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INHIBITION OF LYMPHOCYTE FUNCTION BY 9-DEAZAADENOSINE*

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Abstract—9-Deazaadenosine (c^9Ado), a novel C-nucleoside, has been found to inhibit lymphocyte-mediated cytolysis (LMC) in a time-dependent manner. c^9Ado inhibited LMC by 50% at concentrations of 10 and 0.07 μM after drug-pretreatment periods of 3 and 22 hr, respectively, although a 1-hr pretreatment of cytolytic lymphocytes with 100 μM c^9Ado had no effect upon this lymphocyte function. c^9Ado was metabolized rapidly and extensively to 9-deazaadenosine 5′-triphosphate (c^9ATP) both by mouse cytolytic lymphocytes and by human erythrocytes. Adenosine kinase purified from rabbit liver phosphorylated c^9Ado with a K_m of 200 μM and a V_{max} of 8% that for adenosine. The metabolic buildup of c^9ATP in lymphocytes was accompanied by a large, time-dependent decrease in cellular ATP and by smaller percentage decreases in CTP, UTP and GTP. Among other biochemical effects examined, c^9Ado was found to cause a decrease in lymphocyte cAMP content and appeared to be neither an inhibitor nor a substrate for *S*-adenosylhomocysteine hydrolase. Consistent with this latter result, L-homocysteine thiolactone had no effect on the inhibition of LMC by c^9Ado. Neither the inhibition of LMC by c^9Ado nor the metabolic formation of c^9ATP in lymphocytes was affected by *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), indicating that c^9Ado is not a substrate for adenosine deaminase. 5-Iodotubercidin, a non-competitive inhibitor (K_{is} = 9 nM, K_{ii} = 20 nM) of adenosine kinase, prevented the above effects of c^9Ado on lymphocyte function, c^9ATP formation, and ATP levels. Either complete preservation (with coformycin) or partial replenishment (with adenosine plus EHNA) of ATP levels in c^9Ado-treated lymphocytes resulted in partial restoration of cytolytic function to cells containing large amounts of c^9ATP. These results suggest that c^9Ado is inhibitory to LMC both because it causes a decrease in the absolute concentration of ATP within the cytolytic lymphocytes and because it permits the establishment within these cells of an unfavorable c^9ATP:ATP ratio which impedes the utilization of ATP in a reaction essential to the execution of this lymphocyte function.

The cellular metabolism of 9-deazaadenosine (c^9Ado)‡ (Fig. 1), a recently synthesized and highly cytotoxic adenosine analog [1], has not been described previously. Adenosine and many of its structural analogs are inhibitory to lymphocyte-mediated cytolysis (LMC) *in vitro* [2–5]. Evidence has been obtained in support of several different mechanisms of action of these inhibitory adenosine analogs: stimulation of adenylate cyclase and consequent elevation of cAMP within the cytolytic lymphocytes [2]; metabolism to cAMP analogs [3]; and elevation of cellular *S*-adenosylhomocysteine (AdoHcy), or metabolism to AdoHcy analogs, with resultant inhibition of one or more methyltransferases vital to lymphocyte function [4, 5]. The present report documents the inhibition of lymphocyte function by c^9Ado and describes the metabolism of this adenosine analog in mouse lymphocytes.

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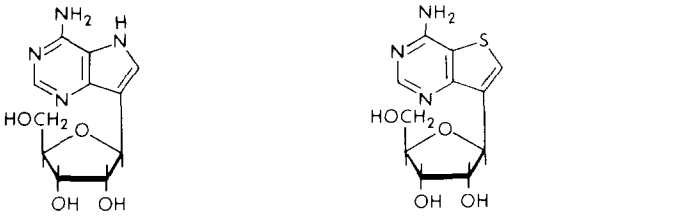
‡ Abbreviations: c^9Ado, 9-deazaadenosine or 4-amino-7-β-D-ribofuranosyl-5*H*-pyrrolo[3,2-*d*]pyrimidine; c^9ADP and c^9ATP, the 5′-di- and -triphosphates of 9-deazaadenosine; AdoHcy, *S*-adenosylhomocysteine; CoF, coformycin; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; HPLC, high-performance liquid chromatography; ITu, 5-iodotubercidin or 4-amino-5-iodo-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine (NSC 113939); LMC, lymphocyte-mediated cytolysis; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

MATERIALS AND METHODS

5-Iodotubercidin was provided by Dr. Leroy B. Townsend of the University of Michigan, Ann Arbor, MI, and coformycin by Dr. Hamao Umezawa of the Microbial Chemistry Research Foundation, Tokyo, Japan. c^9Ado [1], 9-deazainosine, or 7-(β-D-ribofuranosyl) - 4-oxo-3*H*,5*H* - pyrrolo[3,2-*d*]pyrimidine [6], and 7-thia-7,9-dideazaadenosine, or 4-



9-DEAZAADENOSINE 7-THIA-7,9-DIDEAZAADENOSINE

Fig. 1. Chemical structures of 9-deazaadenosine and 7-thia-7,9-dideazaadenosine.

amino-7-β-D-ribofuranosylthieno[3,2-*d*]pyrimidine,* were synthesized at the Sloan–Kettering Institute for Cancer Research, Rye, NY. Other materials were from sources identified elsewhere [2–5, 7].

