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ANALYSIS OF THE INHIBITION OF COMMITMENT OF MURINE ERYTHROLEUKEMIA (MEL) CELLS TO TERMINAL MATURATION BY N^6-METHYLADENOSINE

IOANNIS S. VIZIRIANAKIS,* WILLIE WONG† and ASTERIOS S. TSIFTSOGLOU*‡

*Laboratory of Pharmacology, Department of Pharmaceutical Sciences, School of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, 540 06, Greece; and †Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, MA 02215, U.S.A.

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Abstract—Treatment of cultured murine erythroleukemia (MEL or Friend) cells with N^6-methylated derivatives of adenosine inhibited erythroid cell differentiation induced by various agents. N^6-Methyladenosine (N^6mAdo) inhibited initiation of commitment to terminal maturation and prevented accumulation of hemoglobin in a concentration-dependent manner. Treatment with N^6mAdo slowed cell growth without causing substantial inhibition in the rate of DNA synthesis and a marked decrease in viability and clonogenic potential of MEL cells. Furthermore, N^6mAdo decreased the cytoplasmic accumulation of β^{major} globin mRNA and affected its structural integrity in MEL cells. Cells pre-exposed to N^6mAdo failed to initiate commitment as early as control cells upon challenge with the inducer dimethyl sulfoxide. N^6mAdo-induced inhibition of commitment was not reversed but rather was potentiated by the presence of adenine, L-homocysteine and/or L-methionine, agents involved in the active methylation cycle. To this respect, N^6mAdo-induced inhibition of commitment was found to be different from that caused by cordycepin (3′-deoxyadenosine, an inhibitor of RNA methylation and mRNA polyadenylation). The latter inhibition was fully reversed by the addition of L-methionine. These findings indicate that N^6-methyladenosine: (a) blocks a central process that is required for initiation of commitment; and (b) decreases accumulation of β^{major} globin mRNA, causes mRNA degradation and prevents hemoglobin synthesis. Due to the differential sensitivity of N^6mAdo- and cordycepin-induced blockade of commitment to L-methionine, these agents inhibit commitment by acting via two different mechanisms impinging on the final pathway of MEL erythroid cell maturation.

A powerful experimental approach to delineate the cellular and molecular events of leukemic cell differentiation has been to treat cells with inhibitors having a known mechanism of action, monitor their effects and, based on their performance, try to identify those cellular processes which regulate hematopoietic cell differentiation at the individual cell level. Such an approach has been applied successfully thus far for the analysis of murine erythroleukemia (MEL§) cell differentiation and other systems as well [1–4]. We have learned, for example, that initiation of commitment of MEL cells depends on protein and mRNA synthesis [1, 5] as well as on DNA replication [6], that inducer-treated cells express "memory" to early signals which also depends on protein synthesis [7], and that commitment is a pivotal process which governs the expression of a series of genes [5]. Cordycepin, an adenosine analog, and other metabolic inhibitors were useful tools in such studies [1, 3, 5, 8].

The observations made by Christman *et al.* that MEL cell differentiation is associated with hypomethylation of DNA [9] and more recent studies, which indicate that adenosine analogs including 5′-methylthioadenosine [10, 11] and 3-deazaadenosine [12], acting as methylation modulators, inhibit MEL cell differentiation induced by various agents, prompted us to readdress the potential role of alterations in nucleic acid methylation during MEL cell differentiation. In a preliminary report, we observed that N^6-methyladenosine (N^6mAdo), a natural constituent of mammalian and viral mRNAs, inhibits commitment of MEL cells to terminal maturation [13].

In this study, we further investigated these observations and performed kinetic analysis of N^6mAdo-induced blockade of commitment, in an effort to uncover the possible mechanism(s) by which N^6mAdo inhibits MEL cell differentiation. Information obtained from these studies can be valuable in identifying molecular processes involved in hematopoietic cell differentiation.

MATERIALS AND METHODS

Chemicals. Dimethyl sulfoxide (DMSO) was purchased from Mallinckrodt, Inc., St. Louis, MO. *N,N*-Dimethylacetamide (DMA) and butyric acid was from Fluka Chemie, Buchs, Switzerland. 3,3′-Dimethoxybenzidine was from the Eastman Kodak Co., Rochester, NY. 2-Methyladenine hemi-sulfate, N^6-methyladenine, N^6-methyladenosine, N^6,N^6-dimethyladenine, N^6,N^6-dimethyladenosine, 3′-

‡ Corresponding author. Tel. (031) 991473; FAX (031) 991492.

§ Abbreviations: MEL, murine erythroleukemia; N^6mAdo, N^6-methyladenosine; DMSO, dimethyl sulfoxide; DMA, *N,N*-dimethylacetamide; HMBA, hexamethylene-bis-acetamide; PBS, phosphate-buffered saline; N^6mAde, N^6-methyladenine; and UDP-42-(3-ethylureido)-6-methylpyridine.

deoxyadenosine (cordycepin), adenine, L-methionine, L-homocysteine, hexamethylene-bis-acetamide (HMBA) and hypoxanthine all were purchased from the Sigma Chemical Co., St. Louis, MO. 2-(3-Ethylureido)-6-methylpyridine (UDP-4), a ureido derivative of pyridine, was prepared in our laboratory as described elsewhere [14]. Radiolabeled compounds for hybridization experiments were purchased from NEN, Boston, MA.

Biological materials and cell cultures. Cells employed throughout this study were a clone (MEL-745PC-4A) derived from the original MEL-745 cells [15] after subcloning and subsequent testing of the clones for high degree of inducibility. All cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) bovine serum (GIBCO, Grand Island, NY) and the antibiotics streptomycin and penicillin (100 μg/mL). Fresh medium was used to replenish the cultures every 2 days and to maintain cells in exponential growth. Only exponentially growing cells were used in the present study. Cultures were incubated at 37° in a humidified atmosphere containing 5% CO_2. Drug-treated cultures were diluted with fresh medium containing the agent. The cell number was determined with the use of a hemocytometer under a light microscope.

