EFFECT OF ADENOSINE AND ADENOSINE 5'-MONOPHOSPHATE ON CELL DIVISION OF CULTURED MASTOCYTOMA P-815 CELLS*

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The growth of mouse mastocytoma P-815 cells in culture (37°, 42 hr) was inhibited by exogenous adenosine (0.2 to 1.0 mM) and more effectively by AMP (0.01 to 0.1 mM), but not by adenine. The inhibited growth (a 25% inhibition by 0.5 mM adenosine and a 80% inhibition by 0.25 mM AMP) was restored to a near control level by the addition of uridine (0.5 mM) to the medium. The pretreatment (37°, 3 hr) of the cells with adenosine or AMP caused a 60% inhibition of incorporation (37°, 2 hr) of [U-¹⁴C]aspartate into uracil nucleotides, accumulating ¹⁴C-orotate and orotidine. Both dipyridamole, an inhibitor of adenosine uptake, and exogenous adenosine deaminase suppressed the growth inhibition induced by not only adenosine but also AMP. 2-Chloroadenosine, which is resistant to the action of adenosine deaminase, was a more potent growth inhibitor, while 3'-AMP and 2'-AMP, which are not hydrolyzed to adenosine by membrane 5'-nucleotidase, were ineffective. Adenosine 5'-sulfate and other 5'-substituted adenosines were also ineffective. These observations indicate that AMP inhibits the growth of mastocytoma P-815 cells as a result of its continuous conversion to adenosine and a constant exposure of the cells to a low concentration of adenosine which readily permeates the cell membrane. In addition, adenosine, AMP and their agarose-linked forms rapidly (37°, 20 min) elevated cellular levels of cAMP. This effect was not suppressed by dipyridamole. Apparently adenosine and AMP also act extracellularly for growth inhibition by regulating cAMP levels.

Keywords—mastocytoma P-815 cells; cell division; adenosine; AMP; dipyridamole; adenosine deaminase; adenosine kinase; cAMP levels; uracil nucleotides

Adenosine is known to have many biological effects on a variety of tissues and cells.¹⁾ It inhibits the growth of several mammalian cells in culture²⁻⁵⁾ apparently partly blocking the pyrimidine biosynthesis.⁶⁻⁸⁾ Accumulated evidence also indicates that adenosine is a regulator of cellular levels of adenosine 3', 5'-monophosphate (cAMP), activating adenylate cyclase⁹⁻¹⁹⁾

and inhibiting cAMP phosphodiesterase²⁰⁻²²⁾ in brain, liver and various other tissues and cells. On the other hand, adenosine produces the opposite effect in fat cells^{23,24)} and lung tissues,²⁵⁾ inhibiting the accumulation of cAMP. Furthermore, adenosine is known to inhibit the binding of cAMP to protein kinase^{26,27)} and protein factors which differ from the subunit of cAMP-depen-

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dent protein kinase.²⁸⁾

In addition, both adenosine and cAMP are implicated in the modulation of mast cell functions. Adenosine at physiologic concentrations is known to enhance the mediator release from mast cells induced by various liberators,²⁹⁾ whereas cAMP suppresses the histamine release induced by several chemical liberators.³⁰⁾ Since mast cells can be differentiated from thymus lymphocytes, as suggested by Ishizaka and her coworkers,^{31,32)} which are very sensitive to cytotoxic effect of adenosine,¹⁾ we have examined the effect of adenosine and its related compounds on growth and cAMP content of a neoplastic mast cell, mouse mastocytoma P-815 cell. In this communication, we describe the inhibition of growth and DNA synthesis, and the elevation of cAMP levels of mastocytoma P-815 cells by adenosine and AMP. A preliminary report of this work has appeared in a previous paper.³³⁾

EXPERIMENTAL PROCEDURES

Cell Growth—Mouse mastocytoma P-815 cells³⁴⁾ were supplied by Dr. M. Potter, National Cancer Institute, NIH, Bethesda, Md, USA, and maintained in suspension culture³⁵⁾ in Fischer and Sartorelli's medium³⁶⁾ supplemented with 5% fetal calf serum (standard medium). For experiments, suspensions of cells in the logarithmic phase of growth (4 to 8×10^4 cells/ml, 30 ml) were incubated in 125-ml Erlenmeyer flasks with or without drugs to be tested in an atmosphere of 5% CO₂ and 95% air at 37° in a humidified incubator. The cells were harvested by centrifugation at $280 \times g$ for 5 min at 4° in siliconized centrifuge tubes, resuspended in a given volume of cold phosphate-buffered saline (PBS)³⁷⁾ and used for assays. Cell numbers were determined with a hemacytometer after suspending cells in 0.2% nigrosin in Earl's balanced salt solution.³⁸⁾

