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BIOCHEMICAL MECHANISM OF DRUG RESISTANCE—VII. INHIBITION OF OROTIC ACID METABOLISM BY 5-AZACYTIDINE IN LEUKEMIC MICE SENSITIVE AND RESISTANT TO 5-AZACYTIDINE

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Abstract—Application of 5-azacytidine to leukemic AKR mice results in an inhibition of the utilization of orotic acid in the liver. In 5-azacytidine-sensitive animals, the inhibition is greater than in the resistant ones. Similarly, the excretion of orotic acid and orotidine in the urine is higher after application of 5-azacytidine in the sensitive animals. Cell-free extracts from leukemic cells, both sensitive and resistant to 5-azacytidine, showed a decreased activity of orotidylic acid decarboxylase after application of 5-azacytidine *in vivo*. The differences in the metabolism of orotate are related to the decreased ability of 5-azacytidine-resistant leukemic cells to convert 5-azacytidine to 5-azacytidine 5′-phosphate.

Biochemical alterations of leukemic lymphoid cells of AKR mice resistant to 5-azacytidine involve the utilization of the pyrimidine precursors of nucleic acid components due to a lower activity of uridine kinase.[1] This interference with the salvage pathway, which was observed in other cases of the development of resistance to different pyrimidine antimetabolites,[2, 3] suggested an investigation of the synthesis *de novo* of pyrimidines in these mutant cells.

In the present report the metabolic transformations and the incorporation of orotic acid into the ribonucleic acids of leukemic liver under various conditions were studied. Changes in the metabolism of orotate in 5-azacytidine-resistant cells might contribute to a more detailed understanding of the mechanism of action of 5-azacytidine[4, 5] and, moreover, might be made use of for a chemotherapeutic application of another analogue that would suppress leukemic cells that have developed resistance to 5-azacytidine.

MATERIALS AND METHODS

Reagents. 5-Azacytidine was prepared[6] by Dr. Pískala and 5-azacytidine-4-^{14}C (4·1c/mole) by Dr. Morávek, both of this Institute. 5-Phosphoribosyl-1-pyrophosphate was obtained from Calbiochem (Los Angeles, Calif.). Orotic acid-6-^{14}C (2·3c/mole) was delivered by the Institute for Research, Production and Use of Radioisotopes, in Prague. Orotidine-6-^{14}C 5′-phosphate of the same specific radioactivity was prepared from orotic acid-6-^{14}C and 5-phosphoribosyl-1-pyrophosphate as described.[7]

Animals. Inbred AKR female mice weighing 22–24 g, kept under standard conditions, were used throughout the experiment. The animals were given i.p. a suspension of leukemic cells (2 × 10^7 per mouse) obtained by disintegrating liver in 0·9% NaCl in a glass homogenizer with a loose-fitting plastic pestle. For most experiments we used animals 5–6 days after inoculation. Leukemic animals resistant to 5-azacytidine were obtained as described before.[8] Differentiation of lymphoblasts in the liver was carried out in smears of sediments of liver cell suspensions in 5% polyvinyl pyrrolidone. Staining was done according to Giemsa.

Utilization of orotic acid for synthesis of liver ribonucleic acid. Orotic acid-6-^{14}C (1 μc/μmole) was applied intraperitoneally together with either the inhibitor or the same volume of physiological saline. The groups contained 4–6 animals and each experiment was done independently twice. Two hr after application of the radioactive compound, the animals were killed, and further treatment of the liver and isolation of uridine-3′(2′)-phosphate and cytidine-3′(2′)-phosphate from the alkaline hydrolysate of liver ribonucleic acid were done in the same manner as before.[9]

Urinary orotic acid and orotidine. Groups of 4 female mice 5 days after inoculation were given (between 8.00 and 10.00 a.m.) an i.p. injection of either 5-azacytidine or an equal volume of physiological saline. The animals were left for 24 hr in metabolic cages without food, with water *ad libitum*, and their urine was collected on a strip of Whatman No. 3 paper. Orotic acid and orotidine from aliquots of the urine were isolated as described earlier.[9] The spectroscopically pure compounds were estimated in 0·01N HCl with a Unicam SP 700 spectrophotometer (the extracted amount is expressed in μmole/24 hr).