Determination of benzidine-positive cells and hemoglobin content. The proportion of hemoglobin-containing MEL cells accumulated in culture was evaluated microscopically by scoring more than 250 cells stained with benzidine/H_2O_2 as described elsewhere [16]. The content of cellular hemoglobin was determined spectrophotometrically as previously described [17].

Measurement of viability and clonogenic potential of MEL cells. The number of viable MEL cells treated under various conditions was determined by incubating cells with a buffer of 0.4% (w/v) trypan blue in phosphate-buffered saline (PBS) (pH 7.4) as previously described [18]. The ability of MEL cells (control and drug-treated) to form colonies was determined by subculturing a known number of cells into drug-free plasma clots and assessing the number of colonies that outgrow as described below.

Clonal analysis for the determination of committed cells. To determine the number of control and drug-treated cells which commit to terminal erythroid maturation, cells were removed from culture, washed twice with drug-free medium, and then plated in drug-free plasma clots as previously described [17]. A constant number of cells (400) were plated in 0.1-mL clots in microliter wells (96 wells/plate) (Linbro Scientific, Inc., Hamden, CT) that had been sterilized by UV irradiation; the cells were then incubated at 37° in a humidified 5% CO_2 atmosphere. After 96–120 hr, clots were transferred to microscopic slides, dehydrated with filter paper (Whatman 1MM), fixed with glutaraldehyde, stained with 3,3'-dimethoxybenzidine (1%, w/v) (3 min), treated with hydrogen peroxide solution (2%, v/v) (3 min), counterstained with hematoxylin (Harris Hematoxylin, Fisher Scientific Co., Fair Lawn, NJ) (5 min), covered with permount and a coverslip, and examined under a light microscope (magnification: 100×). Colonies outgrown from the plated cells were classified as committed and uncommitted. The first type contained only benzidine-positive cells and numbered less than 32. The second class of colonies contained only benzidine-negative cells and were greater than 32 cells in size. The number of colonies was directly proportional to the number of committed and uncommitted cells, respectively.

Isolation of RNA and assay of cytoplasmic accumulation of β^{major} globin mRNA by Northern blot hybridization analysis. Cytoplasmic RNA was prepared from control and drug-treated MEL cells at different time intervals during differentiation. MEL cells were harvested from culture, washed three times with ice-cold PBS (pH 7.4), and collected in a tube by centrifugation (2000 *g* for 5 min at 4°). The pellet was suspended in 0.2 mL of lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris·HCl, pH 6.8, 0.4% NP-40, 10 mM Vanadyl ribonucleoside complex), vortexed for 10 sec, overlayered on an equal volume of lysis buffer containing 24% sucrose and 0.2% NP-40 and centrifuged at 6000 *g* for 10 min (4°) in an Eppendorf microcentrifuge equipped with a swing out rotor head. The supernatant was collected, digested with proteinase K (200 μg/mL) for 30 min at 37°, extracted with phenol/chloroform, precipitated with ethanol in the presence of 0.3 M sodium acetate, dried and finally dissolved with TE buffer (10 mM Tris·HCl, pH 7.0, 1.0 mM EDTA). Samples of RNA (20 μg) were electrophoretically separated on a 1.2% agarose gel in the presence of 2.2 M formaldehyde, transferred onto a nitrocellulose filter, and hybridized with a nick-translated ^{32}P-labeled 7.0 kb DNA coding for β^{major} globin mRNA, as previously described [19]. Filters were washed twice, air-dried, and then autoradiographed using Kodak XR-5 film.

RESULTS

Inhibition of erythroid differentiation of MEL cells in vitro by N^6-methylated derivatives of adenosine. As we previously reported, among the various methylated derivatives of both cytosine and adenosine tested for modulation of DMSO-induced MEL cell differentiation, only N^6-methyladenine (N^6mAde) and N^6mAdo inhibited the accumulation of hemoglobin-producing MEL cells (80–85%) [13]. Cells treated with N^6mAdo in the presence and absence of DMSO continued to grow but at slower rates as compared to those cells exposed to no drugs (control) or to DMSO alone (Fig. 1A). Cells exposed to N^6mAdo under conditions shown in Fig. 1A exhibited rates of DNA synthesis similar to those of control and DMSO-treated cells (Fig. 1B). This indicates that N^6mAdo treatment neither suppressed DNA synthesis substantially in control cells nor intensified inhibition of DNA synthesis beyond that caused by DMSO alone. These observations are consistent with the data of Table 1, which indicate that treatment with N^6mAdo for 24 or 36 hr caused only a slight decrease (15–20%) in plating efficiency of MEL cells grown in the absence and presence of DMSO. That N^6mAdo exerts a concentration-dependent inhibitory effect on MEL cell differentiation is shown in Fig. 2. The maximum degree of inhibition of MEL cell differentiation was observed

