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Adenosine kinase initiates the major route of ribavirin activation in a cultured human cell line

(lymphocyte/purine synthesis/purine excretion/futile cycle/deoxyadenosine kinase)

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ABSTRACT **Inhibition of IMP dehydrogenase (EC 1.2.1.14) by ribavirin causes the normal human lymphoblast to excrete increased amounts of newly formed purine into the culture medium. In order for ribavirin to be active as an inhibitor of the dehydrogenase, this synthetic nucleoside must be phosphorylated. The effect of ribavirin on purine excretion has been determined with a normal lymphoblast line, and with lymphoblast lines deficient in hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyl-transferase, EC 2.4.2.8), in adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20), and in both hypoxanthine phosphoribosyltransferase and adenosine kinase. Resistance to the effect of ribavirin on purine excretion was associated only with those cell lines deficient in adenosine kinase activity. These cell lines have normal deoxyadenosine kinase (ATP:deoxyadenosine 5′-phosphotransferase, EC 2.7.1.76) activity. Therefore, the nucleoside kinase activity responsible for ribavirin phosphorylation is adenosine kinase.**

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) inhibits IMP dehydrogenase (EC 1.2.1.14) (1) which catalyzes the conversion of inosinate to xanthylate. Ribavirin also is a potent inhibitor of the growth of a wide variety of RNA and DNA viruses (2). These effects, which may be dissociable, require conversion of the nucleoside to the 5′-phosphate (1). It has been implied that phosphorylation of ribavirin results from deoxyadenosine kinase (dAdo kinase; ATP:deoxyadenosine 5′-phosphotransferase, EC 2.7.1.76) activity. However, this kinase copurified with adenosine kinase (Ado kinase; ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20) and therefore the results are inconclusive (3). We have used cultured human lymphoblasts deficient in Ado kinase activity to establish that Ado kinase and not dAdo kinase is the significant activity *in vivo* for phosphorylation of ribavirin.

MATERIALS

Ribavirin was provided by R. K. Robins and R. Sidwell, ICN Pharmaceuticals, Inc., Irvine, CA. All other chemicals and reagents were obtained as described by Hershfield and Seegmiller (4, 5).

Cell Lines and Cultures. The lymphoblast lines used in this study were the splenic-derived line WI-L2 described by Levy *et al.* (6, 7) and by Lever *et al.* (8) and derivatives of WI-L2 selected *in vitro*: (*i*) AGR_9Cl_{35}, deficient in hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) (8); (*ii*) 107A, deficient in Ado kinase (4, 5); and (*iii*) MTI-TG, deficient in both Ado kinase and HPRT (4, 5). The purine nucleoside phosphorylase (Puo phosphorylase; purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) deficient line was derived from a peripheral blood sample of a patient with immunodeficiency (9) by the procedure of Sly *et al.* (10).

The cells were routinely cultured as described by Lever *et al.* (8). Prior to (14 hr) and during purine synthesis and excretion experiments, the cells were cultured in medium prepared with dialyzed 10% fetal calf serum. For studies with the Puo-phosphorylase-deficient lymphoblast line, medium containing 0.1% fetal calf serum, 0.005% transferrin, and 0.4% bovine serum albumin was used (11).

Purine Synthesis and Excretion. The assay for incorporation of [^{14}C]formate was performed as described by Hershfield and Seegmiller (4, 5). Two-milliliter samples of exponentially growing cultures (10^6 cells/ml) were transferred to 16 × 125-mm plastic screw-cap tissue culture tubes and incubated in a shaking-water bath at 37° under a 5% CO_2 atmosphere for 30 min. In these studies, the test compound, ribavirin, was added and incubation was continued for the times stated. After incubation of samples with and without ribavirin, 10 μl of [^{14}C]formate (1 mCi/ml, 60.7 mCi/mmol) was added to each tube and incubation was continued for 60 min. Assays were terminated by centrifugation at 4° for 4 min at 500 × *g*. Medium was removed and added to glass tubes containing 0.2 ml of 4.4 M perchloric acid; cell pellets were resuspended in 2 ml of 0.4 M perchloric acid and transferred to glass tubes. Medium and cell samples were incubated for 60 min in a boiling water bath to convert purines to bases and were then centrifuged at 1500 × *g* for 10 min. The purine bases were separated from formate by batch absorption onto Dowex 50 × 4 ion exchange resin and elution with 5 ml of 5 M HCl. Radioactivity in purines was measured in 1 ml of the eluate to which was added 10 ml of Triton toluene liquid scintillation counting solution.

