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USE OF ENZYME-DEFICIENT CELL CULTURE LINES AS A BIOCHEMICAL SCREEN FOR STUDY OF PURINES, PURINE NUCLEOSIDES AND RELATED COMPOUNDS*

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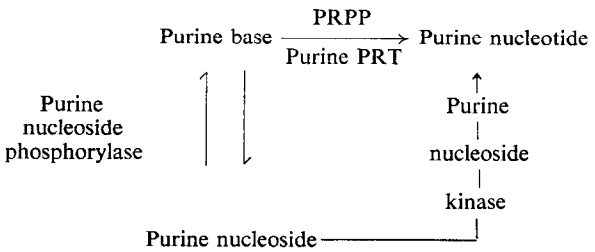
Abstract—From H.Ep. No. 2 cells in culture, two new sublines have been selected for resistance to certain cytotoxic purines and purine nucleosides. These new sublines, together with five resistant sublines previously described, constitute a series of cell lines deficient in the following enzymes, either singly or in combinations: adenine phosphoribosyltransferase (EC 2.4.2.7), hypoxanthine (guanine) phosphoribosyltransferase (EC 2.4.2.8) and adenosine kinase (EC 2.7.1.20). Data are presented illustrative of the usefulness of these cell lines as a convenient biochemical screen that provides an indication of the pathways of anabolism of cytotoxic purines, purine nucleosides or analogs of purines and purine nucleosides.

Most cytotoxic purines and purine nucleosides become active only after their intracellular conversion to nucleotides.[1] Formation of the nucleotides takes place by one of the three pathways shown below: (1) direct phosphorylation of the nucleoside catalyzed by a kinase; (2) conversion of the base to the nucleotide by reaction with 5-phosphoribosyl-1-pyrophosphate (PRPP) in the presence of a purine phosphoribosyltransferase (PRT); or (3) the conversion of a nucleoside to the nucleotide by the sequential action of a nucleoside phosphorylase and a purine PRT. Previously we have reported[2–4] the isolation of cell culture lines deficient in: (a) adenine PRT (EC 2.4.2.7), (b) hypoxanthine (guanine) PRT (EC 2.4.2.8), (c) adenosine kinase (EC 2.7.1.20), (d) hypoxanthine PRT plus adenosine kinase, or (e) adenine PRT plus adenosine kinase. We report now the isolation of two new cell lines, one lacking both adenine PRT and hypoxanthine PRT and the other lacking both of these enzymes and



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adenosine kinase. Also presented are illustrations of the use of these cell lines as a biochemical screen for study of the metabolism of purines, purine nucleosides and their analogs.

MATERIALS AND METHODS

Cell cultures. The cell cultures were derived from the human epidermoid carcinoma (H. Ep. No. 2) line established in culture by Moore *et al.*[5] and carried in our laboratory on SRI-14 medium,[6] which is devoid of purines and pyrimidines except for small amounts that might be present in the calf serum. We have already described the isolation and characterization of five lines of H. Ep. No. 2 cells selected for resistance to various cytotoxic purines and purine nucleosides. These are the lines listed in Table 2 as H. Ep. No. 2/MP;[3] H. Ep. No. 2/FA;[2] H. Ep. No. 2/MeMPR;[4] H. Ep. No. 2/MP/MEMPR;[4] and H. Ep. No. 2/FA/FAR.[4] Each of these lines is essentially devoid of activity of adenine or hypoxanthine PRT or adenosine kinase or of two of these enzyme activities (e.g. H. Ep. No. 2/MP/MeMPR and H. Ep. No. 2/FA/FAR). The two new lines added to this spectrum are those designated H. Ep. No. 2/MP/FA and H. Ep. No. 2/MP/MeMPR/FA. The H. Ep. No. 2/MP/FA line was derived from H. Ep. No. 2/MP cells by culturing the latter in serial passage in the presence of gradually increasing concentrations of 2-fluoroadenine. The H. Ep. No. 2/MP/Me MPR/FA line was similarly derived by culturing H. Ep. No. 2/MP/MeMPR cells in the presence of 2-fluoroadenine. Each of the new cell lines was cultured in the presence of 2-fluoroadenine until a high degree of resistance was reached, and was carried thereafter in the absence of drug, except for periodic checks for retention of resistance.

Responses of cell cultures to inhibitors were determined by cloning methods described earlier[4] and given in some detail in Table 3.

