6	2.8	<1	<1
Me_2SO	1.0	2.1	62	78
Me_2SO + MTA (100 μM)	0.7	1.7	13	24
Me_2SO + MTA (400 μM)	0.7	0.8	8	7
Me_2SO + MTA (100 μM) + SP (2.5 μM)	0.5	1.5	8	26
Me_2SO + MTA (100 μM) + SP (10 μM)	0.7	0.8	16	12
Me_2SO + MTA (100 μM) + SP (100 μM)	0.4	TOX[c]	11	TOX
Me_2SO + MTA (100 μM) + SPD (2.5 μM)	0.7	1.2	9	24
Me_2SO + MTA (100 μM) + SPD (10 μM)	0.6	0.6	13	12
Me_2SO + MTA (100 μM) + SPD (100 μM)	0.5	TOX	11	TOX
Me_2SO + MTA (400 μM) + SP (2.5 μM)	0.5	0.7	13	4
Me_2SO + MTA (400 μM) + SP (10 μM)	0.6	0.5	6	5
Me_2SO + MTA (400 μM) + SP (100 μM)	0.5	TOX	8	TOX
Me_2SO + MTA (400 μM) + SPD (2.5 μM)	0.6	0.6	4	6
Me_2SO + MTA (400 μM) + SPD (10 μM)	0.5	0.6	4	3
Me_2SO + MTA (400 μM) + SPD (100 μM)	0.6	TOX	6	TOX

[a] Measured at the fifth day of treatment as described in "Materials and Methods."
[b] HS, horse serum; FCS, fetal calf serum; SP, spermine; SPD, spermidine; TOX, toxic.
[c] Treatment with Me_2SO and MTA plus spermine or spermidine (100 μM) was toxic. Sp, spermine; Spd, spermidine.

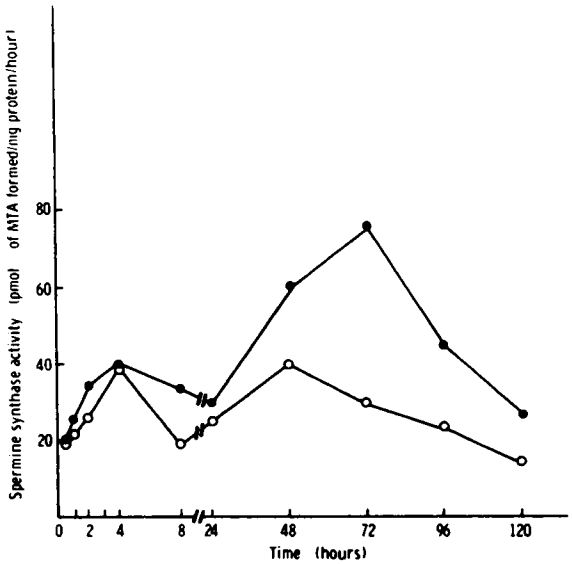

Chart 5. Effect of Me_2SO on the levels of spermine synthase in differentiating FLC. Log-phase cells were plated as described in "Materials and Methods," and the inducer was added at the time of cell seeding. At the times indicated, cells were harvested and cell-free extracts were prepared to assay the enzymatic activity. ●, control cells; ○, Me_2SO, 1.5% (v/v).

obtained with the K 562 cell line indicate that, unlike FLC, erythroid differentiation induced in this cell system by hemin, which is one of its most powerful *in vitro* inducers (1), cannot be inhibited by the same concentrations of MTA which completely block FLC-induced differentiation (400 μM). Higher concentrations of MTA, however (up to 2500 μM), exert a slight inhibitory effect (Table 2).

DISCUSSION

In the present paper, we report that MTA and its synthetic analogue, SIBA, act in FLC as inhibitors of the Me_2SO-induced differentiation. This inhibitory effect has been monitored using 2 different methods, the benzidine staining procedure and the dot-blot hybridization analysis of globin mRNA. Using the former technique, we observed a striking inhibition (80% of the induced differentiation) at a MTA concentration of 400 μM; however, a clear-cut effect was already observable at a concentration as low as 10 μM (36% of the induced differentiation) and 1 μM (13% of the induced differentiation). At the latter concentrations, no appreciable cytostatic effects were exerted by MTA on FLC. It is also of interest to note that the effect of MTA has been

Table 2
Effect of MTA on hemin-induced differentiation of the K 562 cell line

Treatment	Induction (% of benzidine-positive cells)[a]	No. of cells at the fifth day of culture ($\times 10^{-6}$/ml)
None	2.8	14
Hemin (50 μM)	71.0	13
Hemin (50 μM) + MTA (100 μM)	64.3	13
Hemin (50 μM) + MTA (400 μM)	68.2	10
Hemin (50 μM) + MTA (1000 μM)	71.1	7.7
Hemin (50 μM) + MTA (1500 μM)	69.7	7.6
Hemin (50 μM) + MTA (2000 μM)	66.1	4.7
Hemin (50 μM) + MTA (2500 μM)	58.1	3.2

[a] Measured at the fifth day of treatment.

observed at a concentration (1 μM) which is of the same order of magnitude as the physiological levels reported for the nucleoside (12, 48). The dot-blot analysis of globin mRNA confirmed these results and suggested that the block induced by MTA resides at the level of β-globin mRNA transcription.