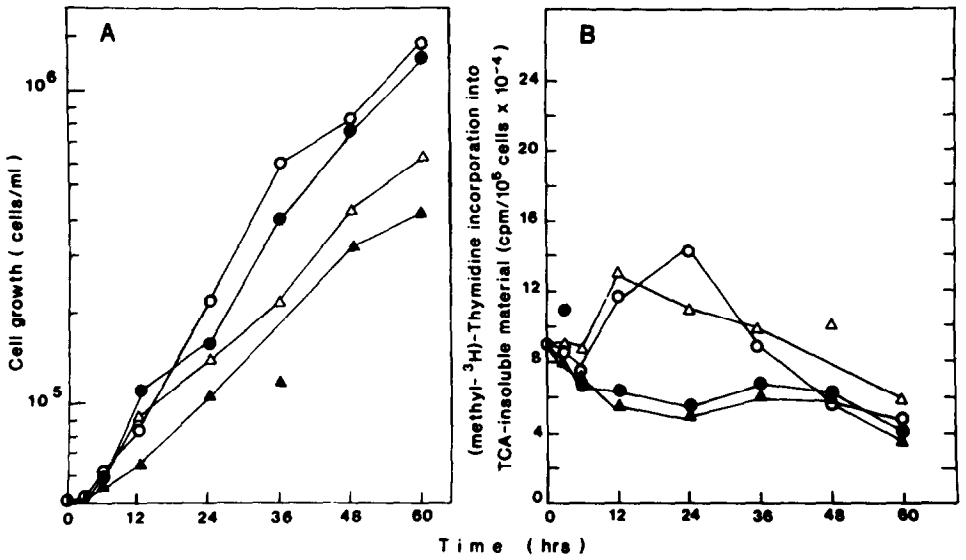


Fig. 1. Effects of N^{6}mAdo on the growth and DNA synthesis of MEL-745PC-4A cells incubated in the presence and absence of DMSO. Exponentially growing cells were incubated with (●—●) and without (○—○) DMSO (1.5%, v/v) in the presence (▲—▲) and absence (△—△) of N^{6}mAdo (0.5 mM). Panel A: Cell growth was determined by measuring cell number with the use of a hemocytometer. Panel B: The rate of DNA synthesis was determined by the incorporation of [*methyl*-^{3}H]thymidine into trichloroacetic acid insoluble material in pulse-labeled cells for 90 min according to a method reported elsewhere [20].

Table 1. Effects of N^{6}mAdo on the clonogenic potential of murine erythroleukemia cells grown in the presence and absence of DMSO

Treatment	Concentration (mM)	Time (hr)	Plating efficiency (% of colonies grown)
None		24	88.2 ± 4.5
DMSO	210	24	82.7 ± 3.0
DMSO + N^{6}mAdo	210 + 0.5	24	77.3 ± 4.9
N^{6}mAdo	0.5	24	69.0 ± 4.1
None		36	93.7 ± 12.4
DMSO	210	36	96.8 ± 8.8
DMSO + N^{6}mAdo	210 + 0.5	36	73.8 ± 1.6
N^{6}mAdo	0.5	36	75.5 ± 4.6

Exponentially growing MEL cells were incubated with and without DMSO in the presence and absence of N^{6}mAdo as described in the legend of Fig. 1. At the times indicated, cells were removed from cultures, washed two times with PBS (pH 7.4) and plated in drug-free plasma clots. The plating efficiency of control and drug-treated cells was determined by counting the number of colonies outgrown per plasma clot, multiplying it by 100 and dividing the product by 400 (number of cells plated per clot) as follows:

$$\text{Plating efficiency} = \left(\frac{\text{number of total colonies}}{400}\right) \times 100 \pm \text{SD (N = 4–6)}.$$

at 0.5 mM as shown by the accumulation of differentiated cells and hemoglobin content. A slight decrease in cell viability was observed under these conditions (Fig. 2A).

To determine whether the effects of N^{6}mAdo on MEL cell differentiation were specific to DMSO or to a central process induced by various agents, we treated cells with a number of structurally unrelated inducers in the presence and absence of various concentrations of N^{6}mAdo and measured the proportion of benzidine-positive cells in each culture. According to the results shown in Table 2, N^{6}mAdo inhibited MEL cell differentiation induced by any of the agents employed; that is, butyric acid, hypoxanthine, UDP-4, DMA and HMBA. On several occasions, coexposure of MEL cells to

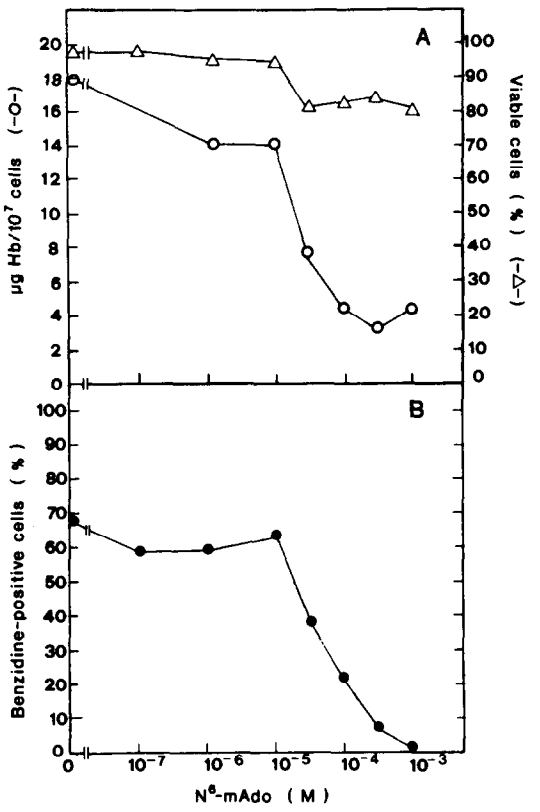


Fig. 2. Concentration-dependent effect of N⁶mAdo on cell viability, hemoglobin production (A) and erythroid differentiation (B) in MEL cells. Exponentially growing MEL-745PC-4A cells were incubated with various concentrations of N⁶mAdo in the presence of 1.5% (v/v) DMSO for 96 hr. By the end of this period, cultures were scored for cell viability (△—△), proportion of benzidine-positive cells (●—●) and for hemoglobin production (O—O) as described under Materials and Methods.

an inducer and N⁶mAdo affected cell growth substantially (Table 2). This implies that N⁶mAdo prevents the function of a central process that is essential for induction of MEL cells to erythroid differentiation.