Enzyme assays. Cells grown in suspension culture were collected by centrifugation and washed free of medium with 0·9% NaCl solution. The packed cells were suspended in 3 vol. of water and homogenized in a glass–Teflon homogenizer; the homogenate was centrifuged at 25,000 *g* for 45 min. The supernatant, which contained 15–25 mg protein/ml, was used immediately for assay of purine PRT and adenosine kinase activities as described in Table 1. Activities of purine PRT's were determined by measurement of the conversion of ^{14}C-labeled adenine, hypoxanthine or guanine to the nucleotides.[3] Adenosine kinase activity was assayed with 6-methylthiopurine ribonucleoside (6-MeMPR) as substrate, since this nucleoside is a good substrate for adenosine kinase and is not subject to the degradative enzymes acting on adenosine.[7–9]

Compounds. Adenine-8-^{14}C, hypoxanthine-8-^{14}C and guanine-8-^{14}C were purchased from New England Nuclear Corp. ^{35}S-6-MeMPR was prepared as described earlier.[4] 6-Mercaptopurine (6-MP) was obtained from Nutritional Biochemicals Corp. and 4-aminopyrazolo(3,4-*d*)pyrimidine (4-APP) from the Drug Development Branch, Chemotherapy, National Cancer Institute. The other unlabeled purines and nucleosides were synthesized in our laboratories by Dr. J. A. Montgomery and his associates.

RESULTS AND DISCUSSION

The enzyme activities of the two new cell lines are compared with those of the parent cell line in Table 1. Table 2 contains a qualitative summary of the enzyme activities of all of the resistant cell lines. Each of the new cell lines was obtained by

selecting, from populations of one of the previously described resistant cell lines, a subline resistant to 2-fluoroadenine. In each of the new lines, resistance to 2-fluoroadenine was associated with loss of adenine PRT, and in each line this new resistance was achieved without any marked change in the activities of hypoxanthine PRT or adenosine kinase of the line from which it was derived. Thus, H. Ep. No. 2/MP/FA cells lack both adenine and hypoxanthine PRT but retain adenosine kinase activity, whereas H. Ep. No. 2/MP/MeMPR/FA cells have lost adenosine kinase activity as well as both purine PRT's. As shown in Table 1, the loss of these enzyme activities was essentially complete.

TABLE 1. SOME ENZYME ACTIVITIES OF H.Ep. NO. 2 CELLS AND TWO SUBLINES RESISTANT TO CERTAIN PURINE ANALOGS

Substrate	Enzyme	Enzyme activity (nmoles nucleotide/30 min/mg protein)*		
		H. Ep. No. 2	H. Ep. No. 2/MP/FA	H. Ep. No. 2/MP/MeMPR/FA
Hypoxanthine-8-^{14}C	Hypoxanthine PRT†	249, 280, 344	1, 1, 5	9, 2
Guanine-8-^{14}C	Guanine PRT†	195, 371, 393	0, 0, 9	16, 0
Adenine-8-^{14}C	Adenine PRT	139, 200, 262	0, 1, 17	12, 0
6-Methylthiopurine ribonucleoside-^{35}S	Adenosine kinase	54‡	48	0, 0

* The assays were carried out with crude 25,000 *g* supernatants. The resistant lines were grown in the absence of drugs. Activities of the purine PRT's were measured by determination of the amount of nucleotide formed upon incubation of the supernatant with PRPP and the ^{14}C-labeled purine in Tris buffer, pH 7·9.[3] The activity of adenosine kinase was determined by measurement of the amount of nucleotide formed when 6-methylthiopurine-^{35}S ribonucleoside was incubated with the supernatant, Mg^{2+} and ATP in phosphate buffer, pH 7·0.[4] The nucleotides were isolated by paper chromatography and assayed in a liquid scintillation spectrometer.[3,4] Individual values indicate determinations on separate batches of cells grown at different times over a period of several years.

† Hypoxanthine and guanine are converted to nucleotides by the same PRT; values are given for each of these natural substrates.

‡ Average of eight determinations over a 3-yr period. The activities of the kinase varied considerably, but six of the eight values were in the range 42–60.

