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N^6-METHYLADENOSINE INHIBITS MURINE ERYTHROLEUKEMIA CELL MATURATION BY BLOCKING METHYLATION OF RNA AND MEMORY VIA CONVERSION TO S-(N^6-METHYL)-ADENOSYLHOMOCYSTEINE

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Abstract—We have shown earlier that N^6-methyladenosine (N^6mAdo) and other methylated derivatives block commitment of murine erythroleukemia (MEL) cells to terminal erythroid maturation. In this study, we further investigated the mechanism of this blockade. Treatment of MEL cells with N^6mAdo inhibited cell growth, prevented accumulation of committed cells, suppressed methylation of total cytoplasmic RNA, and erased the expression of "memory" response, an event that precedes initiation of commitment. Furthermore, N^6mAdo increased the level of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) and altered the SAH/SAM ratio that influences methylation of ribonucleic acid (RNA). Moreover, analysis of the intracellular extracts revealed that N^6-mAdo is converted into S-(N^6-methyl)-adenosylhomocysteine (N^6-SAH) in MEL cells, an active intermediate that affects methylation of RNA. Therefore, we conclude that N^6-mAdo prevents induction of MEL cell differentiation by affecting methylation of critical RNA transcripts involved in expression of "memory" and initiation of commitment. It is likely that this inhibition occurs *via* conversion of N^6mAdo into N^6-SAH.

Key words: MEL; differentiation; RNA; methylation; SAM; SAH; N^6-methyladenosine; SAH hydrolase; S-(N^6-methyl)-adenosylhomocysteine

The precise cellular and molecular mechanisms *via* which chemical and natural agents initiate commitment of MEL† or Friend cells to terminal erythroid maturation are still not very clear regardless of the remarkable progress made in the field [1–3]. Earlier studies with metabolic inhibitors revealed that initiation of commitment to terminal maturation depends on the synthesis of new RNA and proteins [2, 4]. In addition, differentiation of MEL cells was shown to be associated with DNA hypomethylation [5] as well as posttranscriptional modifications of RNA [6, 7] and other biochemical events reviewed elsewhere [1]. Exposure of MEL cells to an inducer for several hours and subsequently plating them in inducer-free medium leads to expression of "memory" response (*i.e.* the acquired ability of cells to respond and commit immediately upon rechallenge with the inducer) [8].

As we have shown recently, induction of erythroid differentiation of MEL cells is associated with hypermethylation of RNA and agents such as N^6mAdo, neplanocin A, 3-deazaneplanocin A, and cycloleucine, all of which affect the methylation cycle and inhibit the differentiation process [9, 10]. N^6mAdo and its derivatives inhibit initiation of commitment and decrease the cytoplasmic accumulation of globin RNA transcripts by blocking a central process required for commitment to terminal maturation [9].

In the present study, we have extended these and more recent observations [6, 7, 11], and investigated the possible mechanism by which N^6mAdo inhibits initiation of commitment in MEL cells. Specifically, we investigated the effect of N^6mAdo on both the synthesis and methylation of RNA, on the expression of "memory" and accumulation of 2 active methylation cycle intermediates SAM and SAH. Radiolabeled N^6-[methyl-^{14}C]-adenosine (N^6-[methyl-^{14}C]Ado) synthesized in our own laboratory was used to explore the metabolic fate of N^6-methylated derivatives of adenosine in MEL cells. Here, we present evidence indicating that treatment of MEL cells with N^6mAdo: (a) leads to intracellular formation of N^6-SAH; (b) erases expression of "memory" response to inducer DMSO in these cells; (c) inhibits initiation of commitment in DMSO-treated cells; (d) reduces the extent of methylation of total cytoplasmic RNA; and (e) alters the intracellular accumulation of both SAM and SAH (the latter affects the SAH/SAM ratio which, in turn, influences the rate of RNA methylation). These data indicate that N^6mAdo is converted into N^6-SAH in MEL cells, an intermediate that might be responsible for blocking methylation of RNAs involved in expression of "memory" and initiation of commitment in MEL cells. Overall, these results suggest that RNA methylation appears to be one of the key regulatory events in the process of erythroid differentiation.

MATERIALS AND METHODS

Chemicals and biochemicals

DMSO was purchased from Mallinckrodt, Inc. (St. Louis, MO, U.S.A.). N^6-mAdo, SAM and SAH were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N^6-SAH was synthesized in our laboratory ac-

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† Abbreviations: MEL, murine erythroleukemia; DMSO, dimethylsulfoxide; N^6mAdo, N^6-methyladenosine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; and N^6-SAH, S-(N^6-methyl)-adenosylhomocysteine.

cording to methods described previously [12, 13]. Radiolabeled N^6-[methyl-^{14}C]-Ado was prepared by using [methyl-^{14}C]-iodide as methylating agent and known methods reported for the synthesis of the unlabeled compound [14, 15] (the synthesized compound was identified by melting point, R_f values obtained in liquid-solid chromatography, and analysis by the HPLC system described in this paper). The resulting radiolabeled compound has a specific activity of 5000 cpm/μmole. Analytical grade potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany). L-[methyl-^{3}H]-methionine (80.0 Ci/mmol) and [5,6-3H]-uridine (39.6 Ci/mmol) were obtained from NEN Research Products (Boston, MA, U.S.A.) and [^{35}S]-methionine (1000 Ci/mmol) from Amersham (England).