Cytolytic lymphocytes were obtained from immunized CD-1 mice, and C57BL leukemia EL4 cells were maintained, harvested, and labeled with $Na_2{}^{51}CrO_4$ as previously described [3]. RPMI 1640 (Flow Laboratories, Rockville, MD) supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY; heat-inactivated at 56° for 30 min), penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.01 M HEPES buffer, pH 7.2, was used as the medium for all cell incubations.

The *in vitro* LMC assay was performed as follows. Cytolytic lymphocytes (2.5×10^5 cells/0.95 ml of medium) were incubated with drugs for the specified times and were mixed with an equal number of ^{51}Cr-labeled EL4 cells in 12 × 75 mm plastic tubes (Falcon Plastics, Oxnard, CA) in a total volume of 1.0 ml. The cell suspensions were mixed, centrifuged at 185 *g* for 5 min, and incubated for 60 min at 37° in a moist atmosphere of 5% CO_2 in air. After the addition of 1.0 ml of cold medium, the tubes were shaken to resuspend the cells and centrifuged at 733 *g* for 10 min. The ^{51}Cr released into the supernatant fraction was measured using a Packard Auto Gamma scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). All assays were performed in duplicate, and the ^{51}Cr released in the drug-treated cultures was compared with the radioactivity released in control (untreated) cultures. Spontaneous release from [^{51}Cr]EL4 cells incubated alone and the maximum ^{51}Cr release by target cells frozen and thawed three times were determined for each experiment. LMC assay results were calculated as follows:

$$\text{Percent lysis} = \frac{\text{cpm released in presence of cytolytic lymphocytes} - \text{cpm released in absence of cytolytic lymphocytes}}{\text{cpm released by freeze-thaw of EL4 cells}} \times 100$$

$$\text{Percent of control lysis} = \frac{\text{percent lysis in presence of drug}}{\text{percent lysis in absence of drug}} \times 100$$

For nucleoside triphosphate determinations, 15–20 × 10^6 cytolytic lymphocytes were suspended in 10 ml of medium and incubated for the specified times at 37° with the indicated drugs. Incubations were terminated by chilling the cell suspensions on ice for 10 min and by collecting the cells in a refrigerated centrifuge. The cell pellets were each extracted with 5.0 ml of cold 0.5 M perchloric acid containing 3.0 μM ITP as a recovery marker. These extracts were clarified by centrifugation, neutralized with KOH, filtered through glass wool to remove the insoluble potassium perchlorate, evaporated to dryness under reduced pressure (in a Buchler Evapo-Mix apparatus), and reconstituted with 300 μl of deionized water. Residual insoluble material was removed by centrifugation and was discarded. These samples were stored at −20° until their analysis for nucleotides by anion-exchange high-performance liquid chromatography (HPLC) [3].

Human erythrocytes (5% suspensions) were incubated for 3 hr at 37° in the absence or presence of 100 μM c^9Ado as described previously [7]. These cells were then harvested and acid-extracted for nucleotide analysis as described above for the lymphocytes.

cAMP present in acid-soluble extracts of lymphocytes was quantitated by radioimmunoassay after purification of the extracts on sequential columns of aluminium oxide and Dowex 1-X8 and subsequent 2′-*O*-succinylation of the resultant samples [3]. The effect of c^9Ado on the relative cellular level of [^{3}H]AdoHcy was assessed by reversed-phase HPLC after prelabeling cytolytic lymphocytes for 1 hr with L-[2-^{3}H]methionine (100 μCi/2.3×10^8 cells in 10 ml of medium) and then incubating the washed cells (2.3×10^7 cells/5.0 ml of medium) for 30 min with saline or test compound [4, 5]. Hexokinase-catalyzed peak-shift of putative c^9ATP was carried out as before [3]. Ultraviolet absorption spectra were recorded with a Beckman model Acta III spectrophotometer.

Adenosine kinase was purified from rabbit liver, and kinetic measurements were obtained using spectrophotometric and radiochemical methods [8].

