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INHIBITION OF LYMPHOCYTE-MEDIATED CYTOLYSIS BY 2-FLUOROADENOSINE—

EVIDENCE FOR TWO DISCRETE MECHANISMS OF DRUG ACTION

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Abstract—2-Fluoroadenosine (F-Ado) is a potent, irreversible inhibitor of lymphocyte-mediated cytolysis (LMC) *in vitro*: the irreversibility of this inhibition has been attributed to the metabolism of F-Ado to 2-fluoroadenosine 5′-triphosphate (F-ATP) and 2-fluoroadenosine 3′,5′-monophosphate (F-cAMP) within the cytotoxic lymphocytes [T. P. Zimmerman, J. L. Rideout, G. Wolberg, G. S. Duncan and G. B. Elion, *J. biol. Chem.* **251**, 6757 (1976)]. The present study was undertaken to define better the biochemical events intrinsic to the inhibition of LMC by F-Ado. Several purine ribonucleosides, which are themselves non-inhibitory toward LMC, have been found to inhibit the metabolism of F-Ado to F-ATP and F-cAMP by the cytotoxic lymphocytes. The reduction in F-cAMP formation caused by these ribonucleosides was counterbalanced by their augmentation of the elevation of lymphocytic cyclic AMP (cAMP) caused by F-Ado. While interference with the metabolism of F-Ado had little or no effect on the immediate inhibitory activity of F-Ado toward LMC, prevention of the cellular formation of F-ATP and F-cAMP did allow most of the inhibitory activity of F-Ado to be reversed after washing the lymphocytes free of exogenous F-Ado. The relative efficacy of these ribonucleosides in allowing reversibility of the inhibitory activity of F-Ado toward LMC followed the same order as did their efficacy in preventing the metabolism of F-Ado by the cytotoxic lymphocytes: 8-aza-adenosine > inosine > guanosine. Cytotoxic lymphocytes which had been preloaded with nucleotides of F-Ado (via prior incubation with F-Ado and subsequent washout of residual extracellular drug) exhibited increased inhibition of their cytolytic activity upon subsequent incubation with an inhibitor (Ro 20-1724) of cAMP phosphodiesterase. Under these latter experimental conditions, Ro 20-1724 caused a 2- to 3-fold elevation of F-cAMP in the cytotoxic lymphocytes but did not raise cAMP above control levels. These results suggest that F-Ado can inhibit LMC by either of two distinct mechanisms: (1) an extracellular mechanism, wherein F-Ado binds reversibly to an adenosine receptor present on the plasma membrane of the cytotoxic lymphocytes and reversibly activates a functionally associated adenylate cyclase, thereby causing an elevation of cellular cAMP; and (2) an intracellular mechanism, wherein F-Ado is metabolized irreversibly (during the 1- to 2-hr experimental period) by the cytotoxic lymphocytes to F-cAMP which, by reason of its ability to activate cAMP-dependent protein kinase, mimics the effect of elevated cellular levels of cAMP.

Adenosine [1] and a number of its structural analogs [2] have been reported to inhibit the destruction *in vitro* of tumor cells by specifically sensitized mouse lymphocytes. The inhibitory activity of most of these nucleosides appeared to be correlated with their ability to elevate cyclic AMP (cAMP)* levels, in a reversible manner, within the cytotoxic lymphocytes. Among the adenosine analogs studied, 2-fluoroadenosine (F-Ado) was particularly notable in that its potent inhibitory effect was essentially irreversible after exposure of the cytotoxic lymphocytes to this agent. Subsequently, it was shown that F-Ado is metabolized extensively and irreversibly (during the 1- to 2-hr experimental period) to both 2-fluoroadenosine 5′-triphosphate (F-ATP) and 2-fluoroadenosine 3′,5′-monophosphate (F-cAMP) within the cytotoxic lymphocytes [3]; this latter metabolic event was suggested to be responsible for the irreversible nature of the inhibition of lymphocyte-mediated cytolysis (LMC) produced by F-Ado.

The above interpretation of results implied that an agent which could prevent the lymphocytic metabolism of F-Ado to F-ATP and F-cAMP might also prevent the irreversible phase of the inhibition of LMC observed with F-Ado; under such a circumstance, F-Ado would be expected to remain inhibitory to LMC but in a reversible manner, similar to that of adenosine [1]. This report presents evidence that F-Ado can indeed inhibit LMC by either of two discrete mechanisms: (1) reversible, involving extracellular, non-metabolized F-Ado; and (2) irreversible, involving intracellular F-cAMP formed metabolically from F-Ado.

