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INHIBITION OF LYMPHOCYTE-MEDIATED CYTOLYSIS BY ADENOSINE ANALOGS

BIOCHEMICAL STUDIES CONCERNING MECHANISM OF ACTION

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Abstract—A number of adenosine (Ado) analogs have been found to inhibit lymphocyte-mediated cytolysis (LMC) *in vitro* at μM concentrations. Those analogs which are substrates for adenosine deaminase were more inhibitory to LMC in the presence of erythro-9-(2-hydroxy-3-nonyl)adenine, an inhibitor of the deaminase. The inhibitory activity of most of these analogs (the exceptions being 2′-deoxyadenosine, 9-β-D-arabinofuranosyladenine and 7-deazaadenosine) was markedly enchanced by an inhibitor (Ro 20-1724) of cyclic AMP phosphodiesterase. With the exceptions of 2-fluoroadenosine, 7-deazaadenosine and formycin A, the inhibition of LMC caused by the Ado analogs was fully reversible upon removal of the analogs from the medium. In general, the Ado analogs did not cause a reduction in the pool sizes of endogenous ribonucleoside 5′-triphosphates in the lymphocytes. Some inhibitory and non-inhibitory analogs were metabolized to their corresponding 5′-triphosphates. Lymphocytes pretreated with a reversible inhibitor of LMC, 2-aminoadenosine, retained most of the resultant 2-amino-ATP during subsequent incubation in analog-free medium. Most of the Ado analogs which were inhibitory to LMC caused a substantial elevation of lymphocyte cyclic AMP; the magnitude of this elevation was enhanced by Ro 20-1724. Collectively, these results suggest that Ado and many of its structural analogs inhibit LMC by reason of their ability to stimulate the formation and consequent build-up of cyclic AMP in the cytotoxic lymphocytes. This stimulation of adenylate cyclase appears to result from the binding of an appropriate nucleoside to an adenosine receptor located on the membrane of the lymphocytes.

Adenosine* (Ado), when protected against enzymatic deamination, is a potent inhibitor of lymphocyte-mediated cytolysis (LMC) studied *in vitro*; this inhibition is associated with an elevation of cyclic AMP within the cytotoxic lymphocytes [1]. The inhibition of LMC, as well as the elevation of lymphocytic cyclic AMP, was reversible after removal of exogenous Ado from the medium. These results and those of others [2–9] have indicated an important role for the cyclic nucleotides in the modulation of lymphocytic function.

A large number of structural analogs of Ado have now been examined for their effect on the cytolytic function and on certain aspects of the metabolism of murine lymphocytes. The present paper describes the results obtained with these Ado analogs and compares their effects with those of Ado itself.

MATERIALS AND METHODS

Materials. Ado, 2-chloro-Ado, N^6-benzyl-Ado, N^6-(Δ^2-isopentenyl)-Ado, N^6-methyl-Ado, N^6-dimethyl-Ado, 3′-dAdo, N^6-hydroxy-Ado, 8-bromo-Ado, N^6-furfuryl-Ado, purine ribonucleoside and 6-methylmercaptopurine ribonucleoside were obtained from Sigma Chemical Co., St. Louis, MO. 2′-*O*-methyl-Ado and 3′-*O*-methyl-Ado were products of Research Plus Laboratories, Denville, NJ. Zeatin ribonucleoside [N^6-(*trans*-4-hydroxy-3-methylbut-2-enyl)-Ado]. 7-deaza-Ado (tubercidin) and 2′-dAdo were from CalBiochem, Wankegan, IL. Ara-A† was obtained from Pfanstiehl Laboratories, La Jolla, CA and α-Ado from Terra-Marine Bioresearch, La Jolla, CA. Inosine, ara-ATP and 2′-dATP were products of P-L Biochemicals, Milwaukee, WI. Formycin A (7-amino-3-(β-D-ribofuranosyl)-pyrazolo[4,3-*d*]-pyrimidine) was purchased from Meiji Seika Kaisha Ltd., Kawasaki, Japan. The following compounds were synthesized at the Wellcome Research Laboratories: 5′-chloro-5′-dAdo and 5′-dAdo by Dr. Lowrie M. Beacham, III, and Mr. Jerald J. Haggerty; N^6-phenyl-Ado, 2-fluoro-Ado, 2-amino-Ado, 2-methyl-Ado, 2-methylthio-Ado, 2-hydroxy-Ado and 8-aza-Ado by Dr. Thomas A. Krenitsky and Mr. George E. Koszalka; erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) by Dr. James L. Kelley. 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20–1724) was generously provided by Dr. Herbert Sheppard of Hoffmann-LaRoche Inc., Nutley, NJ. [2-^{3}H]Ara-A (6.7 mCi/m-mole) and [U-^{3}H]2′-dAdo (6.7 mCi/m-mole) were from New England Nuclear, Boston MA.

Mice, C57BL leukemia EL 4, $Na_2{}^{51}CrO_4$, (Dulbecco's) phosphate-buffered saline and fetal calf

* Names of many of the structural analogs of adenosine have been abbreviated by designating the position and nature of each structural modification as a prefix to the abbreviation for adenosine; e.g. 8-bromoadenosine is abbreviated as 8-bromo-Ado.