RESULTS

Inhibition of lymphocyte cytolytic function by c^9Ado. c^9Ado inhibited the capacity of cytolytic

* W-Y. Ren and R. S. Klein, unpublished data.

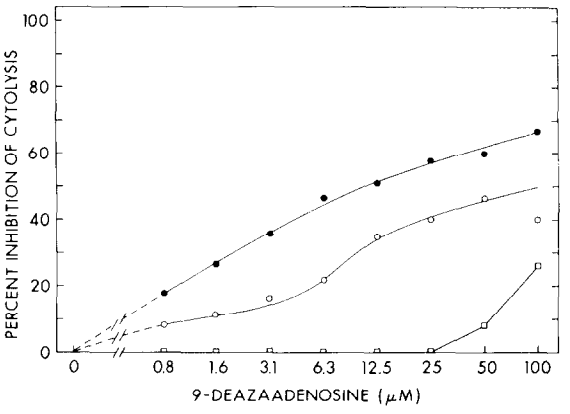


Fig. 2. Effect of c⁹Ado on lysis of ^{51}Cr-labeled EL4 cells by cytolytic lymphocytes. Cytolytic lymphocytes (2.5×10^5 cells/ml of medium) were pretreated for 3 hr at 37° with the indicated concentrations of c⁹Ado (●), for 3 hr with the indicated concentrations of c⁹Ado in the presence of 0.1 μM ITu (□), or for 1 hr with the indicated concentrations of c⁹Ado followed by washing of the cells and a 2-hr incubation in drug-free medium (○). These various lymphocyte suspensions were then mixed with an equal number of ^{51}Cr-labeled EL4 cells, and the 1-hr cytolysis assay was carried out as described under Materials and Methods. Of the total radioactivity (21,094 cpm) released by freeze-thaw treatment of the ^{51}Cr-labeled EL4 cells, 5370 cpm was released in the cytolysis assay in the presence of non-drug-treated cytolytic lymphocytes and 1540 cpm was released nonspecifically when ^{51}Cr-labeled EL4 cells were incubated for 1 hr at 37° in the absence of lymphocytes.

mouse lymphocytes to lyse tumor cells in a time-dependent manner. A 1-hr pretreatment of cytolytic lymphocytes at 37° with concentrations of c⁹Ado as high as 100 μM had no effect on the function of these cells in subsequent 60-min LMC assays. However, pretreatment of these cells for 3 or 22 hr with various concentrations of c⁹Ado revealed 50% inhibitory concentrations for this nucleoside of 10 and 0.07 μM, respectively, in subsequent LMC assays. The effect of c⁹Ado concentration on the inhibition of LMC after a 3-hr pretreatment of cytolytic lymphocytes with this agent is shown in Fig. 2*.

In addition to its slow onset, the inhibition of lymphocyte function caused by c⁹Ado appeared to be somewhat persistent. When cytolytic lymphocytes were pretreated with various concentrations of c⁹Ado for 1 hr at 37° and were then washed free of drug and incubated for another 2 hr in drug-free medium, cytolysis was still inhibited to a considerable extent (Fig. 2).

Several pharmacological agents were examined for their possible effects on the inhibition of LMC resulting from a 3-hr pretreatment of cytolytic lymphocytes with c⁹Ado. *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, 7.9 μM), a potent inhibitor of adenosine deaminase [9], did not enhance the inhibition of LMC observed with c⁹Ado. Similarly, L-homocysteine (200 μM), a co-substrate with adenosine and many adenosine analogs for AdoHcy hydrolase [4, 5], did not alter the activity of c⁹Ado towards LMC. However, 5-iodotubercidin (ITu, 0.1 μM), an inhibitor of adenosine kinase [10–12], was found to protect the cytolytic lymphocytes substantially from the LMC inhibitory effect of c⁹Ado (Fig. 2). This protection by ITu was complete at lower concentrations of c⁹Ado but became less effective at c⁹Ado concentrations $\geq$ 50 μM. Higher concentrations of ITu could not be employed in this type of experiment because ITu itself becomes inhibitory to LMC at concentrations $>$ 0.1 μM.