Structure–activity relationships involved in N⁶mAdo inhibition of differentiation. To demonstrate whether certain structural features are involved in the N⁶mAdo-induced blockade of erythroid differentiation, we treated MEL cells with several methylated derivatives of adenosine and adenine (Fig. 3) in the presence of DMSO and assessed their effects on differentiation by measuring benzidine-positive cells. These included 2-methyladenine, N⁶mAde, N⁶mAdo, N^6,N^6-dimethyladenine, N^6,N^6-dimethyladenosine and N^6-cyclohexyladenosine at various concentrations. The effects of each treatment on both cell growth and differentiation are shown in Fig. 4. Although the clone MEL-745PC-4A employed in this experiment was not as inducible as in the first passages due to drifting of cellular response to inducers by time in culture, we observed that the N⁶-methylated derivatives of adenosine and adenine inhibited cell differentiation with the following decreasing order in potency: N^6,N^6-dimethyladenosine > N⁶mAdo > N^6,N^6-dimethyladenine > N⁶mAde > N⁶-cyclohexyladenosine > 2-methyladenine (Fig. 4). Comparatively, the N⁶-methylated derivatives of adenosine were more potent inhibitors than the corresponding derivatives of adenine. These data indicate that methylation of adenosine and adenine at the N⁶ position leads to products that inhibit MEL erythroid cell differentiation. Notably, N^6,N^6-dimethyladenosine was 100- to 1000-fold more potent an inhibitor of cell growth and maturation than N⁶mAdo. This finding implies that methylation of both hydrogens of the N⁶ position amino-group of adenosine increases the inhibitory activity by several-fold. Whether this substantial difference is attributed to an increase in hydrophobicity of methylated adenosines or to unknown factors is not known.

N⁶mAdo inhibition of the initiation of commitment of MEL cells to terminal erythroid maturation: Kinetic analysis by clonal assessment. To demonstrate that N⁶mAdo not only inhibits accumulation of hemoglobin synthesis in inducer-treated cells like other inhibitors (e.g. imidazole) [21], but more importantly blocks commitment of MEL cells to terminal erythroid maturation, we performed clonal assays in cells exposed to DMSO in the presence and absence of N⁶mAdo. These assays allowed us to determine the kinetics of N⁶mAdo-induced blockade of commitment at the level of individual cells prior to the appearance of hemoglobin. As shown in Fig. 5, cells exposed only to the inducer DMSO began to commit to terminal erythroid maturation after completion of a 12- to 18-hr latent period. In contrast, the majority of cells that were exposed to both DMSO and N⁶mAdo from the beginning failed to commit to terminal erythroid maturation. Colonies outgrown from such cultures were large in size and contained undifferentiated cells without hemoglobin. These observations indicate that N⁶mAdo prevented initiation of commitment to terminal erythroid maturation.

N⁶mAdo could prevent commitment to terminal maturation either by preventing early events that must occur or by abrogating initiation of the commitment process itself. To distinguish between these possibilities, we performed a set of studies shown in Fig. 5 as follows: (a) cells pretreated with N⁶mAdo for 24 hr were challenged with DMSO alone thereafter; (b) cells exposed to both DMSO and N⁶mAdo for 24 hr (throughout the entire latent period) were released from the blockade and rechallenged with DMSO alone. Cells treated with both agents (c) or with DMSO alone (d) during the entire course of incubation (72 hr) served as negative and positive control experiments, respectively. Recapitulation of the entire or a large part of the latent period in cells pre-exposed to N⁶mAdo (see Fig. 5) suggested that such cells did not respond to DMSO alone as rapidly as control cells exposed to DMSO alone but instead responded only after several hours. This delay in the onset of commitment taken together with the lower rate of accumulation of committed cells in culture indicates that N⁶mAdo treatment may prevent early events from occurring

Table 2. Inhibition of MEL cell differentiation by N⁶mAdo in the presence of different inducing agents

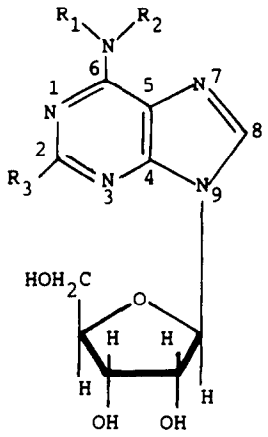
Treatment	Concentration (M)	Cell growth (% of control)	Benzidine-positive cells (%)
None		100	<1
DMSO	0.210	98	77.0
DMSO + N⁶mAdo	$0.210 + 5 \times 10^{-4}$	16.7	26.5
DMSO + N⁶mAdo	$0.210 + 1 \times 10^{-3}$	22.6	23.3
HMBA	4×10^{-3}	95.0	66.9
HMBA	5×10^{-3}	86.4	83.7
HMBA + N⁶mAdo	$4 \times 10^{-3} + 5 \times 10^{-4}$	52.5	21.0
HMBA + N⁶mAdo	$5 \times 10^{-3} + 5 \times 10^{-4}$	14.8	19.1
HMBA + N⁶mAdo	$5 \times 10^{-3} + 1 \times 10^{-3}$	15.5	17.6
UDP-4	1×10^{-4}	57.1	71.3
UDP-4 + N⁶mAdo	$1 \times 10^{-4} + 5 \times 10^{-4}$	28.0	36.2
UDP-4 + N⁶mAdo	$1 \times 10^{-4} + 1 \times 10^{-3}$	22.6	26.0
Butyric acid	1.5×10^{-3}	54.7	76.6
Butyric acid + N⁶mAdo	$1.5 \times 10^{-3} + 5 \times 10^{-4}$	42.3	25.0
Butyric acid + N⁶mAdo	$1.5 \times 10^{-3} + 1 \times 10^{-3}$	22.6	27.9
Hypoxanthine	5.5×10^{-3}	74.0	63.1
Hypoxanthine + N⁶mAdo	$5.5 \times 10^{-3} + 5 \times 10^{-4}$	20.5	20.6
Hypoxanthine + N⁶mAdo	$5.5 \times 10^{-3} + 1 \times 10^{-3}$	15.5	5.3
DMA	2×10^{-2}	56.7	64.7
DMA + N⁶mAdo	$2 \times 10^{-2} + 5 \times 10^{-4}$	7.3	9.4
N⁶mAdo	5×10^{-4}	66.7	<1
N⁶mAdo	1×10^{-3}	48.5	<1