† Ara-A and adenine arabinoside, 9-β-D-arabinofuranosyladenine; ara-ATP, the 5′-triphosphate of 9-β-D-arabinofuranosyladenine.

serum were obtained from sources identified previously [10].

Purity of Ado analogs. With the exceptions noted below, the Ado analogs used in this study were shown by high-pressure reverse-phase chromatography to contain less than 0.05% Ado as an impurity. The commercial preparations of 2′-dAdo, N^6-(Δ^2-isopentenyl)-Ado, N^6-furfuryl-Ado, zeatin ribonucleoside and N^6-hydroxy-Ado were found to contain apparent contaminations of Ado of 0.11, 0.76, 0.67, 0.22 and 0.67 per cent respectively. Also, the 2-hydroxy-Ado contained an impurity of apparent Ado of 0.14 per cent. 2′-dAdo was freed of its suspected impurity of Ado by preparing a 10 mM solution of 2′-dAdo containing 12 mM boric acid, adjusting the pH of this solution to 8.9 with ammonium hydroxide and applying this solution to a column of Dowex-1-Cl^- which had been equilibrated with dilute ammonium hydroxide (pH 10.8). The column was eluted with this dilute ammonium hydroxide (pH 10.8) and the fractions containing ultraviolet (260 nm)-absorbing material were pooled and evaporated to dryness several times in the presence of methanol to eliminate the boric acid [11]. N^6-(Δ^2-isopentenyl)-Ado was purified on a cellulose plate [cellulose PQ2F thick-layer (1 mm) plate, Quantum Industries, Fairfield, NJ] using 1-butanol–water–concentrated ammonium hydroxide (86:10:5) as solvent [12]. R_f values were 0.71 and 0.27 for N^6-(Δ^2-isopentenyl)-Ado and Ado respectively. After purification, 2′-dAdo and N^6-(Δ^2-isopentenyl)-Ado were shown to contain less than 0.02 and 0.06 per cent, respectively, Ado as an impurity. The other adenosine analogs noted above were used without further purification. The purity of 2-amino-Ado and formycin A could not be determined by this methodology due to similar retention times of these two analogs and that of Ado.

Cells. C57BL leukemia EL4 was maintained, harvested and labeled with $Na_2{}^{51}CrO_4$ as described previously [10]. Cytotoxic peritoneal exudate lymphocytes were obtained from CD-1 mice as reported earlier [13]. Phosphate-buffered saline supplemented with 10% heat-inactivated fetal calf serum was used as the medium for all cell incubations.

Assay of lymphocyte-mediated cytolysis in vitro. The assay *in vitro* of cytolysis has been described previously [1, 10]. Briefly, this assay determined the amount of ^{51}Cr released during a 70-min incubation at 37° of ^{51}Cr-labeled EL4 cells and specifically sensitized lymphocytes. When a 1:1 ratio of lymphocytes-to-target cells was used, the actual cellular lysis averaged 20.6 ± 4.4 per cent (range 9.4 to 36.0 per cent) from day to day. For the determination of the inhibitory effects of a compound upon LMC, the following order of addition of reagents was followed: (1) cytotoxic lymphocytes; (2) EHNA, at 7.9 μM final concentration; (3) test compound dissolved in pyrogen-free saline; and (4) ^{51}Cr-labeled EL4 cells. In those experiments involving Ro 20-1724, the latter agent was added (to a final concentration of 50 μM) prior to EHNA.

Incubation and extraction of cytotoxic lymphocytes. The conditions employed for the incubation and acid extraction of lymphocytes, for the purpose of determining cellular pool sizes of nucleoside triphosphates or cyclic AMP, have been described in detail [13]. In these biochemical studies, EHNA was added to the cell suspensions just prior to the addition of the Ado analogs.

Determination of nucleoside triphosphate pool sizes. Acid-soluble extracts of cytotoxic lymphocytes were analyzed by high-performance anion-exchange chromatography as described previously [13]. Nucleotide concentrations in the cell extracts were calculated using response factors (ultraviolet peak area, in square inches, per nmole nucleotide) determined by injecting known amounts of authentic nucleotide standards into the liquid chromatography under the standard operating conditions. Analog ribonucleosides were used to determine both the response factor and the characteristic A_{254}/A_{280} absorbance ratio for each of their corresponding 5′-triphosphates. When an analog 5′-triphosphate was co-eluted from the liquid chromatograph with an endogenous ribonucleoside 5′-triphosphate, one of two mathematical procedures was employed to estimate the amount of analog 5′-triphosphate present in the composite ultraviolet peak. If the analog 5′-triphosphate was co-eluted with either CTP or UTP, the peak area attributable to the analog 5′-triphosphate was obtained simply as the difference between the relatively large area of the composite peak and the relatively small peak area of CTP or UTP found with the extracts from the control cells. If the analog 5′-triphosphate was co-eluted with either ATP or GTP, the amounts of analog 5′-triphosphate and ATP or GTP present in the composite peak were calculated from the following expression:

$$\text{total peak area (at 254 nm)} = [\text{mole fraction}]_A \cdot [\text{response factor (at 254 nm)}]_A \cdot x + [\text{mole fraction}]_B \cdot [\text{response factor (at 254 nm)}]_B \cdot x$$

where x represents the total number of nanomoles of ribonucleotides *A* and *B* present in the composite peak. The mole fractions of ribonucleotides *A* and *B* present in a composite peak were estimated by locating the ratio of peak areas at 254 and 280 nm for the composite peak on a plot of peak area ratio vs mole fraction of *A* (*B*); this latter plot was constructed mathematically, using theoretical fractional mixtures of *A* and *B*, from the response factors determined experimentally for ribonucleotides *A* and *B* at both 254 and 280 nm. In those experiments involving radiolabeled precursors, the effluent of the liquid chromatograph was collected at 1-min intervals and these fractions were monitored by liquid scintillation spectrometry for the content of radioactivity.