Preparation of a cell-free liver extract. Groups of 2 mice 6 days after inoculation were given an i.p. injection of either the inhibitor or physiological saline and the animals were killed 1 hr later. The excised liver was cooled and homogenized with 4 vol. of ice-cold 0·15 M KCl in a Potter–Elvehjem homogenizer and the homogenate was further disintegrated by sonication (MSE ultrasonic oscillator, 1·2 kc, 1 min, 2–5°). After centrifugation (5000 *g*, 20 min, 2°), the supernatant fraction was defatted and kept for, at most, 90 min in an ice-cold water bath at 0°. The protein content was determined according to Lowry *et al.*[10]

Determination of the activity of orotidylic acid decarboxylase. Incubation was carried out at 37° (5 min) in a total volume of 0·5 ml: Tris-HCl buffer (pH 7·5), 40 mM; orotidine-6-^{14}C 5′-phosphate, 0·01 mM; $MgCl_2$, 0·2 mM; reduced glutathione; and 0·1 ml of cell-free liver extract (generally 2·9–3·1 mg protein). Aliquots of the incubation mixture were chromatographed without deproteinization on Whatman No. 1 paper in a solvent system[11] composed of isobutyric acid–ammonium hydroxide–water (66:1·5:33). Radioactivity of the substances formed was eluted and estimated on a proportional 2π flow counter (Frieseke-Hoepfner). The activity of orotidylic acid decarboxylase was estimated from the amount of nonreacted orotidine 5′-phosphate and from the amount of uridine 5′-phosphate, uridine, and uracil formed.

Transformation in vivo *of 5-azacytidine in the liver.* Four leukemic mice, either sensitive or resistant to 5-azacytidine, were used 6 days after inoculation. 5-Azacytidine (1 μc/μmole) was applied i.p. 1 hr before killing the animals. The excised liver was cooled and homogenized twice with 4 vol. of ice-cold 0·2 N $HClO_4$ and the supernatant fractions were combined. Under constant cooling, the pH was adjusted to 7·0–7·5 with 1 N KOH and, after centrifugation, aliquots of the supernatants were separated by paper chromatography on Whatman No. 3 paper in isobutyric acid–

ammonium hydroxide–water. According to the radioactivity record of the chromatogram, the zone of 5-azacytidine-4-^{14}C 5′-phosphate was eluted, as well as the zones of nonreacted 5-azacytidine-4-^{14}C and of its spontaneous decomposition products. The amount of nucleotide formed was expressed in mμmole g liver, fresh weight.

RESULTS

Metabolism of orotic acid in vivo *in leukemic mice sensitive and resistant to 5-azacytidine.* The incorporation of orotic acid into the ribonucleic acids was examined in the liver of leukemic mice, in which, during the course of the disease, the number of leukemic cells increases at the expense of specific liver parenchyma. This process has its metabolic counterpart in the decreased utilization of orotic acid (Fig. 1).

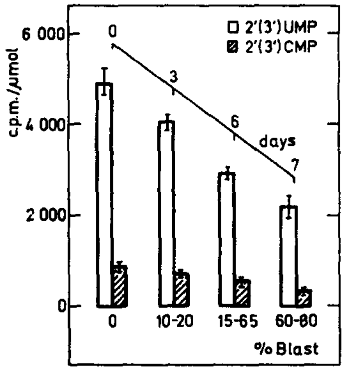

FIG. 1. Utilization of orotic acid for the synthesis of liver ribonucleic acids in mice in different phases of leukemia development. Orotic acid-6-^{14}C (1 μc/μmole/mouse) was administered to groups of 4 leukemic mice at 0, 3, 6, and 7 days after inoculation and 2 hr before killing the animals. Specific radioactivity of uridine-3′(2′)-phosphate (UMP) and cytidine-3′(2′)-phosphate (CMP) isolated from the hydrolysate of liver ribonucleic acid is expressed in cpm/μmole. The percentage of lymphoblasts in the liver, % Blast.

During the terminal stage of mouse leukemia, the utilization of orotic acid for the synthesis of ribonucleic acids by leukemic cells sensitive (AKR/s) and resistant (AKR/r) to 5-azacytidine was similar. When 5-azacytidine was administered concomitantly, the incorporation of orotic acid into liver ribonucleic acids was strongly inhibited. In a number of independent experiments, it has been observed that this inhibition in AKR/s cells was significantly higher than in mutant cells (Table 1).

Analogous differences were found when the excretion of orotic acid and orotidine in the urine was examined. AKR/s and AKR/r leukemic mice excrete, independently of the phase of the leukemic process, approximately equal amounts of both substances. After application of 5-azacytidine, the urinary excretion of orotic acid and orotidine is considerably higher.[12] In leukemic animals sensitive to 5-azacytidine this effect is more remarkable than in 5-azacytidine-resistant mice (Table 2).

Orotidylic acid decarboxylase in