* Abbreviations used in the text are as follows: cAMP, cyclic AMP; 8-aza-Ado, 8-aza-adenosine; 8-aza-ATP, 8-aza-adenosine 5′-triphosphate; EHNA, erythro-9-(2-hydroxy-3-nonyladenine; F-cAMP, 2-fluoroadenosine 3′,5′-monophosphate; F-Ado, 2-fluoroadenosine; F-ATP, 2-fluoroadenosine 5′-triphosphate; Guo, guanosine; Ino, inosine; LMC, lymphocyte-mediated cytolysis; and Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

MATERIALS AND METHODS

Materials. Inosine (Ino), guanosine (Guo), hypoxanthine and guanine were obtained from P-L Biochemicals. 8-Aza-adenosine (8-aza-Ado) and 8-aza-adenine were synthesized at the Wellcome Research Laboratories. 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was generously provided by Dr. Herbert Sheppard of Hoffmann–La Roche Inc. All other materials were obtained from sources identified previously [3].

Methods. All of the experimental procedures employed in this study were carried out as described previously [3].

C57BL leukemia EL4 was maintained, harvested and labeled with $Na_2{}^{51}CrO_4$ as reported earlier [3]. Cytotoxic peritoneal exudate lymphocytes were obtained from CD-1 mice 10, 11 or 12 days after intraperitoneal injection of 2×10^7 EL4 cells [3]; results from other laboratories indicate that between 35 and 80 per cent of the cells in similar lymphocyte preparations possess cytolytic capability [4, 5]. (Dulbecco's) phosphate-buffered saline supplemented with 10% heat-inactivated fetal calf serum was used as the medium for all cell incubations. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (7.9 μM), a potent inhibitor of adenosine deaminase [6], was included in the medium in all experiments involving 8-aza-Ado, Ino and Guo, even though 8-aza-Ado was the only one of these ribonucleosides susceptible to enzymatic deamination.

The assay *in vitro* of cytolysis determined the amount of ^{51}Cr released during a 70-min incubation at 37°C of ^{51}Cr-labeled EL4 cells and specifically sensitized peritoneal exudate lymphocytes [1, 7]. A 1:1 ratio of lymphocytes to target cells yielded an average cellular lysis of 20.6 ± 4.4 per cent (range 9.4 to 36.0 per cent) in different experiments.

A known amount of 8-aza-Ado was injected into the high-performance liquid chromatograph in order to determine the response factor (ultraviolet peak area, in in.^{2}/nmole of nucleotide) for 8-aza-adenosine 5′-triphosphate (8-aza-ATP).

RESULTS

Inhibition of the metabolism of F-Ado by ribonucleosides. From previous work [2] it was known that several purine ribonucleoside derivatives were completely non-inhibitory toward LMC. At the outset of this study, a search was conducted in the hope of finding one or more of these LMC-non-inhibitory ribonucleosides which would prevent the uptake and/or metabolism of F-Ado by cytotoxic lymphocytes. To this end, cytotoxic lymphocytes were preincubated for 30 min with or without each