Table 3 presents data on the responses of the parent cell line, the newly isolated resistant sublines, and the previously described resistant sublines to a selected group of compounds, chosen to illustrate the responses of the various cell lines and also how the pattern of responses may be used to draw conclusions about the anabolism of the analogs. One would predict that H. Ep. No. 2/MP/FA cells would be resistant to analogs of free bases but sensitive to adenosine analogs and this is in fact the observed response. Similarly, H. Ep. No. 2/MP/MeMPR/FA cells should be resistant to all agents that must be converted to nucleotides to be active; this cell line was highly resistant to all of the compounds in Table 3, except 9-cyclopentyl-6-MP and 9-[β-DL-2α,3α,-dihydroxy-4-β(hydroxymethyl)cyclopentyl]adenine (C-Ado).* The reasons for lack of resistance to these two compounds will be discussed below.

The following specific examples have been chosen from the data of Table 3 to illustrate how the responses of these resistant cell lines have been used to gain insight into the pathways of anabolism of purine bases and nucleosides.

* C-Ado is the carbocyclic analog of adenosine.

TABLE 2. ENZYME ACTIVITIES OF PARENT AND RESISTANT LINES OF H. EP. NO. 2 CELLS

Cell line	Selected for resistance to	Enzyme activities*		
		Adenine phosphoribosyl-transferase	Hypoxanthine (guanine) phospho-ribosyltransferase	Adenosine kinase
H. Ep. No. 2/S	Parent line	+	+	+
H. Ep. No. 2/MP	6-Mercaptopurine	+	−	+
H. Ep. No. 2/FA	2-Fluoroadenine	−	+	+
H. Ep. No. 2/MeMPR	6-Methylthiopurine ribonucleoside	+	+	−
H. Ep. No. 2/MP/FA	6-Mercaptopurine 2-Fluoroadenine	−	−	+
H. Ep. No. 2/FA/FAR	2-Fluoroadenine 2-Fluoroadenosine	−	+	−
H. Ep. No. 2/MP/ MeMPR	6-Mercaptopurine 6-Methylthiopurine ribonucleoside	+	−	−
H. Ep. No. 2/MP/MeMPR/FA	6-Mercaptopurine 6-Methylthiopurine ribonucleoside 2-Fluoroadenine	−	−	−

* A plus (+) denotes enzyme activity about the same as that of the parent cell line. A minus (−) denotes either no enzyme activity or an activity less than 10 per cent that of the parent cell line.

(a) The pattern of response to 6-MP and 6-MP-ribonucleoside was the same: cell lines lacking hypoxanthine PRT were resistant to both compounds. This indicates that 6-MP ribonucleoside is not phosphorylated directly but is converted to the nucleotide only, or predominantly, via the free base.

(b) 6-Methylthiopurine ribonucleoside is highly toxic to all cell lines, except those lacking adenosine kinase. The response of the H. Ep. No. 2/MP cell line to this agent was, in fact, the first evidence that it was activated by a pathway not involving conversion to 6-MP and that it was a substrate for a nucleoside kinase.[10]

(c) A nucleoside that is a substrate for adenosine kinase but is not activated to a significant extent via cleavage to the free base should inhibit all lines, except those lacking adenosine kinase. 6-MeMPR and 4-APP-ribonucleoside are such compounds.

(d) The response of the H. Ep. No. 2/MeMPR and H. Ep. No. 2/FA/FAR lines, when considered together, may give considerable information about the metabolism of adenosine analogs. Thus, the fact that H. Ep. No. 2/MeMPR cells are not resistant to 2-fluoroadenosine, whereas H. Ep. No. 2/FA/FAR cells are highly resistant, is good evidence that 2-fluoroadenosine may be activated both by direct phosphorylation and by conversion to 2-fluoroadenine, so that a high degree of resistance is attained only when both adenosine kinase and adenine PRT are lost. This finding, which suggests that fluoroadenosine is a substrate for a nucleoside phosphorylase, is not unexpected in light of the recently reported activity of adenine as a substrate (but a poor one) for purine nucleoside phosphorylase from mammalian cells.[11] The fact that H. Ep. No. 2/FA cells show a small degree of resistance to 2-fluoroadenosine is also in

TABLE 3. RESPONSES OF PARENT AND RESISTANT LINES OF H. EP. NO. 2 CELLS TO PURINES, PURINE NUCLEOSIDES AND RELATED COMPOUNDS*