Evidence for the metabolism of c⁹Ado to 5′-nucleotides. Analysis of acid-soluble extracts of c⁹Ado-treated cells by anion-exchange HPLC revealed a prominent new peak (retention time, 58 min) in the chromatogram between CTP and UTP (Fig. 3B). As a reference, the nucleotide profile of saline-treated lymphocytes is presented in Fig. 3A. A small c⁹Ado metabolite peak (retention time, 32 min) was evident in the diphosphate region of the chromatogram, but no analog nucleotide peak was discernible in the monophosphate region. The novel c⁹Ado-related peak that eluted at 58 min in Fig. 3B was characterized as 9-deazaadenosine 5′-triphosphate (c⁹ATP) on the bases of its elution position in the triphosphate region of the chromatogram, its similar A_{280}/A_{254} absorbance ratio as compared with c⁹Ado, its ultraviolet absorption spectrum which was identical to that of c⁹Ado ($\lambda_{max}^{pH\ 3.5}$ = 273 nm) and which was clearly different from that of 9-deazainosine ($\lambda_{max}^{pH\ 3.5}$ = 261 nm), and its quantitative shift to the retention time (32 min) of the diphosphate metabolite of c⁹Ado after treatment of a portion of cell extract with yeast hexokinase plus glucose.

Human erythrocytes were also shown to metabolize c⁹Ado extensively to c⁹ATP.

c⁹Ado was tested as a substrate for adenosine kinase purified from rabbit liver and was found to be phosphorylated with a K_m of 200 μM and a V_{max} of 8% that for adenosine. As would be expected, c⁹Ado was also found to be a competitive inhibitor (K_i = 190 μM) of adenosine phosphorylation by this enzyme. ITu was found to be a non-competitive inhibitor of adenosine phosphorylation, with a K_{is} of 9 nM and a K_{ii} of 20 nM.

Characteristics of c⁹ATP formation in lymphocytes. The buildup of c⁹ATP in cytolytic lymphocytes incubated continuously with 100 μM c⁹Ado appeared to reach a maximum after 2–3 hr (Fig. 4). Acid-soluble extracts prepared from cells that had been incubated for 22 hr with 100 μM c⁹Ado were devoid of nucleoside triphosphate peaks when analyzed by HPLC, indicating that these cells had lysed in response to drug treatment. The progressive cellular buildup of c⁹ATP in c⁹Ado-treated lymphocytes was accompanied by a reciprocal decrease in cellular ATP (Fig. 4). After this 3-hr treatment of cytolytic lymphocytes with 100 μM c⁹Ado, ATP, GTP, CTP and UTP were decreased by 81, 15, 37 and 37%, respectively, relative to saline-treated cells.

* Although the densities of lymphocytes employed routinely in the LMC assays (2.5×10^5 cells/ml) and in the biochemical experiments (1.5 to 2.0×10^6 cells/ml) described below were almost 10-fold different, it was determined that the 50% inhibitory concentration for c⁹Ado in the LMC assay (following a 3-hr drug-pretreatment of the cytolytic lymphocytes) was independent of lymphocyte density between 2.5 and 25×10^5 cells/ml.

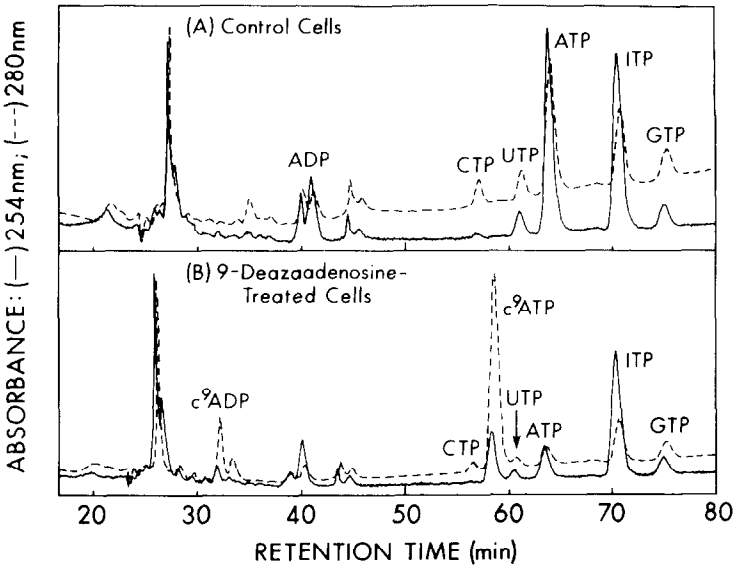


Fig. 3. HPLC nucleotide profiles of cytolytic lymphocytes after incubation with saline or c^{9}Ado. Lymphocytes (19×10^6 cells/10 ml of medium) were incubated for 3 hr at 37° either with saline (A) or with 100 μM c^{9}Ado (B). The cells were then harvested by centrifugation and were extracted for nucleotide analysis.

The cellular accumulation of c^{9}ATP resulting from a 3-hr incubation of lymphocytes with c^{9}Ado was dependent upon the concentration of c^{9}Ado present in the medium (Fig. 5). As in the time–course experiment (Fig. 4), the cellular level of ATP again appeared to be inversely related to the level of c^{9}ATP present in these cells (Fig. 5).