Measurement of cyclic AMP levels. Cyclic AMP present in acid-soluble extracts of lymphocytes was determined by radioimmunoassay after purification of the cell extracts on sequential columns of aluminum oxide and Dowex 1-X8 and subsequent 2′-*O*-succinylation of the resultant samples [13]. This purification scheme, together with the demonstrated specificity of the radioimmunoassay [13], is judged to be sufficient to eliminate any possible interference by analog cyclic nucleotides (see Results) in the determination of cyclic AMP.

Reverse-phase chromatographic analysis of Ado analogs. Concentrated solutions (1.0 to 4.0 mM) of each of the Ado analogs were analyzed in a high-pressure liquid chromatograph (Instrumentation Specialties Co., Lincoln, NE) equipped with a Whatman Partisil-10 ODS column (0.46 × 25 cm). The column effluent was monitored at both 254 nm (Instrumentation Specialties Co. model UA-5 absorbence monitor) and 280 nm (Laboratory Data Control, Riviera Beach, FL, model 1280 flow monitor) with a Honeywell Electronik 194 two-pen recorder. Fifty-μl samples of each analog solution were injected into the liquid chromatograph. The column was eluted with a linear 80-min gradient of 1–75% (v/v) acetonitrile in water. The column flow rate was 60 ml/hr. Full-scale absorbence ranges of 0.05 and 0.08 were employed at 254 and 280 nm respectively. Each solution of analog was analyzed before and after the addition of 20 μM Ado in order to demonstrate separation of the major analog peak from the minor Ado peak as well as to determine whether authentic Ado co-chromatographed with minor peaks of impurities which were present in some of the analog samples. The sensitivity of this analytical procedure was approximately 1 μM Ado present in a sample.

Table 1. Effect of adenosine analogs on lymphocyte-meditated cytolysis *in vitro*

Compound	ID_{50}* (μM)	Inhibitory activity enhanced by: EHNA†	Ro 20–1724‡
Adenosine	6.8 ± 2.5	+	+
2-Fluoroadenosine	1.9 ± 1.2	–	+
2-Chloroadenosine	6 ± 4	–	+
N^6-benzyladenosine	65 ± 43	–	+
N^6-phenyladenosine	55 ± 25	–	+
5′-Chloro-5′-deoxyadenosine	62 ± 9	–	+
5′-Deoxyadenosine	62 ± 22	–	+
2′-*O*-methyladenosine	58 ± 15	+	+
2′-Deoxyadenosine	88 ± 11	+	–
2-Aminoadenosine	102 ± 38	+	+
Adenine arabinoside	120 ± 48	+	–
2-Methyladenosine	118 ± 76	–	+
2-Methylthioadenosine	180 ± 42	–	+
N^6-(Δ^2-isopentenyl)-adenosine	§	NT	+
N^6-methyladenosine	§	NT	NT
N^6-dimethyladenosine	§	NT	NT
N^6-furfuryladenosine	§	NT	+
Zeatin ribonucleoside	§	NT	+
3′-Deoxyadenosine	§	NT	NT
3′-*O*-methyladenosine	§	NT	NT
Formycin A	§	+	+
7-Deazaadenosine	§	–	–
N^6-hydroxyadenosine	§	NT	NT
2-Hydroxyadenosine	§	NT	NT
Purine ribonucleoside	‖	NT	NT
6-Methylmercaptopurine ribonucleoside	‖	NT	–
8-Azaadenosine	‖	NT	NT
α-Adenosine	‖	NT	+
8-Bromoadenosine	‖	NT	NT
Inosine	‖	NT	–

* Determined in the presence of 7.9 μM EHNA. The plot of per cent inhibition of cytolysis vs the logarithm of the inhibitory concentration was approximately linear for 20–80 per cent inhibition; therefore, the ID_{50} values were determined using regression analysis of these values. The averages of at least twenty-five duplicate determinations were used for each analysis.

† A plus sign in this column means that an analog was more inhibitory to LMC in the presence of EHNA (7.9 μM). NT in this column means that an analog was tested as an inhibitor of LMC only in the presence of EHNA.

‡ A plus sign in this column means that an analog was more inhibitory in the presence of Ro 20–1724 (50 μM). NT in this column means that an analog was not tested as an inhibitor of LMC in the presence of Ro 20–1724.

§ When tested at a concentration of 150 μM, these compounds were found to inhibit LMC only 20–35 per cent.

‖ When tested at a concentration of 150 μM, these compounds were found to be completely non-inhibitory toward LMC.