Cell cultures

Cells employed throughout this study were MEL-745PC-4A, a clone of MEL-745 cells obtained by subcloning and subsequent testing of clones derived for a high degree of inducibility. All cultures were maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco, Long Island, NY, U.S.A.) and antibiotics (penicillin and streptomycin 100 μg/mL). Cells were incubated at 37°C in a humidified atmosphere containing 5% v/v CO_2 and maintained at densities that permitted logarithmic growth (1×10^5 to 1×10^6 cells/mL).

Induction experiments for the determination of benzidine-positive cells and clonal assessment of commitment

Cells were incubated with each agent as described under individual figures. At certain timed intervals as indicated in the text, the proportion of differentiated (hemoglobin-producing) cells was assessed cytochemically with benzidine-H_2O_2 solution [16]. The number of committed cells was assessed by the plasma clot clonal assay as described originally by Gusella *et al.* [17].

Assessment of the rate of RNA synthesis

Cells (1×10^6) were removed from cultures and pulse-labeled with 2 μCi/mL [5,6-^{3}H]-uridine (sp. act. 39.6 Ci/mmol) at 37°C for 90 min. At the end of this period, cells were precipitated with an ice-cold solution of TCA (10% w/v) and the insoluble material collected on filters (Whatman GF/B) and washed with 5% w/v TCA solution. The filters were washed with ethanol-ether (1:1), air-dried thereafter, and counted for radioactivity in a scintillation counter.

Determination of intracellular levels of SAM and SAH in MEL cells by HPLC analysis

The procedure used for the detection and quantitation of the intracellular levels of SAM and SAH in MEL cells was the same as previously described [18] and modified by us, accordingly [10]. Briefly, MEL cells at relatively low density (1×10^5 cells/mL) were treated with each agent as indicated in the text. At different times, cells (2×10^6) were removed from cultures and labeled for 3 hr at 37°C with 10 μCi/mL [^{35}S]-methionine (1000 Ci/mmol at reference date) in DMEM supplemented with 5% FCS. At the end of this period, the cells were resuspended in 1M $HClO_4$ solution, sonicated for 10 sec and centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was collected and analyzed by high-performance liquid chromatography (HPLC). The HPLC system was calibrated with the use of standard compounds (adenosine, N^6mAdo, SAM, SAH, and N^6-SAH) with known retention times (see Results). Samples of 20 μL of acid-soluble cell extracts were loaded on the reversed-phase column (RP-18, 5 μm, 25 cm × 4.6 mm I.D.) and analyzed using a linear gradient system consisting of 0.02M KH_2PO_4, pH 3.8 and methanol. Concentrations of methanol ranging from 0% to 35% were used for elution within 30 min. The flow rate was 1 mL/min and detection of SAM and SAH was carried out at 254 nm. Fractions (0.5 mL/30 sec) were collected and counted for radioactivity in a liquid scintillation counter. The column was re-equilibrated for at least 15 min with the low-strength eluent between 2 different runs. Using this procedure, the retention times were reproducible.

Detection of the intracellular intermediate N^6-SAH in MEL cells treated with N^6mAdo

MEL cells were incubated either with N^6-[methyl-^{14}C]-Ado or cotreated with [^{35}S]-methionine and N^6mAdo for times indicated under each figure. At the end of each time interval acid-soluble cellular extracts were isolated and analyzed by HPLC, according to the methods described above for the assessment of SAM and SAH levels in the same cells under similar conditions. N^6-SAH labeled with [^{14}C]- or [^{35}S]- was detected repeatedly as an intracellular intermediate of N^6mAdo in MEL cells and identified by its retention time in HPLC analysis. Unlabeled N^6-SAH prepared by us served as a standard marker (see details under the section Chemicals and biochemicals).

RESULTS

N^6-methyladenosine affects cell growth, commitment, and RNA synthesis in MEL cells

To assess the effects of N^6mAdo treatment on cell growth and RNA synthesis, we exposed cells to N^6mAdo, DMSO, and/or both agents and determined the rate of cell growth and RNA synthesis (by incorporation of [5,6-^{3}H]-uridine into RNA). Cells grown in the absence of any agent served as control experiment. As shown in Fig. 1, continuous exposure of MEL cells to N^6mAdo (0.5 mM) decreased cell growth by 40%–50% (Fig. 1A) and reduced the rate of RNA synthesis by 30%–40% (Fig. 1C). However, treatment of cells with both DMSO and N^6mAdo did not depress the rate of RNA synthesis more than DMSO alone (Fig. 1C), whereas cell growth was reduced by 40%–50% (Fig. 1A) and the number of committed cells diminished by 70%–80% (Fig. 1B). These data indicate that, although N^6mAdo suppressed cell growth and abrogated commitment, this agent exerted much less suppressive effect on RNA synthesis as compared to DMSO alone.