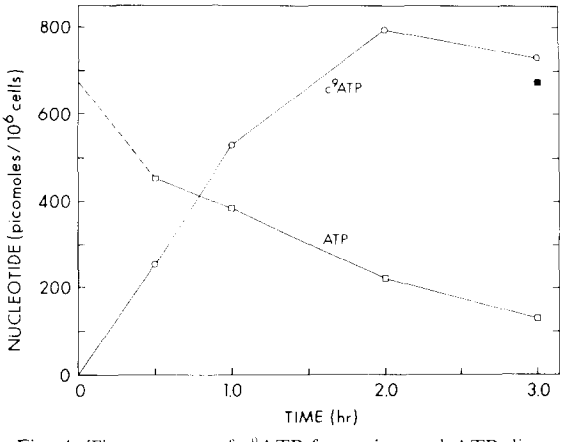


Fig. 4. Time–course of c^{9}ATP formation and ATP disappearance in lymphocytes incubated with 100 μM c^{9}Ado. Cytolytic lymphocytes (17×10^6 cells/10 ml of medium) were incubated with saline for 3 hr or with 100 μM c^{9}Ado for the indicated times, after which the cells were acid-extracted for the quantitation of ATP (□) and c^{9}ATP (○) by anion-exchange HPLC. The closed square represents the ATP content of lymphocytes incubated for 3 hr in the absence of c^{9}Ado. A true zero-time ATP content of lymphocytes was not obtained in this experiment, and the dashed line connecting the 0.5-hr ATP value with the zero-time axis is extrapolated to the cellular ATP content of the 3-hr saline-treated control lymphocytes (■)

EHNA (7.9 μM) did not augment the cellular buildup of c^{9}ATP resulting from a 3-hr incubation of lymphocytes with 100 μM c^{9}Ado.

The adenosine kinase inhibitor ITu was found to be a potent inhibitor of lymphocyte c^{9}ATP formation from c^{9}Ado (Table 1). By itself, 0.1 μM ITu had no significant effect upon the lymphocyte pools of ribonucleoside 5′-triphosphates. The 3-hr incubation of lymphocytes with 50 μM c^{9}Ado alone resulted in a large accumulation of c^{9}ATP and in decreases in cellular pools of ATP, CTP and UTP of 76, 31 and 30% respectively. When added to the lymphocytes together with 50 μM c^{9}Ado, 0.1 μM ITu inhibited by 96% the metabolism of c^{9}Ado to c^{9}ATP.

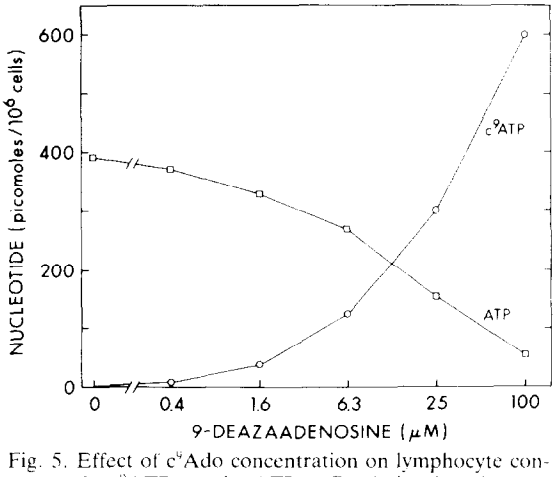


Fig. 5. Effect of c^{9}Ado concentration on lymphocyte content of c^{9}ATP and ATP. Cytolytic lymphocytes (17×10^6 cells/10 ml of medium) were incubated for 3 hr with the indicated concentrations of c^{9}Ado. Acid-soluble extracts of these cells were then prepared and analyzed for ATP (□) and c^{9}ATP (○) by anion-exchange HPLC.

Table 1. Effect of 5-iodotubercidin on the ribonucleoside 5′-triphosphate pools in lymphocytes treated with 9-deazaadenosine*

Additive(s)	(pmoles of nucleotide/10^6 cells*)				
	CTP	UTP	ATP	GTP	c^9ATP
None (saline)	26 ± 3	98 ± 3	546 ± 15	103 ± 11	
ITu (0.1 μM)	28 ± 3	111 ± 20	532 ± 43	113 ± 6	
c^9Ado (50 μM)	18 ± 1	69 ± 2	132 ± 12	102 ± 3	496 ± 12
c^9Ado (50 μM) + ITu (0.1 μM)	31 ± 1	110 ± 2	493 ± 7	112 ± 10	22 ± 2

* Cytolytic lymphocytes (19 × 10^6 cells/10 ml of medium) were incubated for 3 hr at 37° with the specified additives and were then chilled on ice (10 min), harvested by centrifugation, and acid-extracted for nucleotide analysis. This experiment was performed in duplicate, and the results are expressed as the means ± the average deviation of the two analyses.