RESULTS

Effect of Ado analogs on lymphycyte-mediated cytolysis in vitro. A large number of Ado analogs have been tested as inhibitors of LMC *in vitro*, and the more active compounds, together with some closely related inactive compounds, are listed in Table 1 in decreasing order of inhibitory activity. Ado itself is included at the top of the table as a reference compound. The 50% inhibitory concentrations (ID_{50}) presented in Table 1 were all determined in the presence of EHNA (7.9 μM), a potent inhibitor of Ado deaminase [14]. EHNA itself, at the concentration employed, was without effect on cytolysis. The constant use of EHNA in this study allowed the relative intrinsic inhibitory activities of these Ado analogs toward LMC to be determined without interference due to their differing susceptibilities to enzymatic deamination.

Of the Ado analogs tested, eleven (2-fluoro-Ado, 2-chloro-Ado, N^6-benzyl-Ado, N^6-phenyl-Ado, 5'-chloro-5'-dAdo, 5'-dAdo, 2'-*O*-methyl-Ado, 2'-dAdo, 2-amino-Ado, ara-A and 2-methyl-Ado) had an ID_{50} below 150 μM and twelve others were slightly inhibitory at this concentration. Only 2-fluoro-Ado was more inhibitory than Ado, while 2-chloro-Ado was equipotent to Ado. Purine derivatives which, at 150 μM, were non-inhibitory toward LMC included purine ribonucleoside, 6-methylmercaptopurine ribonucleoside, 8-aza-Ado, α-Ado, 8-bromo-Ado and inosine. As indicated in column 3 in Table 1, those inhibitory analogs which are known to be good substrates for Ado deaminase (2'-dAdo [15, 16], 2'-*O*-methyl-Ado [16], 2-amino-Ado [15] and ara-A [15, 16]) were potentiated by EHNA in their inhibition of LMC; with these latter four analogs, ID_{50} values were 2- to 8-fold higher in the absence of EHNA.

The majority of the Ado analogs studied were more inhibitory in the presence of Ro 20-1724, a potent inhibitor of cyclic AMP phosphodiesterase [17] (Table 1, column 4). Ro 20-1724 itself, at the concentration (50 μM) used in this study, was not inhibitory toward LMC. With the exceptions of 2'-dAdo, ara-A and 7-deaza-Ado, Ro 20-1724 caused a 50–90 per cent reduction in the magnitude of the ID_{50} values for the various analogs.

As was the case with Ado [1], the inhibitory activity of most of these purines was fully reversible after a 60-min incubation of the cytotoxic lymphocytes with each analog (in the presence of EHNA) and subsequent removal of extracellular analog. Similarly, most of these analogs did not exhibit increased inhibitory activity toward LMC when preincubated for 60 min with the cytotoxic lymphocytes prior to the addition of the ^{51}Cr-labeled EL4 cells and the start of the LMC assay. However, 2-fluoro-Ado, 7-deaza-Ado and formycin A were

Table 2. Effect of adenosine analogs on pool sizes of ribonucleoside 5'-triphosphates in cytotoxic lymphocytes

Compound*	Concn tested (μM)	Percentage of control value after 60-min incubation with compound			Evidence for analog 5'-triphosphate formation
		UTP	ATP	GTP	
Saline		(100)†	(100)†	(100)†	—
Adenosine	18.8	95	123	109	—
2-Fluoroadenosine	4.7	95	63‡	114	+ (266)§
2-Chloroadenosine	18.8	104	118	147‡	+ (28)§
N^6-benzyladenosine	150	168‡	139	142	—
N^6-phenyladenosine	150	101	79	80	—
5'-Chloro-5'-deoxy-adenosine	150	95	107	82	—
5'-Deoxyadenosine	150	91	96	77	—
2'-*O*-methyladenosine	150	101	98	102	—
2'-Deoxyadenosine	150	100	101	90	—
2-Aminoadenosine	150	74‡	83	114	+ (430)§
Adenine arabinoside	150	113	90	109	+ (9)§
2-Methyladenosine	150	115	93	86	—
2-Methylthioadenosine	150	108	106	101	—
3'-Deoxyadenosine	150	119	114	124	+ (67)§
Formycin A	150	86	77	98	+ (651)§
7-Deazaadenosine	150	?‖	48‡	109	+ (412)§
8-Azaadenosine	150	100	105	100	+ (275)§
Purine ribonucleoside	150	80	71	96	+ (513)§

* All agents were tested in the presence of 7.9 μM EHNA.

† Control cell values of UTP, ATP and GTP were 64–97, 361–583 and 73–121 pmoles/10^6 cells, respectively, on different days. The peak of CTP present on the high-pressure liquid chromatograms was usually too small to quantitate accurately.

‡ $P < 0.05$. All experiments were performed in duplicate and statistical comparisons between values for treated cells and for control cells were made using the two-tailed Student's *t*-test.

§ The parenthetical values represent the estimated cellular content (pmoles/10^6 cells) of analog 5'-triphosphate present at the end of the 60-min experimental period.

‖ The peak corresponding to UTP on the high-pressure liquid chromatogram was obscured by the co-chromatography of the large amount of 7-deaza-ATP.

exceptions to these two generalizations in that each of these three inhibitors of LMC was largely irreversible in its activity and in that each of these agents was more inhibitory toward LMC after preincubation with the cytotoxic lymphocytes.