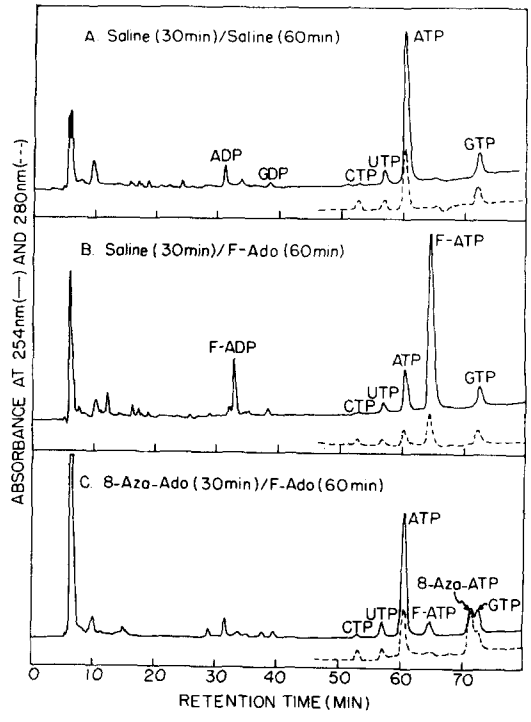

Fig. 1. High-performance liquid chromatographic nucleotide profiles of cytotoxic lymphocytes after incubation with saline, F-Ado or 8-aza-Ado followed by F-Ado. Cytotoxic lymphocytes (1.7×10^7 cells/5.0 ml of medium) were preincubated with either saline (A and B) or 300 μM 8-aza-Ado (C) at 37° for 30 min, after which the cell suspensions were supplemented with either saline (A) or 18.8 μM F-Ado (B and C) and were incubated for another 60 min at 37°. Subsequently, the cells were harvested by centrifugation and acid-soluble extracts of the cells were prepared and analyzed by high-performance liquid chromatography as described previously [3]. The column effluent was monitored both at 254 nm (solid line) and 280 nm (broken line). Full-scale absorbance ranges of 0.04 A unit were employed.

Table 1. Effects of 8-aza-Ado, Ino and Guo on the metabolism of F-Ado to F-ATP by cytotoxic lymphocytes*

Treatment schedule	CTP	UTP	ATP	GTP	F-ATP	8-aza-ATP
	(pmoles nucleotide/10^6 cells)					
Saline (30 min) + saline (60min)	27 ± 2	61 ± 2	535 ± 35	95 ± 5		
Saline (30 min) + F-Ado (60 min)	17 ± 3	42 ± 1	145 ± 1	67 ± 5	721 ± 7	
8-Aza-Ado (30 min) + F-Ado (60 min)	25 ± 1	61 ± 4	381 ± 74	91 ± 16	53 ± 13	228 ± 35
Ino (30 min) + F-Ado (60 min)	24 ± 2	48 ± 4	371 ± 19	87 ± 11	315 ± 21	
Guo (30 min) + F-Ado (60 min)	26 ± 1	50 ± 3	269 ± 25	181 ± 27	453 ± 40	

* Cytotoxic lymphocytes (1.7×10^7 cells/5.0 ml of medium) were preincubated at 37° with 7.9 μM EHNA and with saline or with 300 μM 8-aza-Ado, Ino or Guo for 30 min, after which the cell suspensions were further supplemented with either saline or 18.8 μM F-Ado and were then incubated for another 60 min at 37°. Subsequently, the cells were harvested by centrifugation and acid-extracted and the extracts were analyzed by high-performance anion-exchange chromatography as described previously [3]. The experiment was performed in duplicate and the results are expressed as the mean ± the average deviation of the two analyses.

Table 2. Effects of 8-aza-Ado, Ino and Guo on lymphocytic levels of F-cAMP and cAMP determined at the end of a 30-min incubation of the cells with F-Ado*

Treatment schedule	F-cAMP (pmoles nucleotide/10^7 cells)	cAMP (pmoles nucleotide/10^7 cells)
Saline (30 min) + saline (30 min)	0.05 ± 0.01	0.53 ± 0.04
Saline (30 min) + F-Ado (30 min)	3.65 ± 0.14	1.25 ± 0.06
8-Aza-Ado (30 min) + F-Ado (30 min)	1.18 ± 0.07	3.53 ± 0.14
Ino (30 min) + F-Ado (30 min)	2.80 ± 0.09	1.84 ± 0.09
Guo (30 min) + F-Ado (30 min)	3.09 ± 0.16	1.74 ± 0.11
8-Aza-Ado (30 min) + saline (30 min)	0.12 ± 0.04	0.90 ± 0.03
Ino (30 min) + saline (30 min)	0.15 ± 0.01	0.77 ± 0.05
Guo (30 min) + saline (30 min)	0.10 ± 0.02	0.72 ± 0.04

* Cytotoxic lymphocytes (1.0×10^7 cells/5.0 ml of medium) were preincubated at 37° with 7.9 μM EHNA and with saline or with 300 μM 8-aza-Ado, Ino or Guo for 30 min, after which the cell suspensions were further supplemented with either saline or 18.8 μM F-Ado and were incubated at 37° for a further 30 min prior to their acid extraction. All extracts were column-purified and radioimmunoassayed for cyclic nucleotides as described previously [3]. All incubations were performed in duplicate and each column-purified extract was radioimmunoassayed in duplicate for the appropriate cyclic nucleotide. Each value represents the mean ± the standard error of the mean for four determinations.

of the candidate ribonucleosides (at 300 μM), and F-Ado (18.8 μM) was then added to each of these cellular suspensions. After a further 60-min incubation with the F-Ado, the cells were harvested by centrifugation and acid-soluble extracts of the cells were prepared. These extracts were then analyzed by high-performance anion-exchange chromatography in order to evaluate the ability of each of the purine ribonucleoside derivatives to inhibit the metabolism of F-Ado to F-ATP by the lymphocytes.