Exponentially growing MEL-745PC-4A cells were incubated with and without an inducing agent (DMSO, HMBA, UDP-4, butyric acid, hypoxanthine, DMA) at its optimum inducing concentration in the presence and absence of N⁶mAdo at the concentrations indicated. Cell growth and the proportion of differentiated cells were determined after 48 and 96 hr, respectively. Each value is the mean of two determinations. Abbreviations: DMSO, dimethyl sulfoxide; HMBA, hexamethylene-bis-acetamide; UDP-4, 2-(3-ethylureido)-6-methylpyridine; and DMA, *N,N*-dimethylacetamide.

within the latent period. Alternatively, this delay may represent time required for N⁶mAdo or its active intermediate to be eliminated from the cells, or for its inhibitory actions to be reversed.

Effect of N⁶mAdo treatment on cytoplasmic accumulation of globin mRNA. To determine to what extent treatment of control and DMSO-treated MEL cells with N⁶mAdo affects cytoplasmic accumulation of globin mRNA as expected based on the extinction of Hb (see Fig. 2A), we exposed cells to N⁶mAdo in the presence and absence of DMSO. At various time intervals cytoplasmic RNA was prepared, separated by agarose gel electrophoresis, transferred onto a nitrocellulose membrane and hybridized with a ^{32}P-labeled DNA probe (7.0 kb) coding for mouse β^{major} mRNA, according to conditions employed for human K-562 cellular RNA [19]. According to the data illustrated in Fig. 6, treatment of control and DMSO-treated cells with N⁶mAdo decreased the overall steady-state accumulation of β^{major} globin mRNA but only moderately (Fig. 6, B and C vs A). Such cells continued to produce detectable quantities of β^{major} mRNA, although the steady-state levels of globin mRNA were comparatively lower than those of cells exposed to DMSO alone after 36–60 hr of incubation. It was also noted that, in both cases (Fig. 6, B and C), treatment of cells with N⁶mAdo led to degradation of β^{major} mRNA into lower molecular weight fragments as shown by the hybridization signals in the lower part of the autoradiogram. Considering that the cytoplasmic RNA we prepared was intact as shown by the ethidium bromide-staining pattern of the RNA samples, we tend to conclude that treatment with N⁶mAdo damaged β^{major} globin mRNA. Apparently such fragmented β^{major} globin mRNA fails to support production of hemoglobin.

Effects of L-methionine, L-homocysteine and adenine on N⁶mAdo-induced inhibition of commitment. Earlier studies which indicated that N⁶mAdo is converted into *S*-N^6-methyladenosylhomocysteine which prevents methylation of cellular RNA species [22] prompted us to examine whether N⁶mAdo causes inhibition of commitment via inhibition of methylation of RNA in MEL cells. If this were true, then agents that are involved in the active methylation cycle and contribute to the formation of *S*-N^6-methyladenosylhomocysteine could modulate (potentiate or inhibit) the ability of N⁶mAdo to prevent commitment. In an experiment shown in Fig. 7B, incubation of MEL cells already exposed to both DMSO and N⁶mAdo in the presence of various concentrations of L-methionine, L-homocysteine or adenine did not reverse but instead



Adenosine, 2-methyladenosine and N^6-substituted derivatives

Agent	R_1	R_2	R_3
Adenosine	H	H	H
2-methyladenosine	H	H	CH_3
N^6-methyladenosine	CH_3	H	H
N^6,N^6-dimethyladenosine	CH_3	CH_3	H
N^6-cyclohexyladenosine		H	H

Fig. 3. Structures of N^6-substituted derivatives of adenosine.

potentiated the inhibition of cell differentiation induced by N^6mAdo. In the absence of N^6mAdo, treatment of MEL cells with various concentrations of L-homocysteine and adenine caused only moderate effects on DMSO-induced MEL cell differentiation (Fig. 7A). Such a moderate effect was also noted with L-homocysteine thiolactone by other investigators [12]. Finally, L-methionine caused no detectable effect on MEL cell maturation induced by DMSO (Fig. 7A).

Comparison of N^6mAdo- and cordycepin-induced inhibition of commitment. A comparison of the kinetic patterns of inhibition of commitment obtained by N^6mAdo and cordycepin, a potent inhibitor of commitment [8], RNA methylation [23] and mRNA polyadenylation [24] indicated that N^6mAdo, unlike cordycepin, continued to inhibit commitment regardless of the addition of L-methionine (Fig. 8). Complete recovery of MEL cells from the cordycepin-induced blockade by the addition of L-methionine (Fig. 8) suggested that cordycepin blocks a methionine-sensitive event that is critical to initiation of commitment. This event must occur in time close to initiation of commitment. These findings tend to indicate that N^6mAdo and cordycepin act at different stages of the developmental program of MEL cells by different mechanisms which eventually impinge on the final pathway of hematopoietic cell development.