N^6methyladenosine erases "memory" response of MEL cells to terminal maturation

In a previous report, we showed that N^6mAdo inhibits initiation of commitment of MEL cells by blocking a central process in the differentiation program [9]. To demonstrate whether or not N^6mAdo blocks even biochemical events that precede initiation of commitment such as expression of "memory" response, we exposed

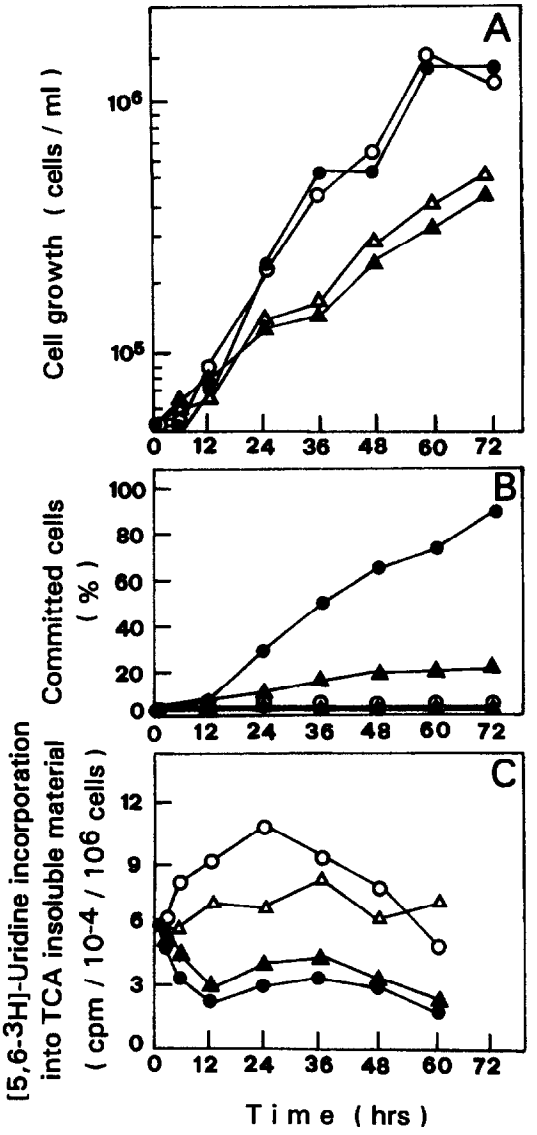

Fig. 1. Effect of N^{6}mAdo on cell growth, commitment, and RNA synthesis in control and DMSO-treated MEL cells. MEL-745PC-4A cells were incubated in DMEM supplemented with 10%v/v FCS with the following additions: with (—●—) and without (—○—) DMSO (1.5%v/v), and with N^{6}mAdo (0.5 mM) in the presence (—▲—) and absence (—△—) of DMSO (1.5%v/v). At times indicated, cell growth (panel A), accumulation of committed cells (panel B), as well as synthesis of cytoplasmic RNA (panel C) were determined as described under Materials and Methods. The values represent the mean values of 2 separate experiments.

cells to N^{6}mAdo in the presence or absence of DMSO in an attempt to determine whether treatment with N^{6}mAdo erases "memory." As shown in Fig. 2A, cells exposed to DMSO alone exhibited a typical kinetics of differentiation determined by gradual accumulation of committed cells. However, removal of the inducer from the culture after 24 hr, reincubation of cells in inducer-free medium in the presence or absence of N^{6}mAdo for 16 hr, and rechallenge of culture with DMSO in fresh drug-free medium thereafter indicated that MEL cells primed with DMSO and exposed to N^{6}mAdo had lost their "mem-