8-Aza-Ado, Ino and Guo were found to inhibit to varying degrees the metabolism of F-Ado to F-ATP by cytotoxic lymphocytes (Table 1). In the absence of these ribonucleosides, F-Ado was metabolized extensively to F-ATP, and cellular levels of ATP, in particular, were diminished greatly (Fig. 1A and 1B). 8-Aza-Ado reduced by 93 per cent the cellular build-up of F-ATP and largely prevented the cellular loss of endogenous ribonucleoside 5′-triphosphates; under these conditions, substantial amounts of 8-aza-ATP* were formed within the lymphocytes from the 8-aza-Ado (Table 1 and Fig. 1C). Ino and Guo were considerably less effective than 8-aza-Ado in blocking the metabolic formation of F-ATP. Incubation of the lymphocytes with 300 μM Guo together with the F-Ado resulted in a doubling of the pool size of GTP; however, Ino was not metabolized detectably to ITP under these same conditions.

8-Aza-Ado, Ino and Guo were subsequently examined for their ability to inhibit the metabolism of F-Ado to F-cAMP. Two different types of experiments were performed to study this question. In the first experiment, lymphocytes were preincubated at 37° with saline or with 300 μM ribonucleoside for 30 min; saline or 18.8 μM F-Ado was then added to each of the cellular suspensions and incubation at 37° was continued for another 30 min prior to acid extraction of the cellular suspensions for cyclic nucleotide determinations. The results of this experiment are presented in Table 2. When F-Ado alone was incubated with the lymphocytes, high levels of F-cAMP (relative to cAMP) were produced by the cells. 8-Aza-Ado inhibited the metabolic formation of F-cAMP by 68 per cent. Under the conditions of this experiment, F-Ado alone caused a 136 per cent increase in cAMP, and 8-aza-Ado alone caused a 70 per cent increase in cAMP; when tested together, these two agents caused a 566 per cent increase in cAMP levels. Ino and Guo were considerably less active than 8-aza-Ado both in inhibiting the formation of F-cAMP and in enhancing the elevation of cAMP caused by F-Ado. It should be emphasized that the lymphocytic levels of both F-cAMP and cAMP determined in the present experiment are elevated above what might be called their steady state values due to continuous stimulation of adenylate cyclase caused by extracellular F-Ado [2, 3]; hence, the cellular levels of F-cAMP presented in Table 2 reflect both the relative cellular content of F-ATP *and* the enhanced utilization of F-ATP by the F-Ado-stimulated adenylate cyclase.

For this latter reason, a second type of experiment was carried out to evaluate the abilities of 8-aza-Ado, Ino and Guo to inhibit the lymphocytic metabolism of F-Ado to F-cAMP. Lymphocytes were preincubated with saline or with 300 μM 8-aza-Ado, Ino or Guo for 30 min prior to the addition of saline or 18.8 μM F-Ado and a subsequent 60-min incubation at 37°. At the end of this latter incubation period, the cells were washed free of exogenous drugs and incubated at 37° for another 30 min prior to their acid extraction. The results of these experiments are presented in Table 3. Under these latter experi-

* The ultraviolet peak labeled as 8-aza-ATP in Fig. 1C was observed only in the chromatograms of extracts from 8-aza-Ado-treated cells. This peak was identified as 8-aza-ATP on the bases of its elution position in the triphosphate region of the chromatogram, its similar A_{254}/A_{280} absorbance ratio as compared with 8-aza-Ado and its shift to the diphosphate region of the chromatogram after incubation of a portion of the cell extract with yeast hexokinase plus glucose.