Effect of Ado analogs on pool sizes of ribonucleoside 5′-triphosphates in cytotoxic lymphocytes. Acid-soluble extracts were prepared from cytotoxic lymphocytes which had been incubated for 60 min with each Ado analog (in the presence of 7.9 μM EHNA), and these extracts were subsequently analyzed by high-performance anion-exchange chromatography in order to determine the pool sizes of the various ribonucleoside 5′-triphosphates. Most of the analogs were tested at a concentration of 150 μM; however, the three most active compounds (Ado, 2-fluoro-Ado and 2-chloro-Ado) were tested at lower concentrations to reflect their markedly lower ID_{50} values toward LMC (cf. Table 1). The results of these experiments are summarized in Table 2. The effects of each analog on the pool sizes of UTP, ATP and GTP are presented as the percentage of the control cell values for these nucleotides. Due to the relatively low cellular content of CTP, this nucleotide could not be quantitated accurately in many of the cell extracts.

The majority of the Ado analogs studied had little or no effect on the lymphocytic pool sizes of endogenous ribonucleoside 5′-triphosphates. Only 2-fluoro-Ado and 7-deaza-Ado caused a marked (> 30 per cent) decrease in the pool size of ATP. Neither Ado nor any of its analogs caused a marked depression of lymphocytic UTP.

Lymphocytic formation of analog nucleoside 5′-triphosphates. In addition to allowing the determination of pool sizes of endogenous nucleotides, analysis of lymphocytic extracts by high-performance liquid chromatography has also provided evidence for the metabolism of some of these Ado analogs (in the presence of EHNA) to their corresponding 5′-triphosphates. In the case of some of these analogs (2-fluoro-Ado, 2-chloro-Ado, ara-A, 3′-dAdo and purine ribonucleoside), discrete new ultraviolet peaks, exhibiting A_{254}/A_{280} absorbance ratios similar to those of the respective analog precursors, were present in the triphosphate region of the chromatograms of the lymphocytic extracts. However, the 5′-triphosphate metabolite of formycin A was co-eluted with CTP, that of 7-deaza-Ado with UTP, that of 2-amino- Ado with ATP, and that of 8-aza-Ado with GTP. In these latter cases, the triphosphate metabolites were apparent in the chromatograms both from the enlargement of a particular triphosphate peak, relative to the control extract, and from the altered A_{254}/A_{280} absorbance ratio of the enlarged peak. The triphosphate character of the putative 5′-triphosphate metabolites of 2-fluoro-Ado, 2-amino-Ado, 7-deaza-Ado, 8-aza-Ado, purine ribonucleoside and formycin A was verified by the ability of yeast hexokinase to change the elution position of these metabolites to the diphosphate region of the chromatograms.

The right-hand column of Table 2 indicates those analogs for which chromatographic evidence was obtained supporting metabolism to 5′-triphosphates; the values listed parenthetically represent the cellular levels (pmoles/10^6 cells) of analog 5′-triphosphates observed. 2-Fluoro-Ado, 2-amino-Ado, formycin A, 7-deaza-Ado, 8-aza-Ado and purine ribonucleoside were all metabolized extensively to their corresponding 5′-triphosphates. 2-Chloro-Ado, ara-A and 3′-dAdo were metabolized to a lesser extent to their 5′-triphosphates.

Although authentic 2′-dATP was shown to elute from the liquid chromatograph as a discrete peak between ATP and GTP, no evidence was observed for the metabolism of 2′-dAdo to its 5′-triphosphate. Furthermore, incubation of cytotoxic lymphocytes with 150 μM [U-^{3}H]2′-dAdo (plus EHNA) resulted in the labeling of the ATP pool without detectable formation of radioactive 2′-dATP. The metabolism of ara-A to ara-ATP was verified by incubating the lymphocytes with 150 μM [2-^{3}H]ara-A (plus EHNA) and demonstrating the formation of a radioactive metabolite which was co-eluted from the liquid chromatograph with authentic ara-ATP.

It has been reported previously [13] that 2-fluoro-Ado, an irreversible inhibitor of LMC, is metabolized irreversibly (i.e. for the duration of the experiment) to 2-fluoro-ATP within the cytotoxic lymphocytes. It was therefore of interest to learn whether the large amount of analog 5′-triphosphate formed from a reversible inhibitor of LMC would also be retained by the lymphocytes after removal of the analog from the medium. Accordingly, cytotoxic lymphocytes were first incubated with 150 μM 2-amino-Ado (plus EHNA) for 60 min and were then either centrifuged and acid extracted or washed free

Table 3. Lymphocytic retention of 2-amino-ATP after removal of exogenous 2-amino-adenosine from the medium*

Additive(s)	Incubation time (min)	CTP	UTP	ATP	GTP	2-Amino-ATP
		(pmoles nucleotide/10^6 cells)				
Saline	60	35 ± 1	83 ± 4	468 ± 33	91 ± 7	
2-Amino-Ado (150 μM)	60	31 ± 3	61 ± 3	388 ± 41	104 ± 13	430 ± 60
2-Amino-Ado (150 μM) + Ro 20–1724 (50 μM)	60	27 ± 2	58 ± 7	278 ± 19	74 ± 7	250 ± 5
Saline†	60/wash cells/30	24 ± 1	55 ± 5	440 ± 38	72 ± 5	
2-Amino-Ado (150 μM)†	60/wash cells/30	28 ± 3	50 ± 3	278 ± 18	78 ± 7	298 ± 30

* All experiments were performed in duplicate and each cell extract was analyzed in duplicate by high-performance liquid chromatography. The results are expressed as the mean ± the standard error of the mean for four determinations.