Measurement of cAMP and Its Degradation Products—cAMP was determined by the radioimmunoassay method of Okabayashi *et al.*³⁹⁾ using a rabbit antiserum for O^{2'}-succinyl cAMP conjugated with human serum albumin. Degradation products of [³H]cAMP in the incubation

medium and in the cells were separated by thin-layer chromatography on aluminium sheets (TLC plates, Silica gel 60/Kieselguhr F₂₅₄ pre-coated, E.Merck, Darmstadt, Germany) with isobutyric acid : ammonia water (28%) : H₂O (64:3:33, by volume) as the developing solvent. Labeled products were located with co-chromatographed unlabeled authentic compounds under UV illumination. Radioactive spots were scraped from chromatograms, and counted in 15 ml of a scintillant (4 g of 2,5-diphenyloxazole (PPO) in 1 liter of toluene-Triton X-100 (2:1, v/v)) by liquid scintillation spectrometry.

Assays of Enzymes—Adenosine deaminase [adenosine aminohydrolase, EC 3.5.4.4] and adenosine kinase [ATP: adenosine 5'-phosphotransferase, EC 2.7.1.20] were partially purified from mastocytoma P-815 cells. Cells homogenized in 10 mM Tris-HCl (pH 7.4) containing 4 mM MgCl₂ were centrifuged at $800 \times g$ for 15 min. To the supernatant fraction solid ammonium sulfate was added, and the fraction obtained between 50 and 80% saturation of the salt was dissolved in 0.05 M Tris-HCl (pH 7.0), dialyzed against the same buffer, and then applied to a column (2.5 × 14 cm) of DEAE-cellulose preequilibrated with the same buffer. A linear gradient of NaCl, 0 to 0.5 M, in 0.05 M Tris-HCl (pH 7.0) was used to elute the enzyme. Adenosine kinase (0.0254 unit/mg of protein) and adenosine deaminase (0.0675 unit/mg of protein), free from each other enzyme activity, were eluted with 0.15 M and 0.25 M NaCl, respectively. Protein was determined by the method of Lowry *et al.*⁴⁰⁾

(1) Adenosine deaminase activity was assayed according to Kalckar by following the decrease of absorbance at 265 nm resulting from the deamination of adenosine.⁴¹⁾ The assay mixture (0.3 ml) containing 0.1 mM adenosine, 0.2 M potassium phosphate (pH 7.0) and the enzyme was incubated at 37° for 10 min. One unit of enzyme catalyzed the deamination of 1 μmol of adenosine per min under the assay conditions.

(2) Adenosine kinase was assayed by a slight modification of the method of Divekar and Hakala.⁴²⁾ The assay mixture (0.25 ml) contained

0.06 mM [8-¹⁴C]adenosine (2.5 μ Ci/ μ mol), 2.5 mM ATP, 0.25 mM MgCl₂, 20 mM potassium phosphate (pH 7.2) and the enzyme. After the incubation at 37° for 30 min, the reaction was terminated by heating the mixture in a boiling water bath for 10 min. Aliquots (50 μ l) were subjected to descending chromatography on Whatman No. 3MM paper in butanol:acetic acid:water (20:3:7, by volume). The radioactive spots corresponding to authentic adenine nucleotides were cut from the paper chromatogram, and counted in 10 ml of the scintillant described above. One unit of enzyme catalyzed the phosphorylation of 1 μ mol of adenosine per min under the assay conditions.

Assay of Uracil Nucleotide Synthesis—The effect of adenosine and its related compounds on biosynthesis of uracil nucleotides was examined by applying the method of Ishii and Green.²⁾ Cells (about 10⁸), harvested at the logarithmic phase of growth were incubated in 10 ml of the standard medium containing 0.2 mM adenosine or AMP for 3 hr at 37°, and then for another 2 hr after the addition of [U-¹⁴C]aspartate (1 mCi/ml, 167 mCi/mmol). After centrifuging at 280×g for 10 min, the medium was acidified with ice-cold HClO₄ to a final concentration of 5% HClO₄. Packed cells were resuspended in 2 ml of distilled water, and 60% HClO₄ was added to a final concentration of 5%. The acidified medium and cell suspension were separately centrifuged at 22000×g for 15 min, and each precipitate was washed once with a small volume of 5% HClO₄. Combined HClO₄ extracts of medium and of cells were neutralized with KHCO₃. After centrifuging at 22000×g for 15 min, the clear supernatant fraction of each extract was adjusted to 2 mM with formic acid (a final pH=3.5), and mixed with washed charcoal (2%, w/v). After washing the charcoal with 1 mM formic acid, nucleotides adsorbed were extracted with 5% ethanol containing 0.5 M NH₄OH. Extracts were evaporated to dryness *in vacuo*, dissolved in a small volume of distilled water, and subjected to two-dimensional chromatography on Whatman No. 3MM paper, using isopropanol:HCl:water (70:15:15, by