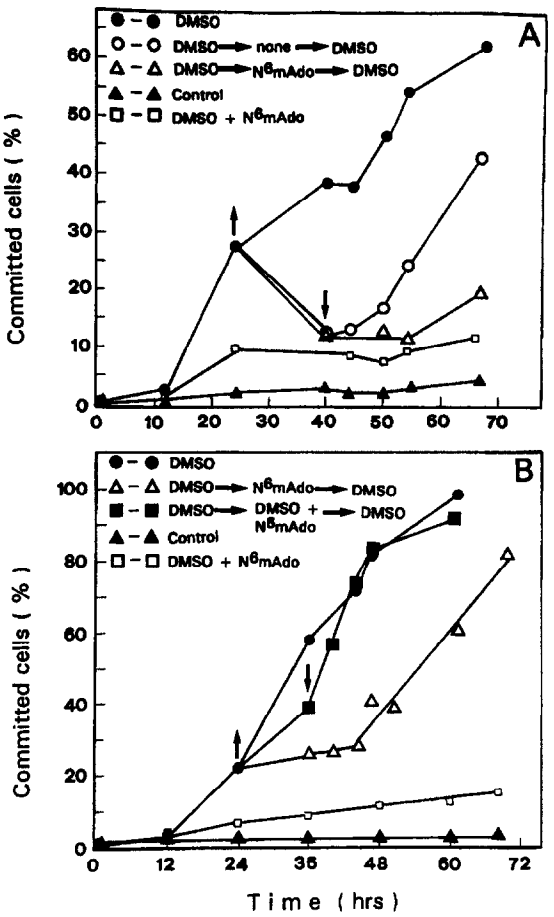

Fig. 2. N^{6}mAdo erases "memory" response expressed in DMSO-treated MEL cells. Panel (A) MEL-745PC-4A cells were incubated in DMEM with DMSO (1.5%v/v) (—●—). At the time indicated by the first arrow, cells were harvested from culture, washed out of DMSO with fresh medium, resuspended in DMEM, and divided into 3 cultures. The first culture was incubated with DMSO alone (1.5%v/v) (—●—), the second with drug-free medium (—○—), and the third with N^{6}mAdo (0.5 mM) in the absence of DMSO (—△—) for 16 hr. By the end of this period (indicated by the second arrow), the cells from either culture were harvested, washed out of any agent, and finally re-incubated with DMSO alone (1.5%v/v). At time intervals indicated, the cells were removed from cultures and cloned in plasma clots as described earlier [17] for the determination of committed cells. Cells exposed continuously to neither (—▲—) or both DMSO and N^{6}mAdo (—□—) served as control experiments. Panel (B) MEL cells were incubated with DMSO alone (1.5%v/v) (—●—). After 24 hr as indicated by the first arrow, the DMSO-treated culture was divided into 3 cultures. The first culture contained DMSO as before (—●—). The second was treated with N^{6}mAdo (0.5 mM) in the absence of DMSO (1.5%v/v) (—△—), and the third with DMSO (1.5%v/v) in the presence of N^{6}mAdo (0.5 mM) (—■—) for 12 hr. At the end of this incubation time (indicated by the second arrow), the cells from each culture were harvested, washed out of any agent with fresh medium, and finally re-incubated with DMSO (1.5%v/v). At time intervals indicated, cells were removed from cultures and cloned in plasma clots as described earlier [17] for the determination of committed cells. Cells exposed continuously to neither (—▲—) or both DMSO and N^{6}mAdo (—□—) served as control experiments.

ory" response (Fig. 2A). Cells exposed to N^6mAdo in absence of DMSO required several hr to recapitulate the latent period upon rechallenge with the inducer and begin to commit. This indicates that N^6mAdo prevents a process responsible for the expression of "memory" response. The next question we then asked was whether or not N^6mAdo was able to do so in the presence of DMSO. As shown in Fig. 2B, cells pretreated with DMSO for 24 hr, then incubated with both DMSO and N^6mAdo for 12 hr and rechallenged with DMSO alone, continue to commit and differentiate like cells exposed to DMSO alone from the beginning. This finding was observed despite the short exposure of cells to N^6mAdo that forced them to recapitulate the latent period in the absence of DMSO for the intermitted period shown in Fig. 2A. Overall, these data indicate that N^6mAdo abrogates the "memory" response by blocking a critical event preceding commitment only in the absence of the inducer.

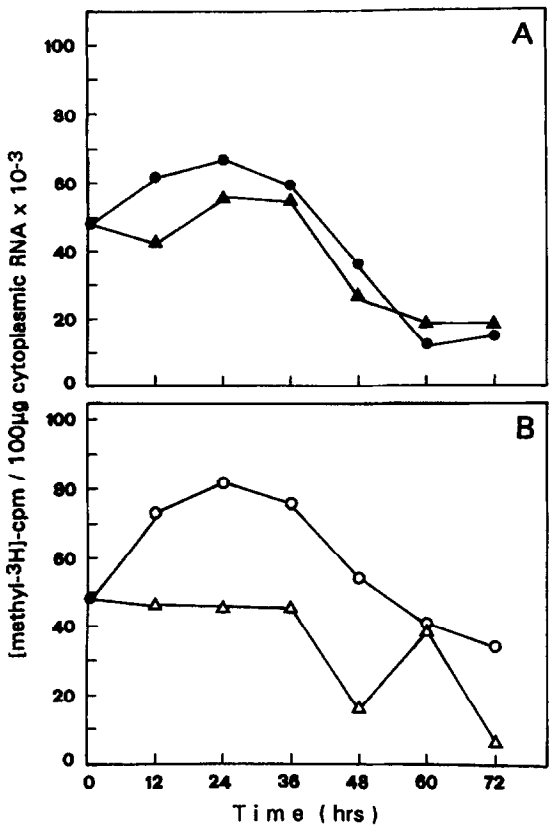

Fig. 3. Effect of N^6mAdo treatment on total cytoplasmic RNA methylation in MEL cells incubated in the presence or absence of DMSO. MEL-745PC-4A cells were incubated in DMEM supplemented with 10%v/v FCS with the following additions: with (—○—) and without (—●—) DMSO (1.5%v/v), and with N^6mAdo (0.5 mM) in the presence (—△—) and absence (—▲—) of DMSO (1.5%v/v). At the times indicated, cells (3–7 × 10^7) were removed from cultures, washed out of any drug, resuspended in fresh DMEM supplemented with 10% FCS, and pulse-labeled with 30 µCi/mL of L-[methyl-^{3}H]-methionine (sp. act. 80.0 Ci/mmol) at 37°C for 3 hr. At the end of this time period the total [methyl-^{3}H]-cytoplasmic RNA was purified and counted for radioactivity in a liquid scintillation counter, as described under Materials and Methods. Each value represents the mean of at least 2 separate experiments.

