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3-DEAZAADENOSINE 5′-TRIPHOSPHATE: A NOVEL METABOLITE OF 3-DEAZAADENOSINE IN MOUSE LEUKOCYTES

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Abstract—Evidence has been obtained for the metabolic formation of small amounts (1–2% of the ATP pool) of 3-deazaadenosine 5′-triphosphate (c^3ATP) from 3-deazaadenosine (c^3Ado) in mouse cytolytic lymphocytes and mouse resident peritoneal macrophages. With intact leukocytes, pharmacological evidence was obtained that adenosine kinase was not the enzyme chiefly responsible for the phosphorylation of c^3Ado. Moreover, in the presence of $MgCl_2$, NaCl and IMP, purified rat liver 5′-nucleotidase catalyzed the phosphorylation of c^3Ado to 3-deazaadenosine 5′-monophosphate (c^3AMP). Two lines of evidence suggest that the metabolic formation of c^3ATP is not involved in the inhibition of leukocyte function caused by c^3Ado. First, the inhibitory action of c^3Ado on antibody-dependent phagocytosis and lymphocyte-mediated cytolysis was reversed markedly upon removal of the drug from the medium. However, the intracellular content of c^3ATP remained constant in lymphocytes and macrophages after removal of c^3Ado. Second, in macrophages and in lymphocytes, similar intracellular amounts of c^3ATP were formed from both c^3Ado and 3-deazaadenine under conditions in which the former was biologically active and the latter was essentially inactive. Thus, it appears unlikely that the novel c^3ATP metabolite is of relevance for the mechanism of action of c^3Ado in mouse leukocytes.

c^3Ado†, a structural analogue of adenosine, exhibits anti-inflammatory and immunosuppressive activity in animal models [1, 2]. Consistent with these observations are results of *in vitro* investigations of the effects of c^3Ado on leukocyte functions. c^3Ado has been found to inhibit macrophage and neutrophil chemotaxis [3], lymphocyte-mediated cytolysis [4], phagocytosis by macrophages [3, 5–7], granule secretion by neutrophils [8], superoxide anion generation in neutrophils [9, 10], and macrophage motility‡. The mechanism of action of c^3Ado in these various leukocytes remains unclear. The initial contention that the biological activity of c^3Ado accrues through its action as an inhibitor of methylation reactions has been questioned recently, and recent data suggest that c^3Ado may exert its biological activity through a mechanism independent of its interaction with *S*-adenosylhomocysteine hydrolase and the resultant accumulation of *S*-adenosylhomocysteine and *S*-3-deazaadenosylhomocysteine [6, 11, 12]. Apart from the formation of the latter, another potential metabolic pathway for this nucleoside analogue is phosphorylation to c^3AMP, c^3ADP and c^3ATP. Previous investigations indicated that c^3Ado is not metabolized detectably to 5′-nucleotides either by intact cells [4, 13, 14] or by purified adenosine kinase [15]. However, the recent availability of [^{3}H]c^3Ado has provided greater sensitivity for detecting cellular c^3Ado nucleotide formation. Here we report evidence for the metabolic formation of low levels of the novel metabolite, c^3ATP, in mouse resident peritoneal macrophages and mouse cytolytic lymphocytes and present evidence that argues against a correlation between c^3ATP formation and inhibition of leukocyte function.

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† Abbreviations: c^3Ado, 3-deazaadenosine; c^3Ade, 3-deazaadenine; c^3AMP, 3-deazaadenosine 5′-monophosphate; c^3ADP, 3-deazaadenosine 5′-diphosphate; c^3ATP, 3-deazaadenosine 5′-triphosphate; PBS-FCS, Dulbecco's phosphate-buffered saline containing 5% fetal calf serum; and Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

‡ K. L. Prus, unpublished observations.

MATERIALS AND METHODS

Materials. [^{3}H(G)]c^3Ado (22 Ci/mmol) and [8-^{14}C]inosine (60 mCi/mmol) were purchased from Moravek Biochemicals, Inc. $Na_2{}^{51}CrO_4$ was obtained from the Amersham Corp. Alkaline phosphatase (EC 3.1.3.1, from calf intestine) and yeast hexokinase (EC 2.7.1.1) were products of Boehringer Mannheim. Alkaline phosphatase (from *Escherichia coli*), 3′-ribonucleotide phosphohydrolase (EC 3.1.3.6, from rye grass), 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5, from *Crotalus adamanteus*), inosine, IMP, and ITP were obtained from the Sigma Chemical Co. ATP was purchased from Pharmacia. PBS and Hepes buffer were products of GIBCO. RPMI-1640 and penicillin/streptomycin were purchased from Flow Laboratories. Polyethyleneimine thin-layer plates were obtained from

EM Science. 5-Iodotubercidin was provided by L. B. Townsend, University of Michigan. c^3Ado and c^3Ade were synthesized in the Wellcome Research Laboratories. c^3AMP and c^3ATP were synthesized by Wayne Miller, according to the procedure cited in Ref. 16. Cytoplasmic 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5) was purified from rat liver according to published procedures [17–19].