volume) for the first dimension and isopropanol:water:ammonium hydroxide (28%) (85:15:1.3, by volume) for the second dimension.²⁾ Labeled products were located with co-chromatographed unlabelled authentic compounds under UV illumination. Areas containing radioactive uridine derivatives were cut into 3-cm wide strips along the second dimension, then cut into 1-cm wide pieces, and counted for radioactivity in 15 ml of a dioxane-based scintillant (5 g of PPO and 100 g of naphthalene in 1 liter of dioxane).

Assay of DNA Formation—Cell suspensions were incubated at 37° for 1 hr in 2 ml of the standard medium containing [methyl-³H]thymidine (³H]TdR, 0.2 μ Ci). After halting the reaction by the addition of 10% trichloroacetic acid (TCA), precipitated cells were sonicated for 1 min. The TCA-precipitable fraction was washed three times with 5% TCA and once with ethanol-ether mixture (3:1, v/v).⁴³⁾ The radioactivity of TCA-insoluble DNA fraction was counted in 0.4 ml of NCS tissue solubilizer and 10 ml of the toluene-based scintillant described above.

Agarose-bound AMP and Adenosine—⁶N-(6-Aminohexyl)-[¹⁴C]AMP-agarose was prepared by applying the method of Mosbach,⁴⁴⁾ using [8-¹⁴C]6-chloro-AMP instead of 6-chloro adenosine riboside.

Materials—cAMP and 8-methylthio cAMP were obtained from Seishin Seiyaku Co., Nodashi; Chiba, Japan; N⁶,O^{2'}-dibutyryl cAMP (Bt₂cAMP), guanosine 3', 5'-monophosphate (cGMP), 5'-nucleotidase (Crotalus atrox venom), adenosine 5'-sulfate, adenosine 5'-mononicotinate, β , γ -methylene adenosine 5'-triphosphate (AMP-PCP) and adenylyl-5'-yl imidodiphosphate (AMP-PNP) from Sigma Chemical Co., St. Louis, Mo, USA; adenosine deaminase of calf intestine (200 U/mg) from Boehringer GmbH, Mannheim, Germany; and 1-methyl-3-isobutylxanthine (MIX) from Aldrich chemical Co., Milwaukee, Wi, USA. 2-Chloro-adenosine and other adenosine analogues were gifts of Dr. K. Kikugawa, Kohjin Co., Hachiwoji, Tokyo, Japan. [8-³H]cAMP (30–50 Ci/mmol), [methyl-

^3H]thymidine (40–60 Ci/mmol), $[8\text{-}^{14}\text{C}]6\text{-chloro-AMP}$ (5 mCi/mmol) and NCS tissue solubilizer were obtained from Amersham Corporation, Arlington, Ill., USA. AMP-Agarose ($2\text{ }\mu\text{mol/ml}$) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Fetal calf serum and horse serum were obtained from Microbial Associates, Bethesda, Md, USA.

RESULTS

Effect of Adenosine and AMP on Cell Division of Mastocytoma P-815 Cells

Both adenosine and AMP inhibited the proliferation of mastocytoma P-815 cells incubated in the standard medium containing fetal calf serum at 37° (Fig. 1A). The inhibition by adenosine was dose-dependent in a range from 0.2 mM to 1 mM (Fig. 1B). By replacing fetal calf serum with horse serum, which lacked in adenosine deaminase activity,²⁾ the growth inhibitory effect of adenosine was approximately doubled.

AMP, at a concentration from 0.01 mM to 0.1 mM, was a more potent suppressor of cell division than adenosine (Fig. 1C). A significant inhibitory

effect was detectable with AMP as low as 0.01 mM. At higher concentrations, AMP became extremely cytotoxic. After 3-hr incubation with 0.5 mM AMP at 37° , 10 to 15% of the inoculated cells became nonviable, and after 20 hr, 80 to 90% of them were not viable. The potency of AMP was slightly higher in horse serum than in fetal calf serum.