Analysis of Purines Excreted. Samples of growth medium were acidified with 0.1 volume of 4.4 M perchloric acid and clarified by centrifugation; and the supernatant was neutralized with Alamine/Freon TF as described by Khym (12). The neutralized samples were then chromatographed by high-pressure liquid chromatography with an RP-18 column and developed with a 5 mM potassium phosphate, pH 4.9/methanol gradient (13).

Nucleoside Kinase Assays. Ado kinase and dAdo kinase activities were assayed as described by Ives *et al.* (14).

RESULTS

Purine synthesis and excretion

When cultures of lymphoblasts are pulsed with [^{14}C]formate,

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Abbreviations: Hyp, hypoxanthine; Ino, inosine; Ado kinase, adenosine kinase (EC 2.7.1.20); dAdo kinase, deoxyadenosine kinase (EC 2.7.1.76); HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); Puo phosphorylase, purine-nucleoside phosphorylase (EC 2.4.2.1).

the rate of purine synthesis *de novo* can be measured (4) and, by separation of cells and media before analysis, purine excretion can be evaluated (5). [^{14}C]Formate can enter the biosynthetic pathway at 2 (the 3rd and 9th) of the 10 steps that begin with the synthesis of phosphoribosylamine from 5′-phosphoribosyl-1 pyrophosphate, the first committed step, and end with the synthesis of IMP, the first completed purine nucleotide. IMP may then be converted in two steps either to AMP or to GMP or catabolized to inosine (Ino) and hypoxanthine (Hyp) by the combination of nucleotidase activity and Puo phosphorylase. The conversion of IMP to AMP is initiated by adenylosuccinate synthetase and to GMP by IMP dehydrogenase. In normal cells, only a small fraction of the [^{14}C]formate label is found in IMP or in the products of IMP catabolism, Ino and Hyp. The major fraction is in adenine and guanine nucleotides (4).

5′-Nucleotidase or phosphatase activity is a very efficient competitor with adenylosuccinate synthetase and IMP dehydrogenase for IMP. In cells deficient in HPRT activity, 20–30% of the newly formed purine is excreted into the culture medium and is identified as Hyp and some Ino (see AGR_9Cl_{35} and MTI-TG results in Table 1). In studies with a Puo phosphorylase-deficient lymphoblast line cultured in medium also deficient in Puo phosphorylase activity (11), all newly formed purine excreted into the medium (20–30% of total newly formed purine as in HPRT-deficient lymphoblasts) was identified as Ino (Willis and Seegmiller, unpublished results). Lymphoblasts with normal HPRT and Puo phosphorylase activities (see WI-L2 and 107A results in Table 1) excrete only 5–10% of the newly synthesized purine into the medium and this purine is identified as Hyp and Ino. The functional significance of what appears to be a futile cycle, i.e., IMP → Ino → Hyp → IMP, in the cultured human lymphoblast is not well understood at this time.

Effect of ribavirin on purine synthesis and excretion

Several compounds which inhibit either IMP dehydrogenase or adenylosuccinate synthetase activity were found to increase the fraction of newly formed purine excreted into the medium. Among the compounds studied was ribavirin which inhibits IMP dehydrogenase activity when phosphorylated *in vivo* (1). As shown in Table 1, incubation of the WI-L2 or normal lymphoblast line with ribavirin for 30 min followed by measurement of purine synthesis and excretion by a 60-min pulse with [^{14}C]formate resulted in 71% of the newly synthesized purine's being excreted into the medium compared to 5% excretion observed with a parallel culture incubated in the absence of ribavirin. Analysis of the distribution of [^{14}C]formate label as described by Hershfield and Seegmiller (4, 5) indicated that, over the period of the [^{14}C]formate pulse, ribavirin completely blocked conversion of IMP to guanine nucleotides while conversion of IMP to AMP was not inhibited significantly. These observations are consistent with observations of the effect of ribavirin on a murine lymphoma cell line (15); however, neither purine synthesis nor excretion was measured in the former study. Interestingly, the total purine synthesized *de novo* is not decreased by ribavirin and the small increase in total purine synthesis observed in the ribavirin-incubated culture is significant (Willis and Seegmiller, unpublished results). The IMP formed in excess of the demands of adenylate and guanylate nucleotide synthesis in the ribavirin-incubated cultures was catabolized and excreted into the medium as Ino and Hyp.