Synthetic procedures. [^{3}H]c^3Ade was prepared by enzymatic phosphorolysis of [^{3}H]c^3Ado. Two hundred microcuries of [^{3}H]c^3Ado (supplied as a solution in 50% ethanol) was evaporated to dryness under a filtered stream of N_2 gas and reconstituted with 90 μl of 10 mM potassium phosphate (pH 7.6). Ten microliters (8.4 units) of purine nucleoside phosphorylase purified from *E. coli* [20], 10 μl (2.0 units) of *E. coli* alkaline phosphatase, and 10 μl (86 units) of calf intestine alkaline phosphatase were added, and the resultant mixture was incubated for 16 hr at room temperature. Reversed-phase HPLC analysis [4] of this reaction mixture revealed that 92% of the radioactivity was eluted with the same retention time as authentic c^3Ade. [^{3}H]c^3Ade was purified to 95% (with < 1% [^{3}H]c^3Ado contamination) by reversed-phase HPLC.

Preparation of cells. Cytolytic lymphocytes and target cells were isolated as previously described [21, 22]. Briefly, cytolytic lymphocytes were harvested by repeated lavages of the peritoneal cavity of CD-1 mice 10 days after i.p. injection of 2×10^7 EL4 cells. The nonadherent cells were collected after passage of the peritoneal exudate cells through a column containing glasswool and used for cytolytic assays as well as for 5′-nucleotide determinations. Target cells were mouse EL4 ascites leukemia maintained by serial i.p. passage in C57BL mice and harvested by repeated lavages of the peritoneal cavity. Cytolytic lymphocytes and target cells were resuspended in PBS-FCS.

Mouse resident peritoneal macrophages were harvested by repeated lavages of the peritoneal cavity of CD-1 mice. Cells were pooled, washed and resuspended in RPMI-1640 containing 25 mM Hepes, 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (referred to hereafter as RPMI medium).

Quantitation of cellular 5′-nucleotides. Cytolytic lymphocytes (2×10^7 cells in 2 ml PBS-FCS) were incubated with the specified concentration of [^{3}H]c^3Ado (174–193 μCi/μmol) or [^{3}H]c^3Ade (9.7 μCi/μmol) for 60 min at 37°. Cells were subsequently placed on ice for 10 min before centrifugation at 200 *g* for 5 min. The supernatant fraction was removed, and the pellet was extracted with 5.0 ml of cold 0.5 M perchloric acid containing 1 μM ITP as a recovery marker. After brief sonication, the cell extracts were clarified by centrifugation, and the supernatant fractions were neutralized with KOH to pH 6.5–7.5. To remove insoluble $KClO_4$, the extract was filtered through glasswool in a Pasteur pipet. The eluate was evaporated to dryness in a Buchler Evapo-Mix apparatus, and the residue was reconstituted with 300 μl of distilled water.

Macrophages in 2.5 ml of RPMI medium were placed in 25 cm^2 tissue culture flasks and incubated for 2 hr at 37°. The medium containing the nonadherent cells (30–35% of the total added) was removed, and 2.5 ml RPMI medium (supplemented with 1 mg/ml D-glucose) was added. These adherent macrophage preparations (10^7 cells/sample) were treated with [^{3}H]c^3Ado or [^{3}H]c^3Ade. After experimental manipulations, the monolayers were extracted with 2.5 ml of cold 0.5 M perchloric acid containing 1 μM ITP, and the extracts were treated as described for lymphocytes.

5′-Nucleotides in cell extracts were analyzed by anion-exchange HPLC as described previously [21]. Isocratic elution with 0.55 M potassium phosphate, pH 3.5, was employed for most analyses. The column effluent was monitored for both absorbance (254 and 280 nm) and radioactivity (present in 1.0-min fractions). Endogenous nucleotides were quantitated from the response factors (ultraviolet peak area per nanomole of nucleotide) determined by HPLC analysis of known amounts of authentic nucleotide standards. [^{3}H]c^3ATP was quantitated by measuring the amount of radioactivity eluted at the retention time of authentic c^3ATP. Each analysis was normalized on the basis of the amount of the ITP recovery marker present in each extract.

Identification of c^3ATP in cell extracts. Samples (100 μl) of cytolytic lymphocyte extracts were treated with 10 μl (14 units) of yeast hexokinase and 10 μl of 0.4 M glucose for 15 min at 31°. After acidification, centrifugation, and neutralization, samples were analyzed by gradient elution anion-exchange HPLC as previously described [21].

For alkaline phosphatase digestion of [^{3}H]c^3ATP formed metabolically in lymphocytes or macrophages, [^{3}H]c^3ATP was purified from the cell extracts by HPLC, desalted by adsorption onto charcoal, and eluted from the charcoal with 5% ammonium hydroxide in 50% ethanol. The eluate was evaporated to dryness under reduced pressure in a Buchler Evapo-Mix apparatus and reconstituted with 50 mM Tris–HCl, pH 8.0. Eighty microliters of the desalted extract was incubated with 10 μl (86 units) of calf intestine alkaline phosphatase and 10 μl (2.5 units) of *E. coli* alkaline phosphatase for 24 hr at room temperature. The reaction was terminated by boiling for 3 min followed by cooling on ice. The sample was clarified by centrifugation, and the supernatant fraction was analyzed for [^{3}H]c^3Ado by reversed-phase HPLC as previously described [4].