Since dipyridamole is known to block cellular uptake of adenosine in isolated perfused guinea pig heart,⁴⁵⁾ and inhibit adenosine deaminase of blood,⁴⁶⁾ the effect of this agent on the growth inhibition induced by adenosine and AMP was examined. Dipyridamole (10 μM) almost completely suppressed the adenosine uptake by mastocytoma cells when the concentration of adenosine was below 60 μM in the medium (Fig. 2), but the suppressive effect of dipyridamole was progressively reduced with increasing concentrations of adenosine. Dipyridamole (10 μM) markedly reduced the strong growth inhibition by AMP and also suppressed the growth inhibition by adenosine without causing any noticeable cell damage (Fig. 1). However, above 10 μM , dipyridamole also became cytotoxic. The ex-

FIG. 1. *Effect of Adenosine and AMP on Cell Division of Mastocytoma P-815 Cells.*

(A) Cells (0.4×10^5 cells/ml) were incubated at 37° . After the addition (indicated by arrow) of 0.5 mM adenosine ($\cdots \circ \cdots$), 0.1 mM AMP ($\cdots \Delta \cdots$), or none (saline alone) ($\text{---} \bullet \text{---}$), cells were incubated for another 40 hr. Values are the mean of duplicate determinations.

(B) and (C) Suspensions of cells (0.8×10^5 cells/ml, 30 ml) were incubated at 37° for 42 hr in the medium supplemented with 5% fetal calf serum ($\bullet \text{---} \bullet$) or 5% horse serum ($\circ \text{---} \circ$) in the presence of different concentrations of adenosine (A) or AMP (B), and with 0.2 unit/ml of adenosine deaminase ($\square \text{---} \square$, +AD in fetal calf serum) or 5 μM dipyridamole ($\Delta \text{---} \Delta$, +DP in fetal calf serum). The cell number in the control flask after 42-hr incubation without drug was 8.22 ± 10^5 cells/ml (= 100%). Values are the mean of triplicate determinations $\pm$ S.E.

genous adenosine deaminase, which was free of AMP deaminase activity, effectively abolished the inhibitory effect of not only adenosine but also AMP.

Effect of Analogues of Adenosine and AMP on Cell Division

Both ADP and ATP were as inhibitory to cell division as AMP (Fig. 3). Neither AMP-PCP nor AMP-PNP, ATP analogues which are resistant to hydrolysis by ATPases in rat liver membranes,⁴⁷⁾ was effective. NADH and FAD were inhibitory, while NMN and FMN, which did not have adenine nucleotide structure,⁴⁾ were without effect. 3'-dAMP and 2'-AMP were ineffective. This may indicate that the hydrolysis of AMP by membrane 5'-nucleotidase might be required for AMP to permeate cell membrane. 2'-Deoxyadenosine, which is as similarly transported and metabolized as adenosine,¹⁾ was also as inhibitory

FIG. 2. Effect of Dipyridamole on Adenosine Incorporation into Mastocytoma P-815 Cells.

Suspensions of cells (5×10^6 cells in 1 ml of PBS) were preincubated with $5 \mu\text{M}$ (◐) and $10 \mu\text{M}$ (●) dipyridamole or without dipyridamole (○) for 15 min at 37° . Incubation was continued for another 15 min after the addition of different concentrations of adenosine containing $0.2 \mu\text{Ci}$ of [^3H]adenosine. The reaction was halted by the addition of 10 ml of PBS, and the mixture was filtered immediately on a glass filter (Whatman, GF/c, 25-mm diameter). After washing the cells on the filter twice with 5 ml of cold PBS, the filter was counted for radioactivity in 10 ml of a Triton scintillant (Experimental procedures).

to cell division as adenosine. Among other adenosine analogues tested, 2-chloro-, 2-amino-, and N^6 -hydroxy-adenosines, which did not serve as the substrate for adenosine deaminase, were very effective in inhibiting cell division. However, adenosine 5'-mononicotinate, adenosine 5'-sulfate, 2',5'-dihydroxyadenosine, 5'-deoxyadenosine and 5'-methylthio-5'-deoxyadenosine were ineffective, indicating a requirement of re-phosphorylation of adenosine to AMP by intracellular adenosine kinase for growth inhibition by exogenous adenosine and its analogues.

cAMP was also inhibitory to cell division. According to dose-response curves, 0.25 mM cAMP was equipotent to 0.05 mM AMP. Thin layer chromatographic analysis showed that about 30% of [^3H]cAMP degraded mainly into [^3H]AMP and a trace amount of [^3H]adenosine during 40 hr incubation at 37° in the standard medium containing fetal calf serum without cells. However, cAMP phosphodiesterase inhibitors, theophylline (1 mM) (Fig. 3) as well as papaverine (0.01 mM) and MIX (0.5 mM) did not augment the inhibitory effect of cAMP. Furthermore, cAMP was without effect in a medium containing heat-denatured fetal calf serum which did not have any residual cAMP phosphodiesterase activity (data not shown). Other nucleotides (GMP, CMP, UMP and IMP), nucleosides (guanosine, cytidine, uridine and inosine), and purine and pyrimidine bases (adenine, guanine, cytosine and hypoxanthine) had no effect on cell division of mastocytoma P-815 cells.