Table 1. Effect of N^6mAdo treatment on cytoplasmic accumulation of SAM and SAH in MEL cells incubated in the presence or absence of DMSO

Time (hr)	Treatment	SAM ([^{35}S]-cpm/2 × 10^6 cells)	SAH ([^{35}S]-cpm/2 × 10^6 cells)	Ratio SAH/SAM
6	None	6838	154	0.022
	N^6mAdo	6384	278	0.043
	DMSO	7621	131	0.017
	DMSO + N^6mAdo	5071	321	0.063
12	None	5402	277	0.051
	N^6mAdo	4336	380	0.088
	DMSO	3704	102	0.027
	DMSO + N^6mAdo	5887	326	0.055
24	None	4568	228	0.050
	N^6mAdo	7784	464	0.060
	DMSO	3196	77	0.024
	DMSO + N^6mAdo	4775	392	0.082
36	None	3377	170	0.050
	N^6mAdo	7493	380	0.051
	DMSO	3110	58	0.019
	DMSO + N^6mAdo	8186	299	0.036
48	None	2498	179	0.072
	N^6mAdo	8094	433	0.053
	DMSO	2626	58	0.022
	DMSO + N^6mAdo	4618	260	0.056
60	None	3122	220	0.070
	N^6mAdo	7620	355	0.047
	DMSO	2006	54	0.027
	DMSO + N^6mAdo	3436	227	0.066

MEL-745PC-4A cells were treated as described in Fig. 1. At times indicated, cells were pulse-labeled with [^{35}S]-methionine and the hydrolysates isolated were analyzed by HPLC, as shown in Materials and Methods. The peak of radioactivity corresponding to SAM and SAH is indicated above and is the mean value of 2 separate measurements.

N^6-methyladenosine inhibits methylation of total cytoplasmic RNA

Earlier studies with adenosine analogues such as cordycepin (3′-deoxy-adenosine), an agent that affects RNA methylation and commitment [19, 20], prompted us to investigate whether the effects of N^6mAdo on MEL differentiation might be related to its effect on RNA methylation. Indeed, as shown in Fig. 3, N^6mAdo suppressed the transient increase in methylation of cytoplasmic RNA observed in DMSO-treated cells, but not in cells treated with N^6mAdo alone. In particular, cells treated with DMSO alone showed an increase in methylation of cytoplasmic RNA after 12 hr incubation, an effect reported earlier [10]. This hypermethylation, however, was not observed in cells treated simultaneously with both DMSO and N^6mAdo (Fig. 3B). Treatment of MEL cells with N^6mAdo alone suppressed the level of methylation of cytoplasmic RNA in control untreated cells, but to a lesser extent than DMSO-treated cells (Fig. 3A and 3B).

N^6-methyladenosine alters the intracellular level of SAM and SAH in MEL cells

The decrease in methylation seen in RNA in differentiating MEL cells could be attributed to depletion of intracellular SAM, to inactivation of RNA methyltrans-