Lymphocyte-mediated cytolysis assay. Lysis of target cells by mouse cytolytic lymphocytes was determined as described previously [22, 23]. Briefly, the amount of ^{51}Cr released from 2.5×10^5 ^{51}Cr-labeled EL4 cells during a 70-min rocking assay with 2.5×10^5 cytolytic lymphocytes, in the presence or absence of drugs, was measured. To shorten the time needed to detect target cell lysis in the drug removal studies, the following modification of the above assay was used. Cytolytic lymphocytes (7.5×10^5 cells) were mixed with ^{51}Cr-labeled EL4 cells (2.5×10^5 cells) in a total volume of 1.0 ml PBS-FCS in 12 × 75 mm plastic tubes. The cell suspensions were mixed, centrifuged at 185 *g* for 5 min, and incubated for 15 min at 37°. After the addition of 1.0 ml of cold PBS-FCS, 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 then quantitated. The same population of lymphocytes was used for the lymphocyte-mediated cytolysis assay and the metabolism studies.

Antibody-dependent phagocytosis. The assay for antibody-dependent phagocytosis was performed, after a 30-min pre-incubation of the macrophages with saline or drugs, as described [6]. The RPMI medium was supplemented with 1 mg/ml glucose. The assay was performed with the same population of macrophages used to investigate c^3Ado and c^3Ade metabolism to c^3ATP.

Enzyme assays. The standard reaction mixture for the assay of [^{3}H]c^3Ado phosphorylation contained 100 mM imidazole-HCl, pH 6.5, 50 mM $MgCl_2$, 10 mM IMP, 500 mM NaCl, 0.1 to 20 mM [^{3}H]c^3Ado and purified rat liver cytoplasmic 5'-nucleotidase (0.01 unit/ml reaction mixture). The products from these reactions were measured using polyethyleneimine thin-layer plates pre-spotted with 10 nmol of the appropriate carriers. The plates were developed with 50% methanol and dried, and the product and substrate spots were located visually by UV absorption. The spots were cut out and quantitated by liquid scintillation spectrometry with ScintiLene (Fisher).

Kinetic analyses were performed as described [19].

Reversed-phase HPLC was used in the analysis of reaction products [4].

RESULTS

Evidence for the cellular formation of c^3ATP. HPLC evidence was obtained for the metabolic formation of small amounts of [^{3}H]c^3ADP and [^{3}H]c^3ATP from [^{3}H]c^3Ado in mouse cytolytic lymphocytes (Fig. 1) and mouse resident peritoneal macrophages (Fig. 2). The putative [^{3}H]c^3ATP which was extracted from mouse lymphocytes or macrophages and separated by anion-exchange HPLC was eluted with the same retention time as that of authentic c^3ATP and UTP. [^{3}H]c^3ATP was shifted to the same retention time as the presumptive [^{3}H]c^3ADP by treatment of the cell extract with yeast hexokinase and glucose. Alkaline phosphatase digestion of [^{3}H]c^3ATP, which was HPLC-purified from cell extracts, yielded a product that was eluted from a reversed-phase HPLC column with the same retention time as authentic c^3Ado (results not shown).

The amount of [^{3}H]c^3ATP formed by mouse cytolytic lymphocytes treated with 20 μM [^{3}H]c^3Ado at 37° increased with time up to 60 min whereupon the levels of [^{3}H]c^3ATP remained constant up to 100 min (Fig. 3). The amount of [^{3}H]c^3ATP found in both lymphocytes and macrophages represented only 1–2% of the cellular ATP levels. Moreover, c^3Ado did not affect significantly the ATP levels of these cells.

The amount of intracellular c^3ATP formed was dependent on the concentration of c^3Ado in the