Effect of Uracil Derivatives on Cell Growth Inhibition induced by Adenosine and AMP

The growth inhibitory effect of adenosine (0.4 mM) and AMP (0.25 mM) was markedly prevented by the addition of uridine (0.5 mM) to the incubation medium (Table I). Uracil and deoxyuridine were less effective than uridine. UMP, UDP and UTP were about equally active as uridine, but all other pyrimidine and purine bases, and their nucleosides and nucleotides tested were ineffective. None of these compounds alone affected the cellular growth. Uridine at an equimolar concentration of adenosine showed a

FIG. 3. *Effect of Adenosine (Ado-) and AMP-analogues on Cell Division of Mastocytoma P-815 Cells*
 Suspensions of cells (0.92×10^5 cells/ml) were incubated at 37° for 42 hr in the standard medium containing various drugs at 0.1 mM (□) and 0.5 mM (■) concentrations. Theophylline (1 mM) was used for cAMP incubation. Values are the mean of triplicate determinations $\pm$ S.E.

maximal activity to restore the growth rate, but even in 2- to 3-fold excess of adenosine or AMP, it did not fully restore the uninhibited growth rate but usually up to 80 to 90% of the control level. These results indicate that adenosine or AMP may function as a growth regulator partly

through its interference with the uridine metabolism in mastocytoma cells.

Effect of Adenosine and AMP on Uridine Nucleotide Synthesis in Mastocytoma P-815 Cells

Pretreatment of cells with 0.5 mM adenosine or 0.25 mM AMP (37°, 3 hr) resulted in 60 to 65%

TABLE I. *Effect of Uracil Derivatives on Growth Inhibition of Mastocytoma P-815 Cells induced by Adenosine and AMP*

Addition (0.5 mM)	Control	Adenosine (0.4 mM)	AMP (0.25 mM)
Cell number ($\times 10^5$ /ml) (% of control*)			
None (control*)	8.32 $\pm$ 0.34	5.32 $\pm$ 0.25 (64)	1.76 $\pm$ 0.22 (21)
Uracil	8.34 $\pm$ 0.24	6.32 $\pm$ 0.33 (76)	3.98 $\pm$ 0.52 (48)
Uridine	8.28 $\pm$ 0.23	7.59 $\pm$ 0.25 (91)	6.92 $\pm$ 0.24 (83)
Deoxyuridine	8.33 $\pm$ 0.24	6.48 $\pm$ 0.23 (78)	3.76 $\pm$ 0.29 (45)

A suspension of mastocytoma P-815 cells (2×10^5 cells/ml, 30 ml) was incubated with 0.4 mM adenosine or 0.25 mM AMP and each 0.5 mM uracil derivatives at 37° for 40 hr. The cell number was counted as described in Experimental procedures. Values are the mean of three incubations $\pm$ S.E. Values in parenthesis are the percentage of the control (8.32).

TABLE II. *Inhibition of Uridine Nucleotide Synthesis by Adenosine and AMP in Mastocytoma P-815 Cells*

Pretreatment	Uridine nucleotides			Orotate and orotidine		
	Medium	Cells	Total	Medium	Cells	Total
(cpm/ 10^8 cells)						
None (control)	435	535	960	203	81	284
Adenosine (0.5 mM)	283	122	405	582	280	862
AMP (0.25 mM)	214	117	331	478	250	728

Mastocytoma P-815 cells (10^8) were pretreated with 0.5 mM adenosine or 0.25 mM AMP at 37° for 3 hr, and then incubated with [U - ^{14}C]aspartate (10μ Ci) for another 2 hr. Labeled products in $HClO_4$ -soluble fractions of the medium and the cells were separated by two-dimensional paper chromatography and counted for radioactivity as described in Experimental procedures.