† After a 60-min incubation with the specified additive, the cells were centrifuged, washed, resuspended in fresh medium lacking 2-amino-Ado, and incubated for a further 30 min prior to their extraction.

Table 4. Effect of adenosine analogs on cyclic AMP levels in cytotoxic lymphocytes

Compound*	Concn compound tested (μM)	Percentage of control cell cyclic AMP level after 30-min incubation with agent	
		− Ro 20–1724	+ Ro 20–1724
Saline		(100)†	128–185 (P < 0.02 to 0.001)‡
Adenosine	18.8	337	1105
2-Fluoroadenosine	4.7	185	551
2-Chloroadenosine	18.8	479	940
N^6-benzyladenosine	150	254	674
N^6-phenyladenosine	150	271	925
5′-Chloro-5′-deoxy-adenosine	150	254 (P < 0.01)	479
5′-Deoxyadenosine	150	181	336
2′-*O*-methyladenosine	150	273 (P < 0.05)	655
2′-Deoxyadenosine	150	160	218
2-Aminoadenosine	150	369	1087
Adenine arabinoside	150	127 (P < 0.01)	201 (P < 0.01)
2-Methyladenosine	150	353	780
2-Methylthioadenosine	150	201	218
N^6-(Δ^2-isopentenyl)-adenosine	150	308	636
N^6-methyladenosine	150	254	556
N^6-dimethyladenosine	150	110 (P < 0.05)	243
N^6-furfuryladenosine	150	201	584
Zeatin ribonucleoside	150	181	450
3′-Deoxyadenosine	150	217	407
3′-*O*-methyladenosine	150	162 (NS)	199
Formycin A	150	215 (P < 0.01)	561
7-Deazaadenosine	150	129 (NS)	179
N^6-hydroxyadenosine	150	160 (P < 0.05)	373
2-Hydroxyadenosine	150	209	324 (P < 0.01)
Purine ribonucleoside	150	106 (NS)	161 (P < 0.02)
6-Methylmercaptopurine ribonucleoside	150	134	191
8-Azaadenosine	150	128 (NS)	201
α-Adenosine	150	110 (NS)	182
8-Bromoadenosine	150	137 (P < 0.01)	179
Inosine	150	130 (P < 0.01)	173

* All compounds tested in the presence of 7.9 μM EHNA.

† Control cell content of cyclic AMP varied over the range 0.67 to 1.67 pmoles/10^7 cells on different days.

‡ All experiments were performed in duplicate and each cell extract, after column purification and 2′-*O*-succinylation, was radioimmunoassayed in duplicate. Comparison between values for treated cells and for control cells were made using the two-tailed Student's *t*-test. Unless otherwise indicated, all numbers are significant to P < 0.001.

of extracellular analog and incubated for a further 30 min in analog-free medium prior to centrifugation and acid extraction. Analysis of these extracts by high-performance liquid chromatography revealed that the 2-amino-ATP formed within the lymphocytes during the 60-min incubation with 2-amino-Ado was largely maintained during the 30-min incubation of the cells in analog-free medium (Table 3); both ATP and 2-amino-ATP decreased approximately 30 per cent during the subsequent 30-min incubation of the 2-amino-Ado-pretreated cells. The data in Table 3 also indicate that 50 μM Ro 20-1724 partially inhibited the uptake and/or metabolism of 2-amino-Ado to 2-amino-ATP in the lymphocytes.

Effect of Ado analogs on lymphocytic levels of cyclic AMP. All of the above-discussed Ado analogs have been studied for their effect on cyclic AMP levels within the cytotoxic lymphocytes. In these studies the cytotoxic lymphocytes were incubated for 30 min,* in the absence or presence of the specified concentration of each analog, prior to acid extraction of the cell suspensions. EHNA (7.9 μM) was present in all of these cellular incubations, and each analog was studied both in the absence and presence of Ro 20-1724 (50 μM). The results of these experiments are summarized in Table 4; cyclic AMP levels are presented as the percentage of control cell cyclic AMP levels observed in the same experiment in which each analog was studied.

* Thirty min was selected as the experimental time of analog treatment in these studies on the basis that this represented approximately one-half of the time span (70 min) of the LMC assay. However, the elevation of cyclic AMP caused by Ado[1] and its analogs (Ref. 13 and unpublished observations) is transient and exhibits unique kinetics with each of these purines. For these reasons, measurement of cyclic AMP levels at a single experimental time point may result in data representing different phases of the time course of cyclic AMP elevation for each analog. However, to perform these studies in more detail than reported herein would have entailed a prohibitive number of cyclic AMP determinations.

As indicated in column 3 of Table 4, nine out of fourteen of the analogs most inhibitory toward LMC raised lymphocytic cyclic AMP levels 150 per cent or more above control levels in the absence of Ro 20-1724. At the concentration tested, Ado itself caused a 237 per cent increase in cyclic AMP. 2-Fluoro-Ado, the most inhibitory analog, caused only an 85 per cent increase in cyclic AMP; however, lymphocytes treated with this analog form large amounts of 2-fluoro-cyclic AMP[13]. Several Ado analogs which were good inhibitors of LMC but which contained a modified ribose moiety (viz. 5'-dAdo, 2'-dAdo and ara-A) caused much smaller (27–81 per cent) elevations of cycle AMP.* Several non-inhibitory analogs (purine ribonucleoside, 6-methylmercaptopurine ribonucleoside, 8-aza-Ado, α-Ado, 8-bromo-Ado and inosine) also caused only small (6–37 per cent) increases in cyclic AMP.