Concomitant with this effect on c^9ATP formation, ITu also prevented most of the c^9Ado-induced decrease in cellular ATP and abrogated completely the smaller decreases in lymphocyte CTP and UTP caused by c^9Ado.

Effect of ATP replenishment on function of c^9Ado-pretreated lymphocytes. An attempt was made to reverse the effects of c^9Ado on lymphocyte ATP levels and cytolytic function by incubating c^9Ado-pretreated lymphocytes in the presence of adenosine. As shown in Table 2, lymphocytes which were treated for 1 hr with 25 μM c^9Ado and were then washed and incubated for 2 hr in drug-free medium exhibited a 49% decrease in ATP and smaller decreases (8–17%) in GTP, CTP and UTP, while accumulating 151 pmoles of c^9ATP/10^6 cells. In a parallel experiment, cytolytic lymphocytes treated in this manner were inhibited 53% in the LMC assay. When these c^9Ado-pretreated lymphocytes were washed and incubated for 2 hr in the presence of 20 μM adenosine plus 7.9 μM EHNA, the ATP pool was expanded to within 13% of control value and, in the parallel LMC experiment, cytolytic function was restored to 78% of control value. Incubation of c^9Ado-pretreated lymphocytes for 2 hr in the presence of 400 μM cytidine plus 400 μM uridine expanded the cellular pools of CTP and UTP greatly beyond control values but did not alter the effect of c^9Ado on lymphocyte ATP levels or cytolytic function. Neither follow-up treatment (adenosine/EHNA or cytidine/uridine) altered the cellular level of c^9ATP present in the c^9Ado-pretreated lymphocytes.

In a separate experiment, it was found that the inhibition of LMC resulting from a 3-hr pretreatment of cytolytic lymphocytes with 25 μM c^9Ado could be prevented completely by co-incubation with 20 μM adenosine plus 7.9 μM EHNA. However, subsequent nucleotide analyses of lymphocyte extracts revealed that the simultaneous addition of adenosine plus EHNA to the cells during treatment with c^9Ado inhibited by 96% the cellular accumulation of c^9ATP.

Influence of coformycin (CoF) on c^9Ado effects. Bagnara and Hershfield [13] have reported recently that CoF, which is an inhibitor of AMP deaminase as well as of adenosine deaminase [14], was able to prevent 2′-deoxyadenosine-induced depletion of ATP in human T lymphoblastoid cells. In view of this report, we have investigated the effect of CoF in mouse cytolytic lymphocytes treated with c^9Ado. As shown in Table 3, a 3-hr pretreatment of cytolytic lymphocytes with 100 μM CoF alone resulted in an

Table 2. Effect of replenishment of ribonucleoside 5′-triphosphate pools in 9-deazaadenosine-pretreated lymphocytes on cytolytic function

Treatment schedule	(pmoles of nucleotide*/10^6 cells)					Cytolysis† (% of control)
	CTP	UTP	ATP	GTP	c^9ATP	
Saline (1 hr)/washed cells/saline (2 hr)	23 ± 1	71 ± 12	580 ± 7	133 ± 7		100
Saline (1 hr)/washed cells/Ado, EHNA (2 hr)	29 ± 1	75 ± 4	863 ± 43	135 ± 7		98
Saline (1 hr)/washed cells/Cyd, Urd (2 hr)	93 ± 2	117 ± 7	613 ± 6	125 ± 7		108
c^9Ado (1 hr)/washed cells/saline (2 hr)	19 ± 1	62 ± 2	295 ± 10	122 ± 1	151 ± 7	47
c^9Ado (1 hr)/washed cells/Ado, EHNA (2 hr)	26 ± 2	75 ± 6	506 ± 21	151 ± 3	157 ± 2	78
c^9Ado (1 hr)/washed cells/Cyd, Urd (2 hr)	53 ± 2	107 ± 2	258 ± 1	121 ± 7	152 ± 11	53

* For the nucleotide measurements, cytolytic lymphocytes (19 × 10^6 cells/10 ml of medium) were incubated for 1 hr at 37° in the absence or presence of 25 μM c^9Ado. These cells were then harvested by centrifugation, washed one time, and resuspended in fresh, drug-free medium to the original cell density. These cell suspensions were then supplemented with saline, with 20 μM adenosine (Ado) plus 7.9 μM EHNA, or with 400 μM cytidine (Cyd) plus 400 μM uridine (Urd) and incubated for another 2 hr at 37° prior to their harvest by centrifugation and their acid-extraction for nucleotide analysis. This experiment was performed in duplicate, and the results are expressed as the means ± the average deviation of the two analyses.