TABLE III. *Effect of AMP on the Incorporation of [3H]Thymidine into Acid-insoluble DNA Fraction of Mastocytoma P-815 Cells*

Addition	Incubation time after AMP (hr)				
	1	2	4	8	18
(cpm/ 10^6 cells)					
None (control)	4.56 $\pm$ 0.23	4.78 $\pm$ 0.43	4.83 $\pm$ 0.36	4.56 $\pm$ 0.44	4.22 $\pm$ 0.25
AMP	4.24 $\pm$ 0.20	4.21 $\pm$ 0.25	2.90 $\pm$ 0.12	1.86 $\pm$ 0.13	0.43 $\pm$ 0.05
AMP+dipyridamole	4.56 $\pm$ 0.45	4.68 $\pm$ 0.32	4.73 $\pm$ 0.33	4.28 $\pm$ 0.29	3.56 $\pm$ 0.21

Cells (2.1×10^5 cells/ml, 40 ml) were incubated at 37° in the standard medium containing 0.1 mM AMP with or without 10μ M dipyridamole. At a given time, aliquots (2 ml) were withdrawn for measuring the incorporation of [3H]thymidine into acid-insoluble fraction as described in Experimental procedures. Values are the mean of three determinations $\pm$ S.E.

reduction of [^{14}C]aspartate incorporation (37° , 2 hr) into uridine nucleotides of acid-soluble fraction of the cells and the medium (Table II). After the pretreatment with AMP, no significant radioactivity was recovered in uridine or thymidine fraction, but most of the radioactivity was present in orotate and orotidine fractions (Fig. 4). A similar inhibition of [^{14}C]aspartate incorporation into uridine and thymidine was observed

FIG. 4. Paper Chromatograms of Pyrimidine Nucleotides and Nucleosides of Mastocytoma P-815 Cells incubated with AMP and then with [^{14}C]Aspartate

Suspensions of cells (10^8 cells in 10 ml) were preincubated in the standard medium with 0.2 mM AMP for 3 hr at 37° , and then with [^{14}C]aspartate ($1 \mu\text{Ci/ml}$) for 2 hr. HClO_4 extracts of the medium and the cells were subjected to two-dimensional chromatography on Whatman 3MM paper as described in Experimental procedures. After the second dimension development and visualization of reference compounds by UV illumination, a 3-cm wide strip was cut from the chromatogram. The radioactivity of 1-cm wide pieces of the strip was counted. ($\circ$ — $\circ$), control (-AMP); ($\bullet$ - - - $\bullet$), + 0.2 mM AMP. cpm = radioactivity per 10^8 cells.

by the treatment of cells with adenosine as in the case reported for fibroblasts.²⁾

Effect of AMP on DNA Synthesis of Mastocytoma P-815 Cells

AMP (0.1 mM), which strongly inhibited the cell growth during 42-hr incubation (Fig. 1), did not significantly suppress the incorporation of [^3H]thymidine into acid-insoluble DNA fraction for 2 hr, but progressively suppressed it thereafter (Table III). The addition of dipyrindamole ($10 \mu\text{M}$) virtually abolished the inhibitory effect of AMP. *Effect of Adenosine and AMP on cAMP Content of Mastocytoma P-815 Cells*

Adenosine and AMP, inhibitors of cell division, rapidly elevated cellular cAMP levels in the presence of MIX (Table IV). Other nucleotides, nucleosides, and purine and pyrimidine bases, which had no effect on cell division, were essentially ineffective. Pretreatment of cells with dipyrindamole augmented the stimulatory effect of adenosine on cAMP levels, but did not affect the effect of AMP. In addition, agarose-linked AMP and adenosine were also effective in elevating cAMP levels in the presence of MIX, although these compounds were less effective than free adenosine and AMP.

DISCUSSION

Our results showed that, as reported for fibroblasts and other mammalian cells in culture,^{2-5,48)} the proliferation of mastocytoma P-815 cells were suppressed by adenosine and/or AMP, but not by adenine. Both adenosine and AMP interfered with the synthesis of uridine nucleotides and DNA (Tables II and III, Fig. 4) as reported for other cultured cells,¹⁻⁴⁾ but the inhibitory mechanism seems to be complicated. Adenosine produced a weaker growth inhibitory effect on mastocytoma P-815 cells than AMP (Fig. 1). However, adenosine is probably an important regulator of proliferation of this cell line by following reasons.

(1) A low concentration of dipyrindamole, a potent inhibitor of adenosine uptake (Fig. 2),⁴⁵⁾ significantly suppressed the growth inhibitory

effect of not only adenosine but also AMP (Fig. 1). This may be partly because the strong growth inhibition by AMP is brought about by exposing the cells to low concentrations of adenosine which will be gradually produced from AMP during incubation, and dipyridamole prevents the uptake of adenosine. As shown in Fig. 2, non-cytotoxic concentrations of dipyridamole (below 10 μ M) effectively suppressed the uptake of low concentrations of adenosine, but it became

ineffective with higher concentrations of adenosine. Furthermore, dipyridamole apparently increases the concentration of adenosine in the incubation medium containing AMP. A preliminary experiment showed that, after 18 hr incubation of mastocytoma P-815 cells (2×10^5 /ml) in the standard medium, 32% of 0.05 mM [3 H]AMP added degraded to [3 H]adenosine in the presence of 5 μ M dipyridamole, while 13% degraded in its absence.