As indicated in the top row of Table 4 (column 4), Ro 20-1724 alone, at a concentration of 50 μM, repeatedly caused small (28–85 per cent) elevations of lymphocytic cyclic AMP levels. When tested in combination with the various LMC-inhibitory analogs, Ro 20-1724 clearly caused greater than additive increases in cyclic AMP levels in the majority of cases; apparent exceptions were 2'-dAdo, ara-A and 2-methylthio-Ado.

During the course of these studies, both 2-fluoro-Ado[13] and 7-deaza-Ado (manuscript in preparation) have been shown to undergo facile metabolism to their corresponding cyclic nucleotide derivatives. Since both 2-fluoro-cyclic AMP[13] and 7-deaza-cyclic AMP[18–20] are known to be highly effective in activating cyclic AMP-dependent protein kinases, the cellular accumulation of these two analog cyclic nucleotides may mimic an elevated cellular level of cyclic AMP.

DISCUSSION

The present work is an extension of our earlier observation[1] that adenosine, when protected chemically against adenosine deaminase, can potently inhibit LMC *in vitro*. This report documents the varying abilities of a large number of Ado analogs to inhibit LMC *in vitro* and describes the results of some biochemical studies concerning the mechanism of action of these agents.

The inhibitory activity of the various Ado analogs toward LMC *in vitro* is highly dependent upon the nature of the structural modification introduced into the Ado molecule. The requirement for the 6-amino or 6-substituted amino group of Ado for LMC-inhibitory activity is demonstrated by the lack of inhibitory activity of purine ribonucleoside, inosine and 6-methylmercaptopurine ribonucleoside. In general, 2-substituted derivatives of Ado were highly inhibitory toward LMC. Among the Ado analogs examined, only 2-fluoro-Ado was more inhibitory than Ado, and 2-chloro-Ado was equipotent to Ado. In all cases examined, substitution of the 6-amino group of Ado resulted in reduced inhibitory activity; however, aryl or aralkyl N^6-substituents, such as the phenyl and benzyl moieties, allowed a greater retention of inhibitory activity than did other N^6-substituents such as methyl, isopentenyl or hydroxyl moieties. 8-Substitution of Ado or replacement of the 8-carbon by nitrogen destroyed inhibitory activity. Modification of the imidazole ring of Ado, as in 7-deaza-Ado and formycin A, resulted in considerable loss of biological activity. On the ribose portion of the Ado molecule, modifications (reduction or methylation) at the 3'-position appeared to be more deleterious to activity than similar modifications at either the 2'- or 5'-positions. Finally, inversion of the glycosidic linkage at the 1'-carbon of Ado (to give α-Ado) resulted in complete loss of activity.

It should be emphasized that the present studies were carried out exclusively in the presence of EHNA; therefore, differential substrate activities of these analogs with adenosine deaminase should not be considered a variable in the above structure–activity discussion. However, the relative abilities of these Ado analogs to inhibit lymphocytic function *in vivo*, under the normal circumstance of abundant adenosine deaminase, would obviously be influenced strongly by their different substrate properties with the deaminase.

Three different aspects of purine metabolism have been investigated in an attempt to correlate the above biological data with an effect of these Ado analogs on the biochemistry of the cytotoxic lymphocyte. Since it is known that the cytolytic activity of the lymphocyte is energy dependent[21], many of these analogs have been examined for their effect on cellular ATP levels. Only one (2-fluoro-Ado) out of the twelve most inhibitory analogs caused a substantial (> 30 per cent) decrease in lymphocytic ATP (Table 2). It therefore seems apparent that the Ado analogs are not acting generally by causing a decrease in metabolic energy levels.

Although some of the inhibitory analogs of Ado were metabolized to their corresponding 5'-triphosphates within the cytotoxic lymphocytes, several observations suggest that this phenomenon is not generally important to the action of these analogs: (a) only a few of the inhibitory analogs could be shown to undergo metabolism to their 5'-triphosphates; (b) lymphocytes which were preloaded with 2-amino-ATP (via prior incubation with 2-amino-Ado) regained their full cytolytic activity after wash-out of extracellular 2-amino-Ado even though they largely retained their cellular content of analog 5'-triphosphate; (c) the 5'-deoxy analogs of Ado obviously lack the potential for metabolism to 5'-nucleotides; (d) while Ro 20-1724 potentiated the inhibitory activity of 2-fluoro-Ado and 2-amino-Ado, Ro 20-1724 had no effect on the lymphocytic build-up

* It was previously reported[13] that 150 μM 2'-dAdo caused moderate increases in the levels of both 2-fluoro-cyclic AMP and cyclic AMP in cytotoxic lymphocytes which had been preloaded with nucleotides of 2-fluoro-Ado. This experiment was carried out with commercial-grade 2'-dAdo prior to our analysis of these analogs for possible contamination by Ado. In view of the apparent contamination (0.11 per cent) of Ado in commercial 2'-dAdo (see Materials and Methods), at least part of the reported effect of 2'-dAdo on 2-fluoro-cyclic AMP and cyclic AMP levels may have been due to a trace impurity of Ado present in the 2'-dAdo.

of 2-fluoro-ATP from exogenous 2-fluoro-Ado [13] and appeared to partially inhibit the metabolism of 2-amino-Ado to its 5'-triphosphate (Table 3); and (e) 8-aza-Ado and purine ribonucleoside, two non-inhibitory analogs, were also metabolized extensively to their 5'-triphosphates.