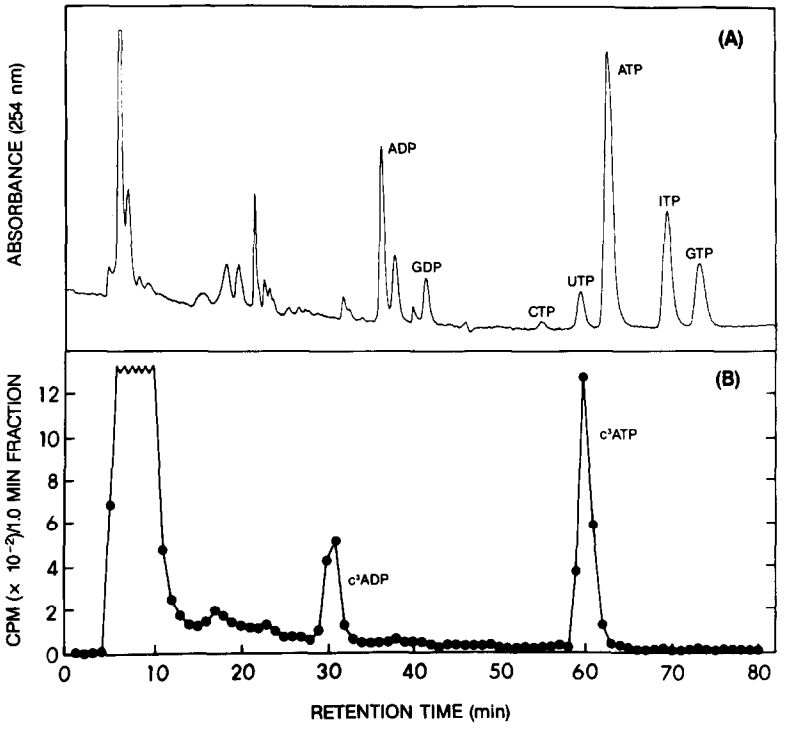

Fig. 1. HPLC elution profile of nucleotides extracted from mouse cytolytic lymphocytes. Cells (2×10^7 in 2 ml of PBS-FCS) were incubated with 20 μM [^{3}H]c^3Ado for 60 min at 37°. Nucleotides were extracted and analyzed by gradient elution HPLC as described in Materials and Methods. (A) UV absorbance; (B) radioactivity elution profile.

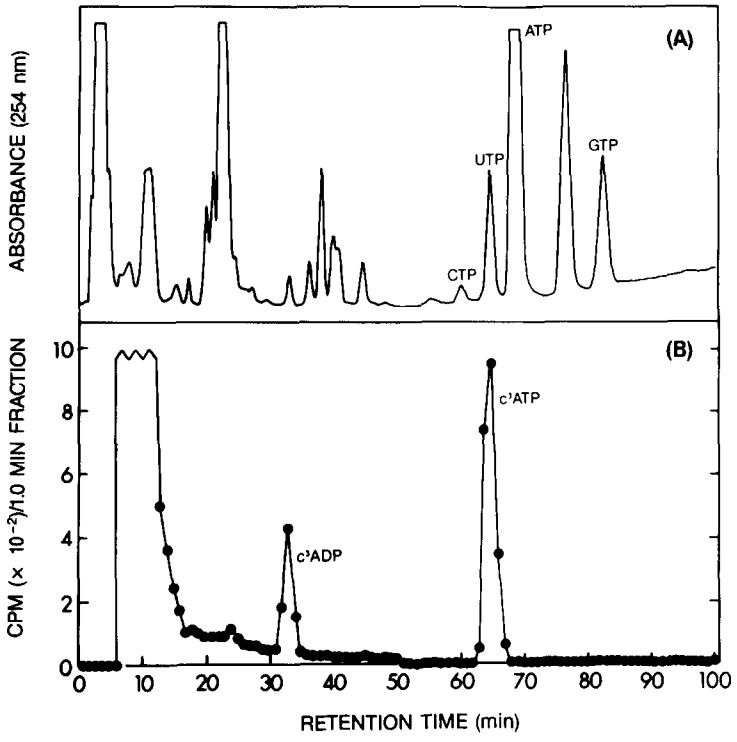

Fig. 2. HPLC elution profile of nucleotides extracted from mouse resident peritoneal macrophages. Adherent macrophages (10^7 in 2.5 ml medium) in tissue culture flasks were incubated with 20 μM [^{3}H]c^{3}Ado for 45 min at 37°. Nucleotides were extracted and analyzed by gradient elution HPLC as described in Materials and Methods. (A) UV absorbance; (B) radioactivity elution profile.

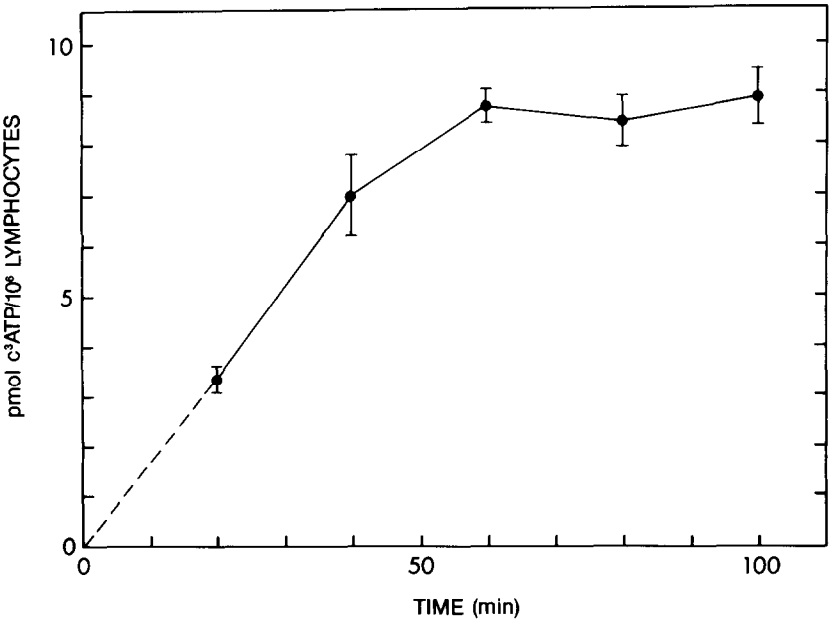

Fig. 3. Time dependence of c^{3}ATP formation from c^{3}Ado in mouse cytolytic lymphocytes. Cells (2×10^7 in 2 ml PBS-FCS) were incubated for the indicated time at 37° in the presence of 20 μM [^{3}H]c^{3}Ado. Nucleotides were extracted and analyzed as described under Materials and Methods. Each point is the mean ± the range of two determinations.