DISCUSSION

Studies over the past several years have indicated that some of the purine derivatives including those of adenine can induce (e.g. xylosyladenine) [25] or inhibit (e.g. 3-deazaadenosine, cordycepin, 5'-methylthioadenosine) [1, 8, 10–12, 26] murine erythroleukemia cell differentiation *in vitro*. The precise mechanism(s) underlying this peculiar mode of action of adenine derivatives on MEL cells has not been fully elucidated. Nevertheless, evidence indicates that some of the adenosine derivatives such as 3'-deoxyadenosine (cordycepin) inhibits MEL cell differentiation [8] by preventing a critical metabolic

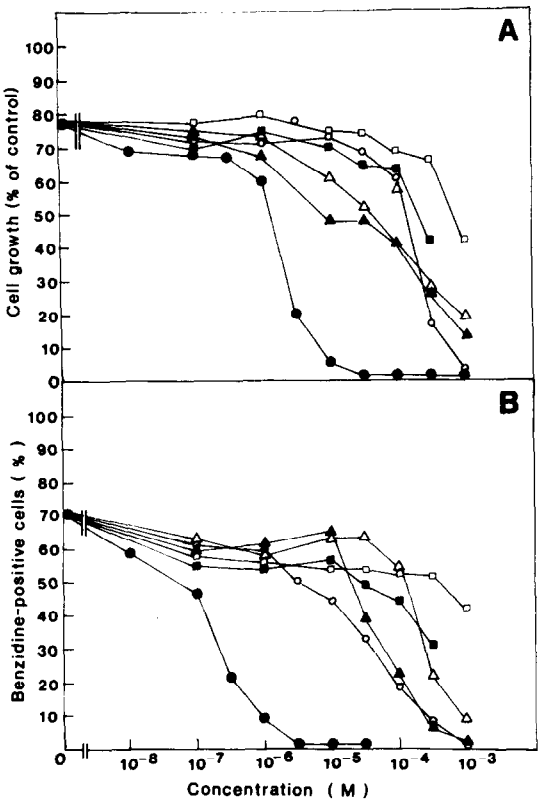


Fig. 4. Concentration-dependent effects of N^6-substituted and 2-methyl-derivatives of adenosine on cell growth and differentiation of MEL cells. Exponentially growing MEL-745PC-4A cells were incubated in culture with various concentrations of each agent shown below in the presence of DMSO (1.5%, v/v). Forty-eight and ninety-six hours after plating, the cell number and the proportion of differentiated cells were determined by measuring cells with a hemocytometer and the scoring for benzidine-positive cells, respectively. The effects of N^6-substituted and 2-methyl-derivatives of adenosine (Ado) and of adenine (Ade) on cell growth (panel A) and erythroid differentiation (panel B) are shown. MEL cells were treated with the following compounds in the presence of DMSO: N^6,N^6-dimethyladenosine (●—●); N^6,N^6-dimethyladenine (○—○); N^{6}mAdo (▲—▲); N^{6}mAde (△—△); N^6-cyclohexyladenosine (■—■); and 2-methyladenine (□—□).

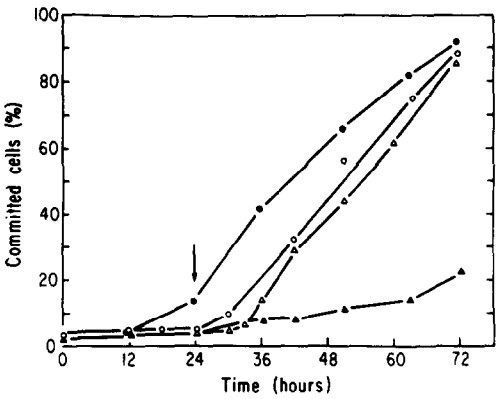


Fig. 5. Effect of N^{6}mAdo on initiation of commitment of inducer-treated MEL cells. Exponentially growing cells were incubated in culture at 37° with DMSO (1.5%, v/v) in the presence (▲—▲) and absence (●—●) of N^{6}mAdo (1.0 mM). After 24 hr of incubation (indicated by the arrow), the culture that was exposed to both agents was divided equally. One part was released from N^{6}mAdo by washing with free medium and reincubated with DMSO alone (△—△). The other continued, as previously, in the presence of both agents (▲—▲). In another culture, cells pretreated with N^{6}mAdo for 24 hr were released from this inhibitor and incubated with DMSO alone at time 0 (○—○). Incubation was continued for 72 hr and the proportion of committed cells was determined at various times.

process(es) such as synthesis and posttranscriptional modifications (e.g. methylation and polyadenylation) of RNA species [23, 24], presumably those needed for initiation of MEL cell maturation.

In a preliminary study presented elsewhere [13], we observed that among a series of methylated nucleoside analogues tested for biological activity, only N^{6}mAdo and its corresponding base N^{6}mAde prevented DMSO-induced erythroid maturation of MEL cells. In the present study, we have investigated the N^{6}mAdo-induced inhibition of commitment kinetically in order to determine the mechanism of inhibition and to uncover the cellular process(es) needed for commitment.

The finding that N^{6}mAdo inhibited cell differentiation induced by structurally unrelated inducers (Table 2) indicates that this agent must prevent a central process that is pivotal to initiation of commitment. Kinetic analysis has shown that N^{6}mAdo acts by disrupting events operating prior to commitment. This conclusion is based on the data of Fig. 5, where cells treated with N^{6}mAdo for 24 hr failed to respond to DMSO as rapidly as untreated control cells. Such cells recapitulated a large part of the latent period to becoming committed to terminal maturation. No substantial lag of time could be observed if N^{6}mAdo inhibited late events, since cells released from N^{6}mAdo had been committed rapidly. Alternatively, one could argue that the lag in time may represent time captured for N^{6}mAdo or its active intermediate to be released from the cells as mentioned in the results. This possibility is currently under investigation.