Table 3. Effects of 8-aza-Ado, Ino and Guo on lymphocytic levels of F-cAMP and cAMP determined after a 60-min incubation of the cells with F-Ado, washout and a subsequent 30-min incubation in drug-free medium*

Treatment schedule	F-cAMP (pmoles nucleotide/10^7 cells)	cAMP (pmoles nucleotide/10^7 cells)
Saline (30 min) + saline (60 min)/washed cells/saline (30 min)	0.04 ± 0.02	0.35 ± 0.04
Saline (30 min) + F-Ado (60 min)/washed cells/saline (30 min)	1.52 ± 0.04	0.17 ± 0.05
8-Aza-Ado (30 min) + F-Ado (60 min)/washed cells/saline (30 min)	0.14 ± 0.01	0.27 ± 0.05
Ino (30 min) + F-Ado (60 min)/washed cells/saline (30 min)	0.57 ± 0.03	0.17 ± 0.04
Guo (30 min) + F-Ado (60 min)/washed cells/saline (30 min)	0.74 ± 0.10	0.14 ± 0.04
8-Aza-Ado (30 min) + saline (60 min)/washed cells/saline (30 min)	0.05 ± 0.01	0.49 ± 0.06
Ino (30 min) + saline (60 min)/washed cells/saline (30 min)	0.03 ± 0.01	0.44 ± 0.06
Guo (30 min) + saline (60 min)/washed cells/saline (30 min)	0.04 ± 0.01	0.41 ± 0.02

* Cytotoxic lymphocytes (0.8×10^7 cells/5.0 ml of medium) were preincubated at 37° with 7.9 μM EHNA and with saline or with 300 μM 8-aza-Ado, Ino or Guo for 30 min, after which the cell suspensions were further supplemented with either saline or 18.8 μM F-Ado and were incubated at 37° for a further 60 min. The cells were then harvested by centrifugation, washed one time, resuspended in fresh, drug-free medium and incubated at 37° for another 30 min prior to their acid extraction. Cell extracts were column-purified and radioimmunoassayed for cyclic nucleotides as described previously [3]. All incubations were performed in duplicate and each column-purified extract was radioimmunoassayed in duplicate for the appropriate cyclic nucleotide. Each value represents the mean ± the standard error of the mean for four determinations.

mental conditions, 8-aza-Ado, Ino and Guo inhibited the cellular formation of F-cAMP from F-Ado by 93, 64 and 53 per cent, respectively. Prior treatment of the cells with F-Ado alone resulted in a 50 per cent decrease in cAMP; the presence of 8-aza-Ado partially prevented this loss of cAMP caused by F-Ado.

Effects of 8-aza-Ado, Ino and Guo on F-Ado inhibition of LMC. When tested alone, at a concentration of 300 μM, 8-aza-Ado, Ino and Guo were each non-inhibitory toward LMC (Table 4, top row). Moreover, this same concentration of 8-aza-Ado had little or no effect upon the ability of F-Ado to inhibit LMC when both agents were present together during the 70-min LMC assay. However, Ino and Guo did partially antagonize the inhibitory effect of F-Ado toward LMC under these conditions. These results show that F-Ado could still exert its full inhibitory activity toward LMC when its uptake and metabolism to F-ATP and F-cAMP by the lymphocytes were largely prevented by an agent such as 8-aza-Ado.

More important to the purpose of the present study was the question of whether agents which prevent lymphocytic formation of F-ATP and F-cAMP also prevent the irreversible nature of the inhibition of LMC associated with F-Ado. To this end, cytotoxic lymphocytes were preincubated for 30 min either with saline or with 300 μM 8-aza-Ado, Ino or Guo prior to a 60-min incubation with either saline or 18.8 μM F-Ado. The lymphocytes were then washed free of extracellular drugs and assayed

Table 4. Effects of 8-aza-Ado, Ino and Guo on the inhibition of LMC by F-Ado when agents are present during the LMC assay*

Concn of F-Ado (μM)	Cytolysis (per cent of control)			
	Saline	8-Aza-Ado (300 μM)	Ino (300 μM)	Guo (300 μM)
0	100	100	100	100
1.2	62	66	70	60
2.4	52	56 ($P < 0.01$)†	62 ($P < 0.001$)	58 ($P < 0.05$)
4.7	44	46	58 ($P < 0.05$)	52
9.4	40	40	56 ($P < 0.05$)	54 ($P < 0.05$)
18.8	36	34	58 ($P < 0.01$)	50 ($P < 0.05$)