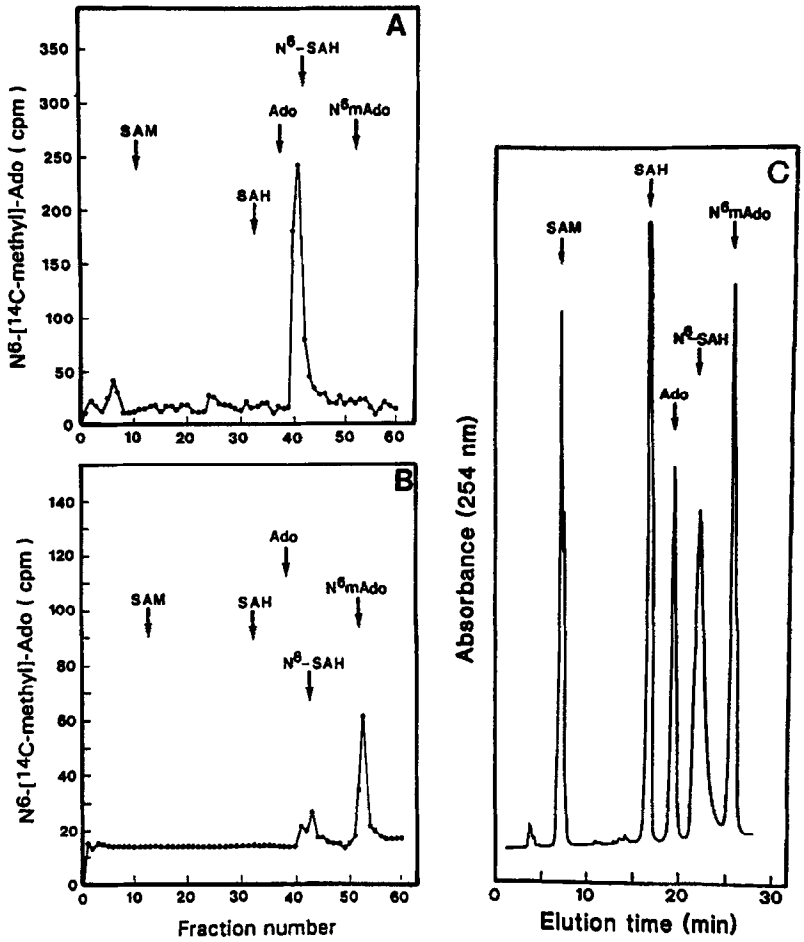

Fig. 4. Detection and identification of N^6-SAH as a metabolite of N^{6}mAdo in MEL cells after their treatment with N^6-[methyl-^{14}C]-Ado. MEL-745PC-4A cells (3×10^7) were incubated in DMEM supplemented with 10%v/v FCS at 37°C with 70,000 cpm/mL N^6-[methyl-^{14}C]-Ado (sp. act. 5000 cpm/μmole) for 6 hr. At the end of this time, cells were washed with PBS, processed for isolation of cellular hydrolysates as described under Materials and Methods, and analyzed by the HPLC system described for the detection of SAM and SAH. The radioactivity profile obtained is shown in panel (A). In panel (B) the same experiment (1×10^7 cells) is represented with one difference. In this study, cell hydrolysates were dissolved in 0.02M KH_2PO_4, pH 3.8 and heated at 55°C for 30 min before HPLC analysis. The arrows indicate the position of elution of standard agents chromatographed under the same conditions, as indicated in Panel (C). Panel (C) HPLC elution pattern of a mixture consisting of the standard compounds SAM, SAH, Ado, N^6-SAH, and N^{6}mAdo (1–3 nmoles each) which were chromatographed on a reversed-phase column (RP-18, 5 μm, 25 cm × 4.6 mm) as described under Materials and Methods. (Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Ado, adenosine; N^6-SAH, S-N^6-methyladenosylhomocysteine, and N^{6}mAdo, N^6-methyladenosine.)

ferases, or to alteration of intracellular accumulation of SAH. The ratio of intracellular levels of both SAH and SAM influences the rate of methylation reactions *via* the active methylation cycle [21]. To examine whether treatment with N^{6}mAdo altered the SAH/SAM ratio, we measured the intracellular levels of these intermediates in control and DMSO-treated cells, as well as in those exposed to N^{6}mAdo in the presence or absence of DMSO, by HPLC analysis. The data (Table 1) show that control untreated cells growing from low (1×10^5 cells/mL) to higher density (1×10^6 cells/mL) toward the stationary phase exhibit a gradual increase in the SAH/SAM ratio. This finding suggests that these changes in the SAH/SAM ratio are growth-dependent. Comparing these changes with the rate of total RNA synthesis seen in Fig. 1C, we conclude that the SAH/SAM ratio reaches a relatively high value at the time when RNA synthesis declines in stationary phase cells. Therefore, an inverse relationship between RNA synthesis and the SAH/SAM ratio may exist. However, when cells were treated continuously with DMSO, the SAH/SAM ratio remained lower than that of control untreated cells, as did RNA synthesis (see Fig. 1C). Exposure of cells to N^{6}mAdo in the presence or absence of DMSO kept the SAH/SAM ratio at a relatively high level in both cultures. These data suggest that the changes in the levels of both the SAH/SAM ratio and RNA synthesis in DMSO- and N^{6}mAdo-treated cells occur as part of the differentiation process in MEL cells. DMSO and N^{6}mAdo, an inducer and inhibitor, respectively, of differentiation that modulate RNA methylation, affect the SAH/SAM ratio in 2 opposite directions.