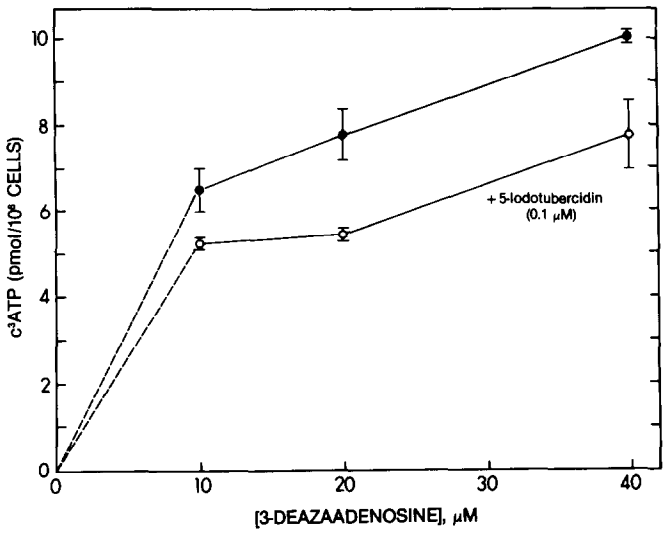

Fig. 4. Concentration dependence of the formation of c³ATP from c³Ado in mouse cytolytic lymphocytes. Cells (2×10^7 in 2 ml PBS-FCS) were incubated for 60 min at 37° with the indicated concentration of [³H]c³Ado in the absence (●——●) or presence (○——○) of 0.1 μM 5-iodotubercidin. Nucleotides were extracted and analyzed as described in Materials and Methods. Each point is the mean ± the range of two determinations.

external medium. Incubation of lymphocytes for 60 min at 37° in the presence of 10–40 μM c³Ado resulted in the accumulation of 6.5 to 10.0 pmol c³ATP/10^6 cells respectively (Fig. 4). In several experiments, the cellular formation of c³ATP from c³Ado was reduced by not more than 30% in the presence of 0.1 μM 5-iodotubercidin (Fig. 4), a potent non competitive inhibitor (K_{is} = 9 nM, K_{ii} = 20 nM) of adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) [24].

Phosphorylation of c³Ado by purified 5′-nucleotidase. It was found that purified 5′-nucleotidase from rat liver, in the presence of 10 mM IMP, 50 mM $MgCl_2$ and 500 mM NaCl, catalyzed the phosphorylation of [³H]c³Ado to [³H]c³AMP. Analysis of this reaction mixture by reversed-phase HPLC revealed the presence of two radioactive peaks corresponding to c³Ado and c³AMP (Fig. 5). The retention time of the radioactive peak which was coeluted with c³AMP was shifted to that of c³Ado upon further incubation with snake venom 5′-nucleotidase, whereas treatment of this reaction mixture with 3′-nucleotidase did not result in this putative c³AMP being converted to c³Ado (data not shown).

The enzymatic formation of c³AMP was dependent on IMP as a phosphate donor since neither ATP nor *p*-nitrophenylphosphate could serve as phosphate donors. An apparent K_m (K'_m) value for c³Ado of 20–50 mM (N = 5) was obtained. Due to limited substrate solubility (25 mM maximum), the K'_m for c³Ado phosphorylation by 5′-nucleotidase could not be determined with greater accuracy. Similarly, c³Ado inhibition of inosine phosphorylation was subject to the same limitations. However, a secondary plot of slope versus c³Ado concentration was consistent with linear competitive inhibition and yielded a K_{is} value of approximately 50 mM (52.5 ± 6.5, N = 3). A K'_m for inosine of 5.0 ± 1.9 mM (N = 6) was obtained.

Further evidence for a common catalytic site on 5′-nucleotidase for inosine and c³Ado was provided by inosine inhibition of c³Ado phosphorylation. Addition of 100 mM inosine to a reaction containing 1 mM c³Ado inhibited c³Ado phosphorylation by 96%.