Other studies with ribavirin indicated that excretion of the newly synthesized purine could be maximally affected by 50 μM ribavirin after 90 min of incubation. Approximately 80% of the newly synthesized purine was excreted by both HPRT-deficient and normal lymphoblast lines.

Table 1. Effects of ribavirin on purine synthesis and excretion by cultured human lymphoblast lines

Cell line	Enzyme defect	Ribavirin,* 0.5 mM	[^{14}C]Formate-labeled purine† Cells‡	Medium‡	Total‡	Excretion, % total
WI-L2	Control	−	17.8	1.1	18.9	6
		+	5.7	13.9	19.6	71
107A	Ado kinase$^-$	−	18.0	1.0	19.0	5
		+	18.6	1.0	19.6	5
AGR_9Cl_{35}	HPRT$^-$	−	14.8	4.0	18.8	21
		+	5.0	15.9	20.9	76
MTI-TG	Ado kinase$^-$ and HPRT$^-$	−	14.4	5.0	19.4	26
		+	14.6	5.4	20.0	27

* Fifty microliters of 0.15 M NaCl (−) containing 20 mM ribavirin (+) was added and incubation was continued for 30 min.

† The values represent the average of triplicate samples. Total purine is the sum of values observed in cell and medium samples. Percentage excretion is the average value observed in medium divided by the average total synthesis value × 100.

‡ cpm × 10^{-4}/hr per 2 ml of culture.

Effects of ribavirin on lymphoblast lines deficient in Ado kinase

The increased excretion of newly formed purines appeared to be a sensitive assay for the determination of inhibitors of IMP dehydrogenase and adenylosuccinate synthetase activities. In previous studies (3), the enzymatic activity responsible for phosphorylation, i.e., "activation," of ribavirin was not clearly established as either Ado kinase or dAdo kinase. Therefore, the effect of ribavirin on purine excretion by cultured lymphoblast lines deficient in Ado kinase activity (4, 5) was evaluated. As indicated in Table 1, ribavirin did not affect purine excretion by lymphoblast lines 107A and MTI-TG, which are resistant to 6-methylmercaptopurine riboside and deficient in Ado kinase activity (4, 5). Table 2 indicates that even though the 6-methylthionosine-resistant lines are deficient in Ado kinase activity, these lines have normal levels of dAdo kinase activity. Therefore, the primary route of ribavirin activation by the cultured human lymphoblast is mediated by Ado kinase activity rather than by dAdo kinase activity.

DISCUSSION

While this study does not eliminate the possibility of some ribavirin being phosphorylated by dAdo kinase, the results indicate that Ado kinase initiates the major phosphorylation required for its metabolic effects in cells possessing both activities. Another possible route of phosphorylation could have been phosphorolysis of the nucleoside by Puo phosphorylase and

Table 2. Nucleoside kinase activities of WI-L2 and a 6-methylthioinosine resistant derivative

Cell line	Enzyme defect	Nucleoside kinase activity, nmol/min per mg Ado kinase	dAdo kinase
WI-L2	Control	0.62	0.38
107A	Ado kinase$^-$	0.01	0.31

phosphoribosylation of the 1,2,4-triazole-3-carboxamide moiety by HPRT activity. However, the ribavirin-dependent excretion of purine was similar in HPRT-deficient and control lines and was also similar to control cell lines in studies with the Puo phosphorylase-deficient line.

The analysis of metabolic routes of drug activation in human cell lines may be greatly facilitated by using the expanding collection of mutant lines, selected *in vitro*, that are deficient in various enzymes of purine and pyrimidine metabolism. For example, 2-amino-1,3,4-thiadiazole is another compound that inhibits IMP dehydrogenase. This analog appears to require activation by the cell and several possible routes of activation have been proposed but not defined (16). The study of effects of aminothiodiazole with cell lines deficient in Puo phosphorylase, Ado kinase, and HPRT may elucidate the pathway of activation.

Results of this study suggest that use of ribavirin for therapy in clinical trials may increase uric acid production and excretion. The magnitude of the increase in uric acid production accompanying the administration of ribavirin needs to be evaluated in order to determine whether or not concomitant therapy with allopurinol is needed.

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