Although direct evidence to indicate or to rule out that N^{6}mAdo is incorporated into DNA is not available, an attempt to digest DNA prepared from N^{6}mAdo-treated MEL cells with Dpn I endonuclease, an enzyme that recognizes and cuts DNA at the sequence G^{m}ATC, did not produce any fragments (data not shown), apparently due to lack of such recognition sites. On the other hand, potentiation of N^{6}mAdo-induced inhibition by agents involved in the active methylation cycle (Fig. 7) suggests that N^{6}mAdo may be converted into an active intermediate like *S*-N^6-methyladenosylhomocysteine or *S*-N^6-methyladenosylmethionine before inhibiting commitment. Conversion of N^{6}mAdo into *S*-N^6-methyladenosylhomocysteine has been observed to occur in mouse liver cells *in*

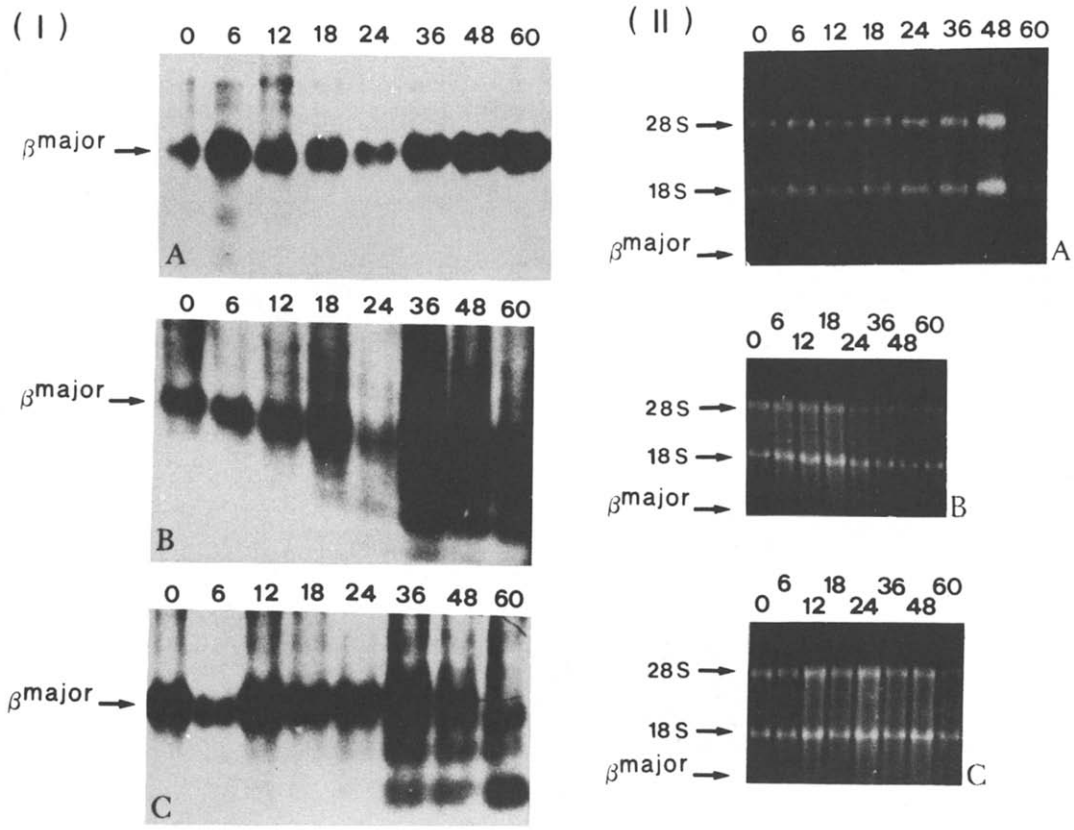


Fig. 6. Effect of N^6mAdo treatment on time-dependent cytoplasmic accumulation of β^{major} globin mRNA in control and DMSO-treated MEL cells. (I) Samples of 20 μg RNA prepared from MEL-745PC-4A cells exposed to DMSO (1.5%, v/v) alone (A) and to N^6mAdo (1.0 mM) in the presence (B) and absence (C) of DMSO, at different times, were electrophoretically separated on an agarose gel, transferred onto a nitrocellulose filter, and hybridized with a ^{32}P-labeled 7.0 kb DNA fragment coding for β^{major} globin mRNA as previously described [19]. The nitrocellulose filters were washed and autoradiographed using Kodak XR-5 film. (II) Corresponding ethidium bromide staining patterns of electrophoresed RNA samples (the positions of 28S and 18S RNAs as well as β^{major} mRNA are indicated).

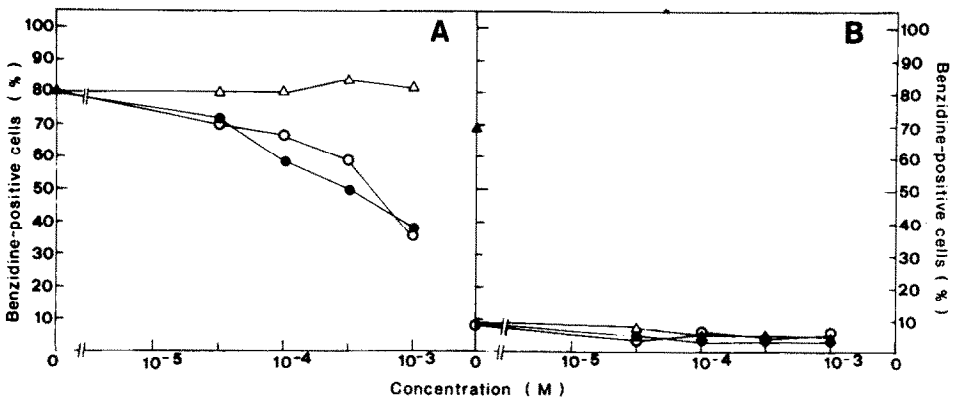


Fig. 7. Effects of L-homocysteine, L-methionine and adenine on DMSO-induced differentiation of MEL cells in the absence (A) and presence (B) of N^6mAdo. MEL-745PC-4A cells exposed to DMSO (1.5%, v/v) in the absence (A) and presence (B) of 0.5 mM N^6mAdo were incubated simultaneously with various concentrations of L-homocysteine, L-methionine, and adenine for 96 hr at 37°. By the end of this period, the proportion of benzidine-positive cells was scored cytochemically. Key: cells exposed to DMSO and various concentrations of L-homocysteine (○—○), adenine (●—●) and L-methionine (△—△) in the absence (panel A) and in the presence (panel B) of N^6mAdo. Cells exposed to DMSO alone (▲—▲) or to DMSO and N^6mAdo (◑—◑) served as positive and negative control experiments, respectively.