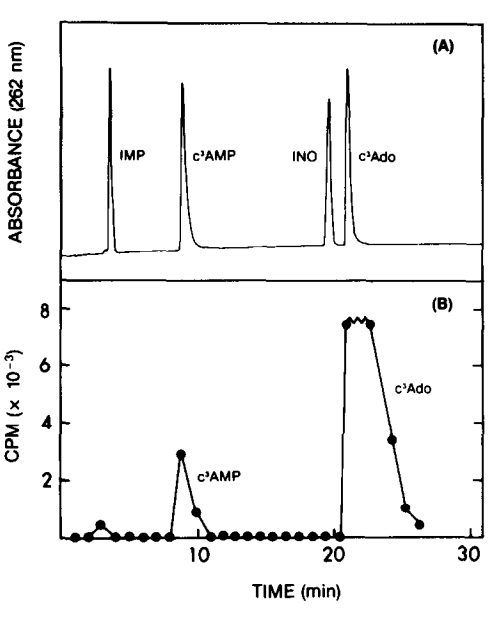

Fig. 5. HPLC profile of (A) authentic standard compounds and (B) the reaction products formed from the phosphorylation of [³H]c³Ado by purified rat liver 5′-nucleotidase. The reaction mixture contained 100 mM imidazole, pH 6.5, 50 mM $MgCl_2$ 10 mM IMP, 500 mM NaCl, purified rat liver 5′-nucleotidase (0.01 unit/ml) and 10 mM [³H]c³Ado. The reaction products were analyzed by reversed-phase HPLC and liquid scintillation spectrometry, as described in Materials and Methods.

Table 1. Effect of c^3Ado on macrophage nucleotide levels and antibody-dependent phagocytosis prior to and following drug removal from the medium

Experimental condition	Nucleotide* (pmol/10^6 cells)				Percent inhibition of phagocytosis
	UTP	ATP	GTP	c^3ATP	
Saline	163 ± 1	910 ± 58	178 ± 13		
20 μM c^3Ado	200 ± 4	1024 ± 4	239 ± 1	17.6 ± 0.6	74 ± 7
20 μM c^3Ado (30 min after removal)	212 ± 28	1039 ± 129	273 ± 33	20.1 ± 0.8	4 ± 3

Mouse resident peritoneal macrophages (10^7 cells/sample in 2.5 ml RPMI medium containing an additional 1 mg/ml glucose) were incubated in the absence or presence of 20 μM [^{3}H]c^3Ado for 45 min at 37°. The cells were then washed and either assayed or acid-extracted immediately or incubated in fresh medium without c^3Ado for 30 min prior to assay or acid-extraction. Nucleotides were analysed by anion-exchange HPLC. The assay for antibody-dependent phagocytosis was performed as described in Materials and Methods.

* Mean ± range of two determinations.

The relative rate of c^3Ado phosphorylation was approximately 20% that for inosine when the concentration of both substrates was either 0.1 or 1 mM in parallel reactions.

Significance of c^3ATP formation for c^3ADO inhibitory activity. c^3ATP formed metabolically in mouse resident peritoneal macrophages incubated with c^3Ado remained constant for at least 30 min after removal of c^3Ado from the medium (Table 1). However, the inhibitory effect of c^3Ado on phagocytosis by these cells was reversed rapidly and almost completely upon removal of the drug from the medium. Phagocytosis was inhibited by 74% in the presence of 20 μM c^3Ado, but by only 4% after removal of c^3Ado and recovery of the cells for 30 min at 37° in fresh medium.

The presence of metabolically formed c^3ATP (1.25 ± 0.13 pmol/10^6 cells) was detected in mouse cytolytic lymphocytes that were treated for 60 min at 37° with 80 μM c^3Ado. These cells demonstrated a 64% inhibition of cytolytic activity (Table 2). Lymphocytes that were treated in the same manner and subsequently washed and allowed to recover in drug-free medium for 15 min still contained the same amount of c^3ATP (1.21 ± 0.10 pmol/10^6 cells); however, these cells exhibited a substantial reduction in percent inhibition of cytolysis (29%). Moreover, cytolytic lymphocytes incubated for shorter times (15 or 30 min) with 80 μM c^3Ado also exhibited greater inhibition of cytolytic activity but lower intracellular accumulation of c^3ATP than did cells incubated for 60 min with c^3Ado and then assayed in drug-free medium.

Further evidence for the lack of correlation between c^3ATP formation and the inhibitory activity of c^3Ado on leukocyte functions was provided by studies with c^3Ade. Incubation of macrophages for 45 min with 100 μM [^{3}H]c^3Ade resulted in the accumulation of 5.8 ± 1.0 pmol c^3ATP/10^6 cells, compared to 9.9 ± 1.9 pmol c^3ATP/10^6 cells resulting from incubation of the cells with 20 μM c^3Ado (Table 3). However, c^3Ade (100 μM) inhibited phagocytosis

Table 2. Effect of c^3Ado on lymphocyte nucleotide levels and lymphocyte-mediated cytolysis prior to and following drug removal from the medium