TABLE IV. *Effect of Adenosine, AMP and related Compounds on cAMP Content of Mastocytoma P-815 Cell*

Experiment	Addition	Concentration (mM)	cAMP content (pmol/ 10^6 cells)
I	None		1.3
	MIX	0.5	7.4
	Adenosine	0.1	3.9
	+MIX	0.5	40.2
II	MIX	0.5	10.1
	+ATP	0.1	22.1
	+ADP	0.1	25.1
	+AMP	0.1	42.8
	+2'(3')AMP	0.2	16.2
	+adenosine	0.1	61.9
	+2''-deoxyadenosine	0.5	16.1
	+adenine	0.5	12.2
	+NAD	0.1	28.9
	+NADH	0.1	32.4
	+FAD	0.1	32.1
	+2-chloroadenosine	0.1	109.2
III	MIX	0.5	7.5
	+dipyridamole	0.01	7.3
	+adenosine	0.05	39.8
	+dipyridamole	0.01	53.8
	+AMP	0.1	19.8
	+dipyridamole	0.01	20.0
IV	MIX	0.5	7.0 $\pm$ 0.2
	+Agarose-AMP	0.02	9.5 $\pm$ 0.5*
	+Agarose-adenosine	0.02	14.2 $\pm$ 0.4**

*Mastocytoma P-815 cells (about 5×10^6) were incubated in 1 ml of PBS containing various reagents at 37° for 20 min. In Experiment III, cells were preincubated with 10 μ M dipyridamole at 37° for 5 min, and further incubated with adenosine or AMP. After the addition of 1 ml of cold 10% TCA to halt the reaction, cAMP content in the cells plus the medium was determined as described in Experimental procedures. Each value represents the mean of two incubations. In Experiment IV, values are the mean of five incubations $\pm$ S.E. * $p < 0.05$ and ** $p < 0.02$.*

(2) 2-Chloroadenosine and other adenosine analogues, which are not hydrolyzed by adenosine deaminase, were very effective growth suppressors (Fig. 3). Furthermore, adenosine was more effective in horse serum lacking adenosine deaminase than in calf serum, and exogenous adenosine deaminase almost completely abolished the growth inhibitory effect of both adenosine and AMP. A similar potent growth inhibitory effect of low concentrations of adenosine on fibroblasts incubated in horse serum, and its ineffectiveness in calf serum were reported also by Ishii and Green.²⁾

(3) NAD, NADH and FAD, which might give rise to AMP as a common degradation product were effective in suppressing the growth of mastocytoma cells as reported for HeLa cells,⁴⁾ while 3'dAMP, 2'-AMP and other derivatives which are not hydrolyzed by membrane 5'-nucleotidase^{49,50)} were without effect. These results (1, 2 and 3) strongly suggest that a continuous exposure of mastocytoma cells to a low concentration of adenosine, which is slowly produced from AMP by enzymes present in serum or plasma membrane, and not much accumulated in the medium, is very effective in suppressing cellular growth as postulated by Hilz and Kaukel for HeLa cells.⁴⁾

(4) In contrast to AMP which does not permeate cell membrane, adenosine is readily taken up by the cells and then can be reconverted to AMP either by direct phosphorylation or by purine salvage pathway.^{1,4)} Therefore, intracellular adenosine kinase and adenosine deaminase must play important roles in regulation of cytotoxicity of adenosine. Ineffectiveness of 5'-deoxyadenosine, 2',5'-dideoxyadenosine and other 5'-substituted adenosine analogues, and effectiveness of 2'-deoxyadenosine in growth inhibition (Fig. 3) also indicate a requirement of re-phosphorylation of adenosine to AMP by adenosine kinase for growth inhibition. In our preliminary experiment, K_m (adenosine) values of adenosine deaminase and adenosine kinase partially purified from mastocytoma P-815 cells were found to be 0.4 mM and 8 μ M, respectively. In addition, adeno-