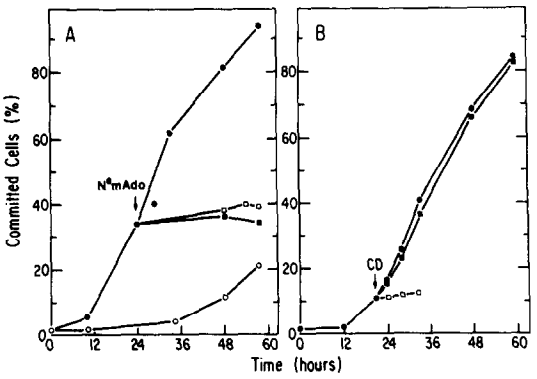


Fig. 8. Effects of the addition of methionine on N⁶mAdo- and cordycepin-induced blockade of commitment of MEL cells to terminal erythroid maturation. MEL-745PC-4A cells were incubated with DMSO (1.5%, v/v) in the absence (●—●) and presence (○—○) of N⁶mAdo (1.0 mM) and cordycepin (□—□). Panel A: At the time indicated by the arrow, cells were exposed to N⁶mAdo (1.0 mM) in the presence (□—□) and absence (■—■) of L-methionine (1.0 mM). Panel B: At the time indicated by the arrow, cells were treated with cordycepin (CD; 2×10^{-5} M) in the absence (□—□) and presence (■—■) of L-methionine (1.0 mM).

vivo [22] and in intact lymphocytes [27] where it acts as a potent inhibitor of RNA methyltransferases, which are responsible for posttranscriptional methylation of RNAs. Potentiation of N⁶mAdo-induced blockade (Fig. 7) indicates that agents like L-homocysteine and L-methionine may facilitate the formation of an internal complex like the *S*-N^6-methyladenosylmethionine or *S*-N^6-methyladenosylhomocysteine which in turn prevents RNA methylation.

Whether N⁶mAdo inhibits initiation of commitment to terminal maturation via an active intermediate which modulates RNA methylation is not known. However, we recently observed that N⁶mAdo inhibits methylation of cytoplasmic RNA substantially in both undifferentiated and differentiated MEL cells [28]. Whether this inhibition in RNA methylation is specific to certain RNA species and whether it is associated with initiation of commitment are currently under investigation.

Comparative kinetic analysis of N^6-Ado-induced blockade of commitment with that obtained by treatment with cordycepin has indicated major differences. According to data illustrated in Fig. 8, the cordycepin-induced blockade was fully reversed by addition of L-methionine in contrast to that obtained by N⁶mAdo which continued to persist despite the addition of L-methionine into the medium. Moreover, we confirmed earlier observations [8] that cells released from cordycepin committed rapidly and synchronously (Fig. 8) in contrast to those released from N⁶mAdo which committed after several hours following incubation with the inducer DMSO (Fig. 5). Based on the kinetic analysis we propose that these agents inhibit commitment by two different mechanisms, although such differences between N⁶mAdo and cordycepin could be attributed in part to different concentrations of agents used and to differential cytotoxicity (cordycepin is more cytotoxic than N⁶mAdo).

Evidence exists to indicate that RNA species like 5.8S rRNA is 2′-O-hypermethylated in differentiating myoblasts [29], an event that may play an important role in mammalian cell differentiation. Alterations in the rate of posttranscriptional methylation of RNA may influence the rate of its cytoplasmic appearance, suggesting that RNA methylation may be required for efficient processing or transport from nucleus to cytoplasm [30]. Alternatively, alterations in RNA methylation at internal N⁶mAdo sites may affect mRNA stability and presumably RNA slicing. Northern blot hybridization analysis with a ^{32}P-labeled 7.0 kb fragment of mouse β^{major} globin DNA indicated that N⁶mAdo inhibited cytoplasmic accumulation of β^{major} globin mRNA and caused degradation of this mRNA into fragments of lower molecular weight in MEL cells (Fig. 6).

N⁶mAdo is a natural constituent present in mammalian and viral RNAs with unknown biological function in cell growth and differentiation [31–34]. Some published reports have claimed that undermethylation of N⁶mAdo internal sites of cytoplasmic mRNAs is related to the rate of transport of mRNAs from nucleus to cytoplasm [30]. Moreover, evidence exists to indicate that alteration at the internal N⁶mAdo sites of RNA which are located at the consensus sequences GAC (exon–intron junction sequences) appears not to affect RNA splicing [35].

The precise mechanism of N⁶mAdo-induced blockade of MEL cell differentiation is unknown. It is possible for N⁶mAdo to prevent initiation of commitment by affecting the methylation and stability of RNA species required for erythroid cell maturation. Alternatively, N⁶mAdo like other adenosine analogues (e.g. 5′-methylthioadenosine [10, 11], 3-deazaadenosine [12]) may modulate polyamine synthesis or alter intracellular cAMP levels as in the case of N^6-phenylisopropyladenosine (PIA) [36]. Unfortunately, at present, lack of experimental data from comparative studies prevents us from drawing any conclusion on how these adenosine derivatives inhibit initiation of MEL cell differentiation.

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