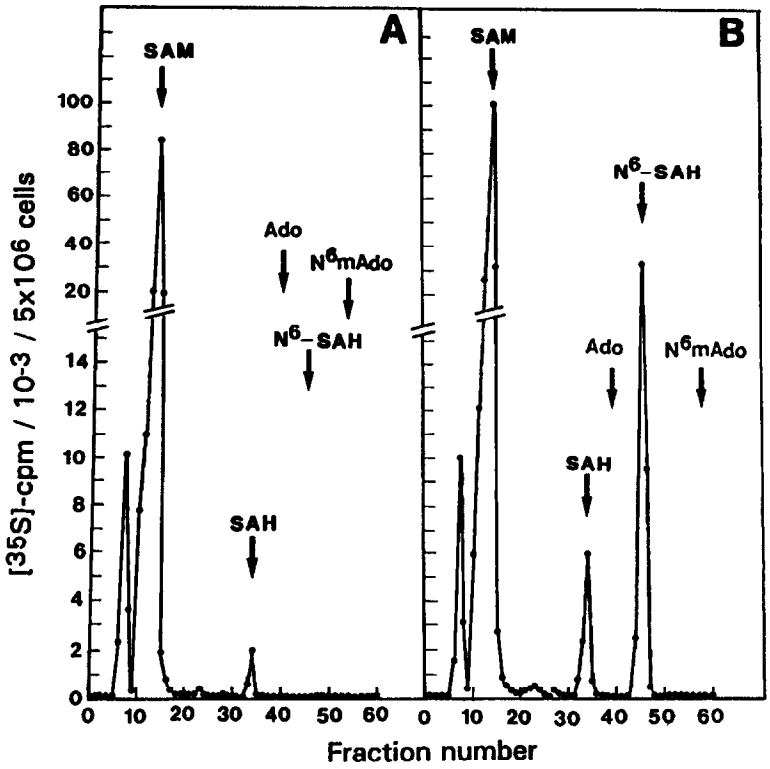

Fig. 5. Detection and identification of N^6-SAH as a metabolite of N^6mAdo in MEL cells cotreated with [^{35}S]-methionine and N^6mAdo. MEL-745PC-4A cells (3×10^7) were incubated in DMEM supplemented with 5%v/v FCS at 37°C with 10 μCi/mL [^{35}S]-methionine for one hr. At the end of this time, the culture was divided into 2 cultures, one treated with no agent, Panel (A), and the other with 1 mM N^6mAdo for 2 additional hours at 37°C. Cellular hydrolysates were isolated and analyzed by HPLC, as described under Materials and Methods for the detection of SAM and SAH as shown in Fig. 4. The arrows indicate the position of elution of standard agents chromatographed under the same conditions (Abbreviations: as shown under Fig. 4).

N^6mAdo is converted into S-(N^6-methyl)-adenosylhomocysteine in MEL cells

The inhibition of RNA methylation as well as the increase in the SAH/SAM ratio seen in MEL cells treated with N^6mAdo, as reported above, might be attributed to intracellular formation of metabolite(s) of N^6mAdo in MEL cells. To explore this possibility, radiolabeled N^6-[methyl-^{14}C]-Ado was employed. Treatment of MEL cells with N^6-[methyl-^{14}C]-Ado led to an intracellular accumulation of N^6-SAH that was detected by applying the HPLC column chromatography system shown in Fig. 4C and identified both by its retention time (Fig. 4A) and the fact that treatment with 0.02M KH_2PO_4 buffer, pH 3.8 at 55°C for 30 min yielded radiolabeled N^6mAdo as expected (Fig. 4B). The latter treatment hydrolyzes thioether compounds such as N^6-SAH and yields N^6mAdo. The formation of N^6-SAH was further confirmed by applying an alternative approach. MEL cells were labeled with [^{35}S]-methionine in the presence or absence of N^6mAdo and the cellular extracts were then analyzed by HPLC as above. As illustrated in Fig. 5A, treatment of cells with [^{35}S]-methionine in the absence of N^6mAdo resulted in the detection of radioactive [^{35}S]-SAM and [^{35}S]-SAH as expected. However, cells exposed simultaneously to both N^6mAdo and [^{35}S]-methionine yielded a peak of radioactivity eluted at the position of N^6-SAH, as well as peaks corresponding to both [^{35}S]-SAM and [^{35}S]-SAH, respec-

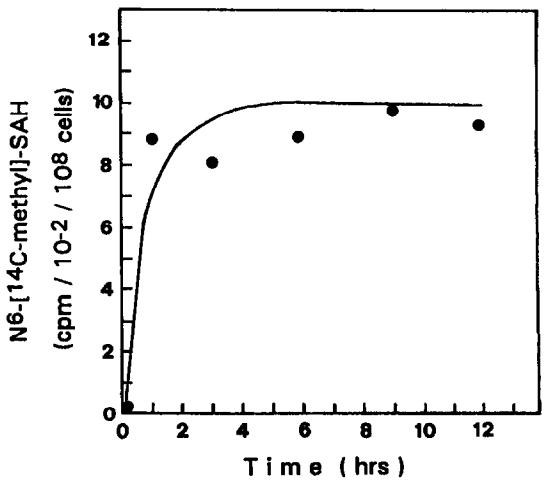

Fig. 6. Kinetics of formation of N^6-SAH in MEL cells treated with N^6-[methyl-^{14}C]-Ado. MEL-745PC-4A cells were incubated in DMEM supplemented with 5%v/v FCS with 70,000 cpm/mL N^6-[methyl-^{14}C]-Ado (sp. act. 5000 cpm/μmole). At time intervals indicated, cells were removed from culture and assessed for the formation of N^6-SAH, as described in Fig. 4. The amount of radioactivity corresponding to N^6-SAH at each time point is indicated above.

tively (Fig. 5B). Kinetic analysis of the accumulation of N^6-SAH in MEL cells showed that this metabolite is formed quite rapidly after treatment with N^6mAdo and reaches a plateau level within an hr of incubation (Fig. 6). Cells treated with DMSO in the absence or presence of N^6mAdo accumulated significantly smaller amounts of N^6-SAH as compared to untreated control and N^6mAdo-treated cells (data not shown).