† For the LMC assays, cytolytic lymphocytes (2.5 × 10^5 cells/1.0 ml of medium) were treated ± the specified agents according to the same schedule as shown at the left of this table for the nucleotide experiment and were then washed and evaluated in the 60-min LMC assay for their relative cytolytic function.

Table 3. Effect of coformycin on ribonucleoside 5′-triphosphate pools and on cytolytic function of lymphocytes treated with 9-deazaadenosine

Additive(s)	(pmoles of nucleotide*/10^6 cells) CTP	UTP	ATP	GTP	c^9ATP	Cytolysis† (% of control)
None (saline)	38 ± 4	92 ± 5	792 ± 3	158 ± 7		100
CoF (100 μM)	32 ± 4	87 ± 2	951 ± 2	144 ± 3		105
c^9Ado (100 μM)	33 ± 3	106 ± 1	153 ± 5	168 ± 6	424 ± 27	36
c^9Ado (100 μM) + CoF (100 μM)	22 ± 3	77 ± 11	817 ± 27	67 ± 2	322 ± 46	59

* For the nucleotide measurements, cytolytic lymphocytes (20×10^6 cells/5.0 ml of medium) were incubated for 3 hr at 37° with the specified additives and were then chilled on ice (10 min), harvested by centrifugation, and acid-extracted. This experiment was performed in duplicate, and the results are expressed as the means ± the average deviation for the two analyses.

† For the LMC assays, cytolytic lymphocytes (2.5×10^5 cells/1.0 ml of medium) were first incubated for 3 hr at 37° with the specified additives. These cell suspensions were then supplemented immediately with equal numbers of ^{51}Cr-labeled EL4 cells and evaluated in the 60-min LMC assay for their relative cellular function.

ATP level 20% above that of the saline-treated control cells, without any significant effect on LMC. Under these same conditions, 100 μM c^9Ado alone caused the expected decreases in lymphocyte ATP (81%) and function (64%) and was metabolized extensively to c^9ATP. The simultaneous addition of c^9Ado and CoF to these 3-hr lymphocyte incubations resulted in complete preservation of lymphocyte ATP content and in a somewhat (24%) reduced accumulation of c^9ATP. Under these conditions, CoF was found to prevent partially (59 vs 36% of control cytolysis) the inhibition of lymphocyte function caused by c^9Ado.

Other biochemical studies concerning c^9Ado. Cytolytic lymphocytes (1×10^7 cells/5.0 ml of medium) incubated for 30 min with 50 μM c^9Ado exhibited a 24% decrease ($P < 0.001$) in cAMP levels. This effect is presumed to be due in part to the decrease in lymphocyte ATP caused by c^9Ado but may also indicate inhibition of cAMP formation due to the binding of c^9ATP to adenylate cyclase.

Lymphocytes prelabeled with L-[2-^{3}H]methionine did not exhibit an increase in their relative content of [^{3}H]AdoHcy when incubated with 50 μM c^9Ado for 30 min. Moreover, no evidence for the metabolic formation of the 9-deaza analog of AdoHcy was obtained in this experiment. Considered in the light of past experience with this experimental protocol [4, 5], these results indicate that c^9Ado is neither an inhibitor nor a substrate for AdoHcy hydrolase.

Comparative studies with 7-thia-7,9-dideazaadenosine. An abbreviated investigation of 7-thia-7,9-dideazaadenosine (Fig. 1), a recently synthesized analog of c^9Ado,* was conducted to compare the biological and biochemical effects of this nucleoside with those of c^9Ado described above. 7-Thia-7,9-dideazaadenosine was found to be more inhibitory towards LMC than c^9Ado, exhibiting a 50% inhibitory concentration of 2.5 μM after a 3-hr drug-pretreatment of the cytolytic lymphocytes. The LMC inhibitory activity of this nucleoside was not enhanced by either EHNA (7.9 μM) or L-homocysteine (200 μM). However, ITu (0.1 μM) reduced the inhibition of LMC resulting from a 3-hr pretreatment of lymphocytes with 5.0 μM 7-thia-7,9-dideazaadenosine from 64 to 11%. Extracts from lymphocytes incubated for 3 hr with 50 μM 7-thia-7,9-dideazaadenosine were analyzed by anion-exchange HPLC and were found to contain a large peak of putative 7-thia-7,9-dideazaadenosine 5′-triphosphate which was eluted on the front side of the UTP peak. This 3-hr treatment with 50 μM 7-thia-7,9-dideazaadenosine resulted in a 66% decrease in lymphocyte ATP content. 7-Thia-7,9-dideazaadenosine was found to be a better substrate (K_m = 100 μM, V_{max} = 16% that of adenosine) than c^9Ado for adenosine kinase purified from rabbit liver.