Experimental condition	Nucleotide* (pmol/10^6 cells)					Percent inhibition of lymphocyte-mediated cytolysis
	CTP	UTP	ATP	GTP	c^3ATP	
80 μM c^3Ado, 60 min + wash	83.7 ± 3.0	263 ± 11	1090 ± 57	285 ± 28	1.21 ± 0.10	29 ± 7
80 μM c^3Ado, 60 min	73.8 ± 7.5	237 ± 6	1135 ± 44	327 ± 14	1.25 ± 0.13	64 ± 6
80 μM c^3Ado, 30 min	68.3 ± 3.0	246 ± 26	1177 ± 2	242 ± 47	0.52 ± 0.21	59 ± 7
80 μM c^3Ado, 15 min	63.9 ± 2.3	208 ± 6	1166 ± 24	221 ± 20	0.43 ± 0.03	50 ± 6

Mouse cytolytic lymphocytes (2×10^7 cells in 25 ml PBS-FCS containing 1 mg/ml glucose) were incubated with 80 μM [^{3}H]c^3Ado for the indicated time at 37°. The cells were either assayed or acid-extracted immediately or incubated in fresh medium without c^3Ado for 15 min ("wash") prior to assay or acid-extraction. Nucleotides were analyzed by anion-exchange HPLC. The assay for lymphocyte-mediated cytolysis was performed as described in Materials and Methods.

* Mean ± SEM for three determinations.

Table 3. Effects of c^3Ado and c^3Ade on macrophage nucleotide levels and antibody-dependent phagocytosis

Additive	Nucleotide* (pmol/10^6 cells)				Percent inhibition of phagocytosis
	UTP	ATP	GTP	c^3ATP	
Saline	121 ± 13	557 ± 27	160 ± 22		
20 μM c^3Ado	155 ± 12	486 ± 23	162 ± 5	9.9 ± 1.9	87 ± 3
100 μM c^3Ade	121 ± 10	532 ± 27	160 ± 11	5.8 ± 1.0	11 ± 8
200 μM c^3Ade	126 ± 2	455 ± 12	132 ± 17	4.9 ± 0.7	45 ± 7

Mouse resident peritoneal macrophages (10^7 cells in 2.5 ml of RPMI medium) were incubated with saline, 20 μM [^{3}H]c^3Ado, 100 μM [^{3}H]c^3Ade or 200 μM [^{3}H]c^3Ade for 45 min at 37° prior to their acid extraction or assay for phagocytosis. The cells were washed twice before acid extraction. Nucleotides were analyzed by anion-exchange HPLC. The assay for antibody-dependent phagocytosis was performed as described in Materials and Methods.

* Mean ± SEM for three determinations.

by only 11%, whereas 20 μM c^3Ado inhibited phagocytosis by 87%. Furthermore, 200 μM c^3Ade inhibited phagocytosis by 45%, even though it did not yield c^3ATP levels greater than those obtained with 100 μM c^3Ade.

c^3Ade, although essentially inactive in inhibiting lymphocyte-mediated cytolysis, was nonetheless metabolized to c^3ATP in mouse cytolytic lymphocytes (Table 4). The levels of c^3ATP formed upon incubation of these cells with 100 or 200 μM c^3Ade for 60 min were 7.3 ± 0.7 and 13.1 ± 1.6 pmol c^3ATP/10^6 lymphocytes respectively. By comparison, similar treatment of lymphocytes with 20 μM c^3Ado resulted in the formation of 5.9 ± 0.6 pmol c^3ATP/10^6 cells. However, while 20 μM c^3Ado inhibited lymphocyte-mediated cytolysis by 46%, 100–200 μM c^3Ade inhibited by only 5–7%.

DISCUSSION

Evidence was obtained for the intracellular formation of the novel metabolite, c^3ATP, from c^3Ado and c^3Ade in mouse cytolytic lymphocytes and mouse resident peritoneal macrophages. Intracellular formation of c^3ATP from c^3Ado was found to be both time- and concentration-dependent. The largest amount of c^3ATP formed in both cell types represented only 1–2% that of normal cellular levels of ATP. c^3ATP in macrophage and lymphocyte extracts was identified on the basis of the following criteria: (1) elution of the putative c^3ATP in the triphosphate region of the HPLC chromatogram with a retention time identical to that of authentic c^3ATP and similar to that of UTP; (2) the quantitative shift of the putative c^3ATP peak to the elution position of c^3ADP after incubation with yeast hexokinase and glucose; and (3) the conversion of the putative c^3ATP to a material having the same retention time as c^3Ado upon treatment with alkaline phosphatase.

Evidence suggests that adenosine kinase is surprisingly not primarily responsible for catalyzing c^3Ado phosphorylation. 5-Iodotubercidin, a potent inhibitor of adenosine kinase, reduced the intracellular formation of c^3 ATP from c^3Ado by only 30% under experimental conditions whereby the metabolism of 9-deazaadenosine to its corresponding 5'-triphosphate was inhibited by 96% [24]. This result is consistent with the lack of detectable substrate activity of c^3Ado with adenosine kinase, purified from rabbit liver, reported previously by Miller *et al.* [15]. No detectable phosphorylation of c^3Ado

Table 4. Effects of c^3Ado and c^3Ade on lymphocyte nucleotide levels and lymphocyte-mediated cytolysis