DISCUSSION

Although it has been shown that MEL cell differentiation is blocked by agents that inhibit methylation of RNA such as cordycepin [19, 20], 3-deazaadenosine [22], S-5′-isobutylthioadenosine, and 5′-methylthioadenosine [23], neplanocin A, 3-deazaneplanocin A, and cycloleucine [7, 10], the precise mechanism(s) of this inhibition is still not clear. In an effort to underline the mechanism(s) by which N^6mAdo prevents erythroid differentiation of MEL cells [9] and uncover the potential role of RNA methylation [10], we determined the effects of this agent on commitment, RNA methylation, and expression of "memory," the unique ability of cells to remember earlier signals for differentiation. Moreover, we examined whether or not N^6mAdo in MEL cells is converted into an active intermediate that affects the active methylation cycle.

It is known that MEL cells induced to differentiate *in vitro* express a "memory" response [8] that is sensitive to cordycepin and cycloheximide, as well as dexamethasone [24, 25]. The data presented in Fig. 2A indicate that N^6mAdo erases the "memory" of MEL cells to a previous exposure to DMSO, while the "memory" in MEL cells exposed to both DMSO and N^6mAdo during the intermitted period of DMSO treatment remained intact (Fig. 2B). This is consistent with the effects of cordycepin and cycloheximide, where the presence of DMSO during the inhibitor-treatment period was sufficient to prevent erasure of "memory" response caused by these agents [24]. Furthermore, the fact that cordycepin and N^6mAdo both inhibit RNA methylation suggests that this process may be essential for some RNAs involved in the expression of "memory," a prerequisite step for initiation of commitment.

We have recently demonstrated that induction of MEL cell differentiation is causally related to hypermethylation of polyA$^+$ RNA at specific sites [10]. N^6mAdo blocks MEL cell differentiation and inhibits methylation of total cytoplasmic RNA (Fig. 3). This inhibition was higher in DMSO-treated than in control untreated MEL cells. N^6mAdo reversed the pattern of hypermethylation seen in DMSO-induced MEL cells without substantially affecting the overall RNA synthesis of these cells (Fig. 1C).

As reported previously, N^6mAdo is a substrate as well as a competitive inhibitor of the enzyme S-adenosylhomocysteine hydrolase (SAH hydrolase) (k_i 2×10^{-4}M) [26, 27], responsible for the hydrolysis of SAH, which inhibits methylation reactions when accumulated intracellularly [21, 28]. Although the reaction catalyzed by SAH hydrolase is reversible to the direction of synthesis, physiologically the reaction proceeds in the direction of hydrolysis due to the existence of enzymes that further metabolize the products of SAH hydrolysis [21, 28]. In addition, it is known that N^6mAdo is not a substrate for adenosine deaminase [29] and is, therefore, not inactivated by this enzyme. Our observation that treatment of MEL cells with N^6mAdo led to the intracellular accumulation of N^6-SAH is consistent with previous studies showing formation of N^6-SAH in mouse liver [29], lymphocytes [30] and, after incubation of N^6mAdo, with purified SAH hydrolase [26]. Inhibition of SAH hydrolase by N^6mAdo may be responsible for accumulation of SAH in MEL cells and reversion in the SAH/SAM ratio seen in DMSO-treated cells. With respect to the latter point, we have observed two types of changes in the SAH/SAM ratio: growth-dependent as seen in control untreated cells where cells undergo changes from the exponential to the stationary phase and differentiation-dependent changes that appear to be affected by DMSO and N^6mAdo. Alternatively, N^6-SAH can act as an inhibitor of RNA methyltransferases, enzymes involved in the methylation of RNA, as has been reported previously [29, 31]. The notion that SAH hydrolase was affected in MEL cells exposed to N^6mAdo so as to influence the extent of RNA methylation during MEL cell differentiation is further supported by the fact that other known inhibitors of SAH hydrolase, such as 3-deazaadenosine, S-5′-isobutylthioadenosine, 5′-methylthioadenosine, neplanocin A, and 3-deazaneplanocin A, or inhibitors of SAM synthetase such as cycloleucine, inhibit MEL cell differentiation as N^6mAdo [10, 21–23]. Changes in the extent of RNA methylation resulting from alterations in the levels of both SAH and SAM may affect the physicochemical behavior of RNAs due to increased hydrophobicity and possible interactions with cytoplasmic proteins that influence RNA stability [11] as seen previously [32–39]. If so, we can then assume that RNA methylation may be a key event in hemopoietic cell maturation, as we proposed elsewhere [6, 10].

Overall, the data presented here strongly support our hypothesis that N^6mAdo and related agents may block initiation of commitment and erase "memory" response in MEL cells *via* inhibition of RNA methylation, a process that may be responsible for induction of differentiation and for differential RNA stability seen in differentiating MEL cells [10, 11, 32, 33, 40, 41].

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