sine kinase was inhibited by high concentration of adenosine (a 50% inhibition by 70 μ M adenosine). This 50-fold difference in K_m value of the two enzymes for the same substrate may influence the cytotoxicity of adenosine depending upon the concentration of exogenous adenosine to which cells are exposed. However, the mechanism of adenosine phosphorylation is apparently very complicated, and influenced also by intracellular nucleotide pool, especially ATP pool⁵¹⁾ and Mg^{2+} concentration.⁵²⁾ Recently S-adenosylhomocysteine hydrolase [EC 3.3.1.1] has been identified as a cytosolic protein with high binding affinity for adenosine in human lymphoblasts and placenta.⁵³⁾ The intracellular concentration and cytotoxicity of adenosine may be regulated by this protein also in mastocytoma P-815 cells. Although rephosphorylation of adenosine to AMP is required for growth inhibition, the inhibitory mechanism also seems to be complicated. The growth inhibition of Chinese hamster cells by 2'-deoxyadenosine, which is readily transported into the cells and metabolized similarly like adenosine,¹⁾ was not prevented by uridine, but overcome by deoxycytidine,⁵⁴⁾ indicating an inhibition of ribonucleotide reductase by accumulated dATP, instead of an inhibition of uridine biosynthesis. AMP may regulate the biosynthesis of nucleotides and the purine salvage as a stronger suppressor than adenosine of phosphoribose pyrophosphokinase.⁵⁵⁾ However, the cytotoxicity of adenosine may not be mediated by nucleotides to which it may be converted, because the adenosine kinase-deficient mutant of human lymphoblast was reported to be still as sensitive to its parent to growth inhibition caused by adenosine when adenosine deaminase was inhibited by erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) in horse serum.⁵⁶⁾ These points certainly need further investigations.

(5) Adenosine was more effective than AMP in elevating cellular levels of cAMP, a known regulator of cell growth, of mastocytoma P-815 cells in the presence of MIX (Table IV). Adenosine is known to inhibit cAMP phosphodiesterase from a variety of sources,²⁰⁻²³⁾ and

might affect the metabolism of cAMP in mastocytoma cells. However, adenosine seems to act extracellularly, because dipyrindamole ($10\ \mu\text{M}$) did not inhibit the increase of cAMP induced by adenosine or AMP in mastocytoma cells as reported in neuroblastoma cells.¹²⁾ It is also known that dipyrindamole did not affect the decrease of cAMP in fat cells.⁵⁷⁾ Furthermore, agarose-linked adenosine and AMP were also able to elevate cAMP levels, although they were less active than free adenosine and AMP (Table IV). It has been reported that adenosine covalently bound to starch was nearly as potent as free adenosine in inhibiting the increase of cAMP in fat cells.⁵⁸⁾ On the other hand, the chemical stability of agarose-linked ligand has been questioned, because Vauquelin *et al.*⁵⁹⁾ observed that the ligand was readily released from cyanogen bromide-activated agarose-linked isoproterenol under physiological incubation conditions (37° , 10 min). In our preliminary experiment, after 10-min incubation of mastocytoma P-815 cells (2×10^6) with $0.8\ \mu\text{mol}$ of [^3H]AMP-agarose 4B ($10\ \mu\text{Ci}$) in the standard medium (1 ml) at 37° , 0.1% of the total radioactivity was recovered in the supernatant fraction ($1500 \times g$, 10 min). Thin layer chromatographic analysis showed that 67% of the released radioactivity was associated with AMP, 25% with adenosine and a trace with adenine. These results correspond to the release of 0.54 nmol of AMP and 0.2 nmol of adenosine from agarose-linked AMP. In contrast to cellular growth during a longer incubation, adenosine was more effective than AMP in elevating cAMP levels, and dipyrindamole augmented the effect of adenosine, but did not affect the effect of AMP on cAMP levels. However, it is not clear yet as to whether adenosine slowly produced from AMP was causing the increase of cAMP levels. The dependence of cAMP elevating effect on adenosine structure was evident, since 2-chloroadenosine, a powerful growth inhibitor (Fig. 3) which is known to increase cAMP levels in rat liver cells, human fibroblasts and neuroblastoma cells,⁶⁰⁾ was very effective in elevating cAMP levels in

mastocytoma P-815 cells (Table IV).

The action of adenosine to elevate cAMP levels seems to be rapid and extracellular. However, the relationship between the action of adenosine to elevate cAMP levels and the growth inhibitory effect of adenosine seems to be complicated. Ullman *et al.*⁶¹⁾ reported that adenosine was always toxic to T cell lymphosarcoma, even when specific mutations were induced to the cells that blocked adenosine-mediated increase of cAMP, but left intact the responsiveness to isoproterenol or cholera toxin to elevate cAMP. These results may imply that alteration of cAMP level is not critical for adenosine toxicity. The relationship between adenosine-mediated increase of cAMP and growth inhibition, and the regulatory mechanism to orient intracellular adenosine to purine salvage pathway or direct phosphorylation certainly needs further investigations.

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