* Cytotoxic lymphocytes (2.5×10^5 cells/1.0 ml of medium) were preincubated at 37° for 30 min with 7.9 μM EHNA and with either saline or with 300 μM 8-aza-Ado, Ino or Guo. The lymphocytes were then supplemented with the specified concentration of F-Ado and with the ^{51}Cr-labeled EL4 target cells, and the LMC assay was conducted for 70 min in the usual manner [1, 7]. Results are expressed as the per cent of ^{51}Cr released specifically by the drug-treated cells as compared with that released specifically by the untreated (saline control) cells. Each value represents the mean of duplicate assays. Of the total radioactivity (13,919 cpm) released by freeze–thaw treatment of the ^{51}Cr-labeled cells, 3,290 cpm was released in the cytolysis assay in the presence of non-drug-treated cytotoxic lymphocytes and 515 cpm was released nonspecifically when ^{51}Cr-labeled EL4 cells were incubated for 70 min at 37° in the absence of cytotoxic lymphocytes.

† Values of P shown in parentheses were calculated by means of the two-tailed Student's *t*-test. At each concentration of F-Ado tested, statistical comparisons were made between the data from the saline-supplemented LMC assays and the data from the ribonucleoside-(8-aza-Ado, Ino or Guo)-supplemented LMC assays.

Table 5. Effects of 8-aza-Ado, Ino and Guo on the irreversibility of the inhibition of LMC caused by F-Ado*

Treatment schedule prior to start of LMC assay	Cytolysis (per cent of control)
Saline (30 min) + saline (60 min)/washed cells	100
Saline (30 min) + F-Ado (60 min)/washed cells	13
8-Aza-Ado (30 min) + F-Ado (60 min)/washed cells	78 ($P < 0.001$)†
Ino (30 min) + F-Ado (60 min)/washed cells	61 ($P < 0.001$)
Guo (30 min) + F-Ado (60 min)/washed cells	37 ($P < 0.001$)
8-Aza-Ado (30 min) + saline (60 min)/washed cells	103
Ino (30 min) + saline (60 min)/washed cells	102
Guo (30 min) + saline (60 min)/washed cells	98

* Cytotoxic lymphocytes (2.5×10^5 cells/1.0 ml of medium) were preincubated at 37° for 30 min with 7.9 μM EHNA and with either saline or with 300 μM 8-aza-Ado, Ino or Guo prior to a 60-min incubation of the cells with either saline or 18.8 μM F-Ado. The lymphocytes were then harvested by centrifugation, washed one time, resuspended in fresh, drug-free medium and mixed with the ^{51}Cr-labeled EL4 cells, and the LMC assay was conducted for 70 min in the usual manner [1, 7]. Results are expressed as in Table 4. Of the total radioactivity (3979 cpm) released by freeze–thaw treatment of the ^{51}Cr-labeled cells, 1068 cpm was released in the cytolysis assay in the presence of non-drug-treated cytotoxic lymphocytes and 168 cpm was released nonspecifically when ^{51}Cr-labeled EL4 cells were incubated for 70 min at 37° in the absence of cytotoxic lymphocytes.

† Values of P shown in parentheses were calculated by means of the two-tailed Student's *t*-test. Statistical comparisons were made between the data for the saline/F-Ado-pretreated cells and the data for the ribonucleoside (8-aza-Ado, Ino or Guo)/F-Ado-pretreated cells.

Table 6. Effects of Ro 20-1724 on the residual cytolytic activity of lymphocytes preloaded with nucleotides of F-Ado (via prior incubation with F-Ado)*

Concentration of F-Ado used to pretreat lymphocytes (μM)	Cytolysis (per cent of control)	
	Ro 20-1724 (50 μM) present during LMC assay	
	Minus	Plus
0	100	97
1.2	85 ($P < 0.001$)†	70
2.4	70 ($P < 0.001$)	55
4.7	58 ($P < 0.001$)	38
9.4	42 ($P < 0.001$)	26
18.8	27 ($P < 0.001$)	15

* Cytotoxic lymphocytes (2.5×10^5 cells/1.0 ml of medium) were preincubated at 37° for 60 min with the specified concentration of F-Ado. The lymphocytes were then harvested by centrifugation, washed one time, resuspended in fresh, drug-free medium, supplemented with saline or 50 μM Ro 20-1724 and mixed with ^{51}Cr-labeled EL4 cells to start the 70-min LMC assay [1, 7]. Results are expressed as in Table 4. Of the total radioactivity (24,343 cpm) released by freeze–thaw treatment of the ^{51}Cr-labeled cells, 5,333 cpm was released in the cytolysis assay in the presence of non-drug-treated cytotoxic lymphocytes and 614 cpm was released nonspecifically when ^{51}Cr-labeled EL4 cells were incubated for 70 min at 37° in the absence of cytotoxic lymphocytes.