Compound	50% Inhibitory concn for parent line (μM)	Ratio of inhibitory concentrations: Resistant line/Parent line						
		/MP	/FA	/MeMPR	/MP/FA	/MP/MeMPR	/FA/FAR	/MP/MeMPR/FA
6-Methylthiopurine ribonucleoside	1	1	1	>300	0·1	>300	>300	>300
6-Mercaptopurine	0·6	>400	1	0·5–1	>200	>1000	0·5–1	>300
6-Mercaptopurine ribonucleoside	0·7	>140	1	1	>100	>400	1	>150
2-Fluoroadenosine	0·02	2	20	1–2	~2†	~2†	>2000	>900
2-Fluoroadenine	0·03	2	>2000	1	>4000	~2†	>2000	>2000
4-Aminopyrazolo-(3,4-*d*)pyrimidine ribonucleoside	0·2	1	1	>300	1	>1500	>300	>700
4-Aminopyrazolo-(3,4-*d*)pyrimidine	3	0·7	>50	2	>40	~5†	>25	>100
9-Cyclopentyl-6-mercaptopurine	9	1	1	2	~2†	0·8	2	2
Carbocyclic analog of adenosine	0·7	1	0·5	2	0·5	2	8	0·5–1

* The 50 per cent inhibitory concentration is that concentration inhibiting colony formation by 50 per cent. Approximately 100 cells were placed in 4-oz prescription bottles containing either 10 ml of SRI 14 medium[6] or 10 ml of this medium in which the candidate inhibitor was present. After the cultures had been incubated for 7–10 days, the medium was decanted and the cells adhering to the glass were washed with buffered 0·85% NaCl solution (pH 7·0), fixed with Bouin's fixative, and stained with Giemsa stain. The colonies were then counted. Cloning efficiencies of control cultures were usually in the range of 40–60 per cent.

† The symbol (~) indicates some lack of reproducibility between experiments.

accord with the presence of a phosphorylase acting on this nucleoside: the cleavage of 2-fluoroadenosine to 2-fluoroadenine, which cannot be converted to the nucleotide in this cell line, would in effect reduce the amount of 2-fluoroadenosine available for nucleotide formation by direct phosphorylation.

(e) Compounds active as free bases or as nucleosides should be about equally inhibitory to all of the cell lines. One of the compounds, 9-cyclopentyl-6-MP, listed in Table 3, gave this pattern of inhibition. This finding corroborates other evidence that metabolic alteration is not a factor in the inhibitory activity of 9-alkyl- or 9-cycloalkyl-purines.[12–14]

Other than 9-cyclopentyl-6-MP, the only compound to which H. Ep. No. 2/MP/MeMPR/FA cells were not highly resistant was the carbocyclic analog of adenosine, C-Ado. The other cell lines (H. Ep. No. 2/MeMPR and H. Ep. No. 2/FA/FAR) deficient in adenosine kinase also had at best a low degree of resistance to this compound. C-Ado is known to be phosphorylated and, in cells that have adenosine kinase, a phosphate apparently is the toxic metabolite[15]; however, the responses of the kinase-deficient cells suggest that C-Ado as such has a potent inhibitory action.

These cell lines, used either singly or in various combinations, have proven of great usefulness as a biochemical screen that provides an indication of the pathways of anabolism of new growth-inhibitory purines, purine nucleosides or analogs of purines or purine nucleosides. The amount of effort required is small relative to that required for more direct methods. For most cytotoxic compounds, 5 mg or less is sufficient for assays in duplicate against the entire series of cell lines; however, for most compounds, it is not necessary that all cell lines be examined. Over a period of several years, indications thus obtained of the metabolism of a new agent have always been confirmed when the metabolism of the agent was studied by tracer techniques in intact cells or when the agent was evaluated as a substrate for particular isolated enzymes. In addition to examples given above, which were drawn from the data of Table 3, two other examples might be cited as illustrations of the reliability of the deductions made from the responses of certain of these cell lines. The first is concerned with the response of H. Ep. No. 2/MeMPR cells to other purine nucleosides; those nucleosides to which this cell line is resistant[4] should be substrates for adenosine kinase, and their substrate activity was confirmed when they were studied as substrates for the isolated enzyme.[7] The second example is drawn from the responses of H. Ep. No. 2/MP cells. The fact that these cells were resistant to 8-azahypoxanthine but fully sensitive to 8-azainosine is evidence that 8-azainosine may be phosphorylated directly; this was found to be so when the metabolism of ^{3}H-labeled 8-azainosine was investigated.[16]

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