* W-Y. Ren and R. S. Klein, unpublished data.

DISCUSSION

The results of the present study provide an initial glimpse of the biochemical properties of the novel adenosine analog c^9Ado. The predominant biochemical effects of c^9Ado observed in mouse lymphocytes were the extensive metabolism of this C-nucleoside to c^9ATP and the reciprocal decrease in cellular ATP content. Consistent with this observation of c^9ATP formation, c^9Ado was found to be a substrate for adenosine kinase purified from rabbit liver. Moreover, both the metabolic formation of c^9ATP and the c^9Ado-induced depletion of lymphocyte ATP were prevented by the adenosine kinase inhibitor ITu. Based upon the lack of effect of EHNA either on c^9Ado metabolism to c^9ATP or on c^9Ado inhibition of LMC, it appears that c^9Ado is not an efficient substrate for adenosine deaminase. Unlike many other adenosine analogs, c^9Ado did not cause an elevation in lymphocyte cAMP [2] and did not appear to act as either a substrate or an inhibitor of AdoHcy hydrolase [4, 5].

Although we have not investigated in depth the mechanism for this c^9Ado-induced loss of ATP from mouse lymphocytes, a recent report by Bagnara and Hershfield [13] suggests a likely explanation for this effect. These authors have delineated the enzymology by which 2′-deoxyadenosine induces a similar depletion of ATP in human T lymphoblastoid

cells [13]. These latter cells accumulate high levels of dATP during incubation with deoxyadenosine and, in the process, generate ADP and AMP. dATP and ATP were both shown to stimulate the activity of AMP deaminase, and dAMP was found to be a less efficient substrate than AMP for this enzyme. As a subsequent step in this catabolic pathway, both dATP and ATP were shown to stimulate the dephosphorylation of IMP by a cytoplasmic nucleotidase. In support of this catabolic mechanism, CoF, which also acts as an inhibitor of AMP deaminase [14], was shown to prevent the deoxyadenosine-induced depletion of cellular ATP [13]. In the present study, CoF was found to prevent the c^9Ado-induced loss of ATP from mouse lymphocytes (Table 3). By analogy with the findings of Bagnara and Hershfield [13], we suggest that the c^9Ado phosphorylation was accompanied by the generation of ADP and AMP and that c^9ATP plus ATP stimulated both the deamination of AMP (but not of 9-deazaadenylate) and the dephosphorylation of the resultant IMP, thereby depleting cells of adenine nucleotides. The results obtained with ITu (Table 1) indicate strongly that phosphorylation of c^9Ado was a prerequisite for adenine nucleotide degradation to occur.

The ability of c^9Ado to inhibit LMC appears to have been due to the metabolism of this nucleoside to c^9ATP and to the accompanying decrease in cellular ATP. The inhibition of LMC by c^9Ado was characterized by its slowness of onset (i.e. requiring >1 hr pretreatment of cytolytic lymphocytes with 100 μM drug), by its persistence after transfer of c^9Ado-treated lymphocytes to drug-free medium (Fig. 2 and Table 2), and by its antagonism by the adenosine kinase inhibitor ITu (Fig. 2). These three characteristics of this biological activity of c^9Ado correlate well with the effects of c^9Ado on lymphocyte c^9ATP and ATP: the cellular accumulation of c^9ATP and concomitant decrease in lymphocyte ATP were progressive with time (Fig. 4), persisted after transfer of c^9Ado-treated lymphocytes to drug-free medium (Table 2), and were antagonized by ITu (Table 1). Moreover, these nucleoside triphosphate effects associated with c^9Ado occurred over the same concentration range of c^9Ado (Fig. 5) as did the inhibition of LMC (Fig. 2). These results indicate that *both* the intracellular presence of c^9ATP *and* the c^9Ado-induced decrease in cellular ATP were individually detrimental to this lymphocyte function. Thus, neither complete preservation (Table 3) nor partial replenishment (Table 2) of ATP levels in c^9Ado-treated lymphocytes was able to restore fully the cytolytic capacity of cells containing large amounts of c^9ATP. Additionally, it is known that the lysis of tumor cells by specifically sensitized lymphocytes is an energy-dependent process requiring adequate cellular levels of ATP [15].

In summary, it is concluded that c^9Ado is inhibitory to LMC both because it induces a decrease in the absolute concentration of ATP within the cytolytic lymphocytes and because it allows the establishment within these cells of an unfavorable c^9ATP:ATP ratio which impedes the utilization of ATP in a reaction essential to the execution of this lymphocyte function.

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