† Values of P shown in parentheses were calculated by means of the two-tailed Student's *t*-test. At each concentration of F-Ado tested, statistical comparisons were made between the data for the LMC assays conducted in the absence of Ro 20-1724 and the data for the LMC assays conducted in the presence of Ro 20-1724.

for residual cytolytic activity (Table 5). Pretreatment of the lymphocytes with F-Ado alone reduced their cytolytic activity to 13 per cent of that of the control cells. However, inclusion of 8-aza-Ado, Ino and Guo during the F-Ado-pretreatment step resulted in recoveries of 78, 61 and 37 per cent, re-

Table 7. Effects of Ro 20-1724 on levels of F-cAMP and cAMP in cytotoxic lymphocytes preloaded with nucleotides of F-Ado (via prior incubation with F-Ado)*

Concn of F-Ado used to pretreat lymphocytes (μM)	Ro 20-1724 (50 μM) present during subsequent 30-min incubation	F-cAMP (pmoles of nucleotide/10^7 cells)	cAMP (pmoles of nucleotide/10^7 cells)
0	−	0.02 ± 0.01	0.64 ± 0.08
0	+	0.03 ± 0.01	1.05 ± 0.11
2.4	−	0.43 ± 0.05	0.48 ± 0.07
2.4	+	1.23 ± 0.10	0.49 ± 0.04
4.7	−	0.67 ± 0.06	0.42 ± 0.04
4.7	+	1.56 ± 0.15	0.63 ± 0.02
9.4	−	0.69 ± 0.06	0.34 ± 0.04
9.4	+	1.54 ± 0.10	0.55 ± 0.01
18.8	−	1.34 ± 0.10	0.34 ± 0.01
18.8	+	3.52 ± 0.41	0.71 ± 0.06

* Cytotoxic lymphocytes (0.81×10^7 cells/5.0 ml of medium) were preincubated at 37° for 60 min with the specified concentration of F-Ado. The lymphocytes were then harvested by centrifugation, washed one time, resuspended in fresh, drug-free medium, supplemented with saline or 50 μM Ro 20-1724 and incubated at 37° for 30 min prior to their acid extraction. Other conditions are as described in the legend for Table 2.

spectively, of control cytolytic activity. 8-Aza-Ado, Ino and Guo allowed reversal of the inhibitory effect of F-Ado on LMC only when added to the lymphocytes prior to, or at the time of, the addition of F-Ado; greater cytolytic activity was recovered when the lymphocytes were preincubated with these nucleosides prior to the addition of the F-Ado. EHNA was required for 8-aza-Ado to be effective in this type of experiment. 8-Aza-adenine, hypoxanthine and guanine were completely ineffective in allowing reversal of F-Ado inhibition of LMC.

Effect of Ro 20-1724 on the residual cytolytic activity of lymphocytes preloaded with nucleotides of F-Ado. In view of the possible role of F-cAMP in the mechanism of F-Ado inhibition of LMC [3], it was of interest to learn whether Ro 20-1724, a potent inhibitor of cAMP phosphodiesterase [8], would affect the irreversible phase of LMC inhibition caused by F-Ado. Accordingly, cytotoxic lymphocytes were preloaded with different cellular levels of nucleotides of F-Ado (via incubation with different concentrations of F-Ado and subsequent washout of residual extracellular drug) and were then assayed for residual cytolytic activity in the absence and presence of 50 μM Ro 20-1724. The data in Table 6 show that the presence of Ro 20-1724 during the LMC assay augmented, in a highly reproducible manner, the irreversible inhibition of cytolysis caused by pretreatment of the lymphocytes with F-Ado. Ro 20-1724 (50 μM) by itself was not inhibitory toward cytolysis.