The third and perhaps most important biochemical parameter monitored in this study was the level of lymphocytic cyclic AMP. This experimental approach was prompted by the previous findings that the inhibition of LMC by Ado is accompanied by a rapid elevation in lymphocytic cyclic AMP [1]. Indeed, the majority of the Ado analogs which were potent inhibitors ($ID_{50} < 150$ μM) of LMC resembled Ado in that they caused a substantial (> 150 per cent) increase in lymphocytic cyclic AMP. Moreover, like Ado, the majority of these analogs were reversible in their activity and appeared to be potentiated by Ro 20-1724 in their ability both to inhibit LMC and to elevate cyclic AMP. Since it is known from other work [2–9] that the elevation of lymphocytic cyclic AMP caused by various non-purine agents (e.g. prostaglandin E_1, isoproterenol and cholera toxin) results in the inhibition of cytolytic activity, it is reasonable to conclude that most of these Ado analogs inhibit LMC by virtue of their ability to cause an elevation of lymphocytic cyclic AMP. The elevation of cyclic AMP is believed to be effected by the binding of Ado, or a suitable structural analog, to an adenosine receptor located on the membrane of the cytotoxic lymphocytes and by the consequent stimulation of a functionally associated adenylate cyclase [13, 22, 23].

The irreversibility of the inhibition of LMC observed with 2-fluoro-Ado, 7-deaza-Ado and formycin A is tentatively attributed to their metabolism both to 5'-triphosphates and to cyclic 3',5'-monophosphates. It has been shown that the 5'-triphosphates of two Ado analogs, 2-fluoro-Ado [13] and 2-amino-Ado (Table 3), once formed within the cytotoxic lymphocytes, are metabolically stable for at least 30 min in the absence of additional extracellular analog. Thus, to the extent that analog 5'-triphosphate formation contributes to the inhibition of LMC, this inhibition would be expected to be essentially irreversible during the 70-min LMC assay. Moreover, two of these irreversible inhibitors, 2-fluoro-Ado [13] and 7-deaza-Ado (manuscript in preparation), have been found to undergo further metabolism to their corresponding 3',5'-monophosphates. Since both of these analog cyclic nucleotides are highly effective in activating cyclic AMP-dependent protein kinases [13, 18–20], their irreversible formation within the lymphocytes would be expected to mimic biologically a persistent elevation of cellular cyclic AMP. No evidence is presently available concerning the possible metabolism of any of the other Ado analogs to their 3',5'-monophosphates in these lymphocytes.

Although there appears to be a reasonably good qualitative correlation between the ability of an Ado analog to inhibit LMC and its ability to increase lymphocytic cyclic AMP, the data presented in Tables 1 and 4 do not provide a quantitative relationship between these two activities. Several possible explanations can be suggested for this lack of a quantitative correlation. First, the time course for cyclic AMP elevation appears to vary with the different analogs (unpublished observatons). Hence, cyclic AMP data derived from a single experimental time point (as in Table 4) are unlikely to represent either the maximum effect or the persistence of this effect for all of the different analogs. Second, there appears to be a threshold of approximately 100 per cent increase in lymphocytic cyclic AMP required for the onset of inhibition of LMC. Ro 20-1724 (50 μM) and several Ado analogs caused elevations of cyclic AMP (after 30 min) of as much as 85 per cent without being inhibitory toward LMC. Third, Ado and several of its structural analogs have been shown to be inhibitory to protein kinase from a variety of tissues [24–28]. It is possible, therefore, that the effect of each of the different Ado analogs on lymphocytic function represents a unique balance between the antagonistic activities of that analog in increasing cellular levels of cyclic AMP and in inhibiting protein kinase. Fourth, 2-fluoro-Ado, the most inhibitory analog studied, had a small effect on cyclic AMP but is metabolized extensively in the lymphocytes to 2-fluoro-cyclic AMP, a biologically active analog of cyclic AMP [13]. Fifth, in view of their relatively small effect on cyclic AMP levels, several of the ribose-modified analogs (viz. 5'-dAdo, 2'-dAdo and ara-A) together with 2-methylthio-Ado appear to inhibit LMC by a mechanism largely independent of cyclic AMP involvement. Sixth, compartmentalization of cyclic AMP, whether subcellular [29–34] or within cells of different types, if present, could obscure any quantitative relationships between cyclic AMP levels and inhibition of LMC.

The mechanism whereby elevated levels of cyclic AMP result in inhibition of the cytolytic function of lymphocytes remains conjectural. Presumably, this physiological effect is brought about through the activation of one or more protein kinases in the lymphocytes by the increased cellular content of cyclic AMP [24]. In particular, it has been shown that a cyclic AMP-stimulated protein kinase can phosphorylate microtubular protein [35]. Since microtubules appear to play a role in LMC [36], it is possible that cyclic AMP modulates the cytolytic function of lymphocytes through its effect on microtubules.

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