Additive	Nucleotide* (pmol/10^6 cells)					Percent inhibition of lymphocyte-mediated cytolysis
	CTP	UTP	ATP	GTP	c^3ATP	
Saline	20.7 ± 0.6	84.2 ± 4.7	563 ± 29	132 ± 11		
100 μM c^3Ade	14.7 ± 1.6	81.3 ± 4.1	536 ± 10	149 ± 6	7.3 ± 0.7	5 ± 3
200 μM c^3Ade	19.6 ± 1.6	85.4 ± 2.9	546 ± 17	149 ± 3	13.1 ± 1.6	7 ± 2
20 μM c^3Ado	32.5 ± 1.7	115 ± 3.5	582 ± 13	173 ± 7	5.9 ± 0.6	46 ± 5

Mouse cytolic lymphocytes (3×10^7 cells in 2 ml of PBS-FCS containing 1 mg/ml glucose) were incubated with saline, 20 μM [^{3}H]c^3Ado, 100 μM [^{3}H]c^3Ade or 200 μM [^{3}H]c^3Ade for 60 min at 37° prior to their acid-extraction or assay for lymphocyte-mediated cytolysis. Nucleotides were analyzed by anion-exchange HPLC. Lymphocyte-mediated cytolysis was assayed according to the procedure described in Materials and Methods

* Mean ± SEM for three determinations.

occurred with calf thymus deoxycytidine kinase.* In contrast, an enzyme that can transfer the phosphate group of a nucleoside 5′-monophosphate to a number of nucleoside analogs [18, 19, 25] did catalyze the phosphorylation of c^3Ado. Incubation of [^{3}H]c^3Ado with purified rat liver 5′-nucleotidase and IMP resulted in the formation of a product which was identified as [^{3}H]c^3AMP on the basis of its elution with authentic c^3AMP on a reversed-phase HPLC system and of its specific enzymatic hydrolysis with 5′-nucleotidase but not with 3′-nucleotidase. In the presence of purified rat liver 5′-nucleotidase, no c^3AMP was formed when either ATP or *p*-nitrophenylphosphate was used as the phosphate donor; however, with IMP as phosphate donor, c^3Ado phosphorylation was readily detected. This monophosphate donor specificity, in addition to a high salt and divalent cation requirement, is indicative of the activity of the cytosolic 5′-nucleotidase. The percent inhibition (96%) observed in a reaction that contained 100 mM inosine and 1 mM c^3Ado is very close to the calculated value (95%)†, using the kinetic constants determined here and assuming simple competitive inhibition. The phosphorylation of other nucleoside analogues via the enzyme-phosphate intermediate of 5′-nucleotidase has been reported [18, 19, 25].

The relevance of this novel c^3ATP metabolite to the expression of the biological activity of c^3Ado was examined in mouse cytolytic lymphocytes and resident peritoneal macrophages. Several lines of evidence suggest that c^3ATP is not an active metabolite of c^3Ado responsible for the inhibition of lymphocyte-mediated cytolysis or macrophage phagocytosis caused by this nucleoside analogue. First, the same low levels of c^3ATP were detected in mouse resident peritoneal macrophages both during incubation of the cells with c^3Ado and after removal of c^3Ado from the external medium, whereas the inhibition of phagocytosis was observed only in the continued presence of c^3Ado and was rapidly and completely reversed upon removal of c^3Ado from the medium. Likewise, the same amount of c^3ATP was found in mouse cytolytic lymphocytes before and after removal of c^3Ado, whereas lymphocyte-mediated cytolysis was inhibited much more extensively in the continued presence of c^3Ado. Second, macrophages treated with 100 μM c^3Ade formed c^3ATP in amounts similar to those observed with c^3Ado and yet were still capable of phagocytosis. Third, mouse cytolytic lymphocytes incubated in the presence of 100–200 μM c^3Ade formed c^3ATP in amounts similar to those found in lymphocytes incubated with 20 μM c^3Ado even though c^3Ade was not significantly inhibitory to lymphocyte-mediated cytolysis.

Since many of the cell functions, including lymphocyte-mediated cytolysis and phagocytosis, that are inhibited by c^3Ado require the involvement of the cytoskeleton, it seemed plausible that the microfilament system may represent the site of action of c^3Ado. In support of this hypothesis, c^3Ado, but not other inhibitors of methylation reactions, induced the disorganization of the microfilament network of mouse resident peritoneal macrophages [6]. This effect of c^3Ado was subsequently found to be indirect with respect to microfilaments, as c^3Ado did not prevent the polymerization of purified skeletal muscle actin or of extracts prepared from P388 cells or mouse splenic leukocytes.‡ Since ATP is hydrolyzed in the process of actin polymerization, the intracellular formation of c^3ATP and interference with actin polymerization initially appeared to provide an attractive mechanism of action for c^3Ado. However, the metabolic data and the rapid reversibility of biological activity suggest that c^3Ado itself, and not its 5′-triphosphate, may be responsible for the inhibition of phagocytosis and lymphocyte-mediated cytolysis.

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* J. Tuttle, unpublished observations.

† $K_i = \dfrac{I(1-i)}{i(1+S/K_m)}$.

‡ K. L. Prus, unpublished observations.

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