MTA has also been tested on the differentiation of K 562 cells. The K 562 cell system was chosen since it can be regarded as the human equivalent of an erythroid *in vitro* differentiating system, such as FLC (1, 10, 36). The results obtained demonstrate that MTA does not exert any inhibitory effect upon hemin-induced K 562 erythroid differentiation. A slight inhibition of the induced differentiation was visible at extremely high concentrations of MTA, such as 2500 μM. It has been reported previously that the main enzyme involved in MTA metabolism, *i.e.*, MTA phosphorylase, is absent in one clone of K 562 cells in which this enzyme was assayed (22). The absence of this enzyme has been confirmed on the K 562 strain used in these experiments (data not shown); therefore, a possible acquired resistance of the K 562 cells to MTA could account for the relative insensitivity of this line to the MTA inhibitory action on induced differentiation. However, other alternative explanations could also be considered.

Since it has been reported that some inhibitors of FLC induced differentiation, such as interferon (13), have a "pendulum" effect; *i.e.*, they behave like either differentiation inhibitors or inducers depending on the concentrations used, we tested MTA alone on FLC to verify whether MTA also shared such an effect. Our results exclude MTA from having a mode of action similar to the above-mentioned substances, since it has no ability to induce FLC differentiation when given alone in a wide range of concentrations (1 to 400 μM).

We have also shown that adenine, which is a catabolite of MTA, is not able to mimic the inhibitory action of the nucleoside, thus suggesting that MTA acts *per se* on erythroid differentiation of FLC. We have demonstrated previously (7) that the thioether, at a concentration near physiological values, is rapidly taken up by FLC and metabolized; indeed, in these conditions, the initial velocity values of transport represent the limiting step of MTA influx, and only small amounts of the nucleoside are detectable intracellularly. Therefore, it is conceivable that the action of MTA at the cellular level should occur at early times after the exposure of FLC to the inducer. Several target sites of action have been attributed to MTA on the basis of its inhibitory effect on isolated enzymes. In fact, MTA may interfere with polyamine biosythesis by inhibiting propylamine synthase (28, 30) and may interfere with transmethylation reactions, either directly, by inhibiting protein methylase I (8), protein methylase II (18), and DNA modification methylases (24, 50) or, indirectly, by inhibiting *S*-adenosylhomocysteine hydrolase (16). If the inhibitory effects on the above-mentioned enzymes occur also *in vivo*, they might allow one to speculate on the involvement of the nucleoside in the differentiation process. In fact, it has been suggested that polyamine synthesis (20) or the diamine:polyamine ratio within the cell (19) might play a role in FLC induced differentiation.

The known inhibitors of FLC induced differentiation may be divided into 2 classes, according to the possibility as to whether their action can be reverted or not by the addition of polyamines (20). The action of inhibitors like dexamethasone or 12-*O*-tetradecanoylphorbol-13-acetate can be reverted by polyamine addition, whereas the inhibitory action of bromodeoxyuridine cannot be reverted by the same addition. Since the MTA effect is not reversible by exogenous polyamines, the nucleoside seems to behave more similarly to bromodeoxyuridine than to dexamethasone or 12-*O*-tetradecanoylphorbol-13-acetate.

As far as the inhibition of transmethylation reactions is concerned, the DNA-methylases inhibition (24, 50) probably is not related to the inhibition of the differentiated phenotype for 2 reasons: it seems that DNA undermethylation is involved in gene expression rather than in repression; and no differences are reported in the pattern of DNA methylation in either the α- or β-globin genes after the induction of FLC to differentiate (41).

The protein methylases block exerted by MTA, on the other hand (8, 18), could provide a working hypothesis for an explanation of the mode of action of the nucleoside on FLC induced differentiation. In fact, it has been demonstrated that in neuroblastoma cells induced to differentiate by Me_2SO there is an increase in the capacity of membrane-bound proteins to be carboxymethylated (23). Furthermore, we have shown that in the FLC system there is an increase of the protein methylase II activity (carboxymethylase) after Me_2SO treatment.[4] Protein methylation, therefore, could play a role in the expression of the differentiative program, which could be affected in a negative fashion by MTA. The recent observation that MTA can inhibit indirectly cellular transmethylation, blocking *S*-adenosylhomocysteine hydrolase and then increasing the intracellular content of *S*-adenosylhomocysteine (a powerful inhibitor of the transmethylation reactions) (43), supports the hyphothesis that MTA can act by inhibiting protein methylation. Further experiments, including the assays of intracellular levels of *S*-adenosylhomocysteine in FLC following exposure to thioether, are necessary to test this hypothesis.

The assays of the enzymatic activities involved in MTA synthesis and catabolism indicate that a MTA decrease might occur in FLC undergoing erythroid maturation. In fact, we noticed that aminopropyl transferase (the enzyme responsible for MTA biosynthesis) activities decreased in Me_2SO-treated cells when compared to the untreated controls, while the activity of the catabolic enzyme, MTA phosphorylase, remained almost unmodified. One can speculate that a MTA decrease might be a signal for the cell to undergo differentiation or, alternatively, that a high level of MTA is incompatible with the occurrence of the maturative process. The exogenous administration of the drug could mimic the latter situation, preventing the Friend cells from differentiating. If this were the case, MTA might be regarded as an endogenous regulator of the Me_2SO-induced erythroid differentiation of FLC.

[4] P. Galletti, D. Ingrosso, C. Manna, M. Grieco, and P. P. Di Fiore, unpublished observations.

Further work is in progress in our laboratory aimed at verifying whether other differentiating inducible systems behave like FLC or the human K 562 cell line upon the simultaneous addition of inducers of differentiation and MTA.

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