Effect of Ro 20-1724 on levels of F-cAMP and cAMP in lymphocytes preloaded with nucleotides of F-Ado. The ability of Ro 20-1724 to diminish the residual cytolytic activity of lymphocytes which had been preloaded with nucleotides of F-Ado (Table 6) prompted a study of lymphocytic levels of F-cAMP and cAMP under these same experimental conditions. Cytotoxic lymphocytes pretreated with different concentrations of F-Ado accumulated F-cAMP in a dose-dependent manner and underwent a reciprocal loss of cAMP (Table 7). The subsequent 30-min incubation of these F-Ado-pretreated cells with 50 μM Ro 20-1724 resulted in a 2- to 3-fold increase in the levels of F-cAMP and smaller increases in cAMP. Ro 20-1724 alone caused a 64 per cent increase in cAMP levels (to 1.05 pmoles/10^7 cells) in the saline-pretreated control cells; in no case examined did Ro 20-1724 raise cAMP levels of the F-Ado-pretreated cells above this latter control value.

DISCUSSION

It has been reported previously [3] that F-Ado causes potent and irreversible inhibition of LMC and that this physiological effect is accompanied by a transient elevation of cAMP and an extensive metabolism of F-Ado to F-ATP and F-cAMP within the cytotoxic lymphocytes. The present report provides evidence that this inhibition of lymphocytic function by F-Ado can be dissociated experimentally into reversible and irreversible components which differ in their biochemical mechanisms.

It is apparent from the present results that F-Ado can potently inhibit LMC (Table 4) even when its uptake and/or metabolism by the lymphocytes is almost totally prevented by nucleosides such as 8-aza-Ado (Tables 1–3). While 8-aza-Ado greatly reduced the lymphocytic formation of F-cAMP from F-Ado, it markedly enhanced the ability of F-Ado to elevate cAMP levels in the lymphocytes (Table 2); this latter effect is attributed largely to the ability of 8-aza-Ado to maintain cellular levels of ATP in the presence of F-Ado (Table 1). When the metabolism of F-Ado to F-ATP and F-cAMP was inhibited, F-Ado resembled more closely adenosine [1] and many of its other structural analogs [2] in that its inhibitory effect toward cytolysis was largely reversible upon washout of extracellular F-Ado from the lymphocytes (Table 5). It is well known that the cytolytic function of lymphocytes is modulated by cyclic nucleotides and that agents which cause an elevation of cAMP within lymphocytes also cause an inhibition of LMC [9–16]. The present results, together with previous evidence [1–3], suggest that F-Ado can inhibit LMC solely by virtue of its ability to bind reversibly to an adenosine receptor located on the membrane of the cytotoxic lymphocytes and thereby stimulate the activity of a functionally associated adenylate cyclase [3, 17, 18].

In addition to this largely reversible mechanism of action, F-Ado can inhibit LMC in an irreversible manner once F-Ado is taken up and metabolized to nucleotides within the cytotoxic lymphocytes. 8-Aza-Ado, Ino and Guo prevented the establishment of this irreversible mode of LMC inhibition to varying degrees (Table 5), in relation to their ability to prevent the lymphocytic formation of F-ATP (Table 1) and F-cAMP (Table 3). Since this irreversible mode of inhibition is expressed in the absence of extracellular F-Ado, it appears to be unrelated to the adenosine receptor mechanism discussed above. F-cAMP has been shown to be highly effective, relative to cAMP, in activating a preparation of protein kinase [3]. The ability of Ro 20-1724 both to enhance the irreversible inhibition of LMC caused by F-Ado (Table 6) and to elevate the levels of F-cAMP in lymphocytes preloaded with nucleotides of F-Ado (Table 7) provides additional support for the proposal [3] that F-cAMP is the biologically active metabolite of F-Ado in these cytotoxic lymphocytes. It is concluded that F-cAMP formed metabolically within the cytotoxic lymphocytes can inhibit LMC by reason of its ability to mimic elevated cellular levels of cAMP.

The mechanism whereby 8-aza-Ado, Ino and Guo inhibit the lymphocytic formation of nucleotides from exogenous F-Ado is uncertain. The observation that these nucleosides were more effective when incubated with the lymphocytes prior to the addition of F-Ado may be interpreted to indicate that these agents require metabolism to their corresponding nucleotides in order to inhibit the uptake and/or metabolism of F-Ado maximally. However, the inability of 8-aza-adenine, hypoxanthine and guanine to prevent the irreversible phase of LMC inhibition caused by F-Ado is not easily reconcilable with this view. It therefore appears that the ribonucleosides prevent the metabolism of F-Ado by inhibiting the transport of F-Ado into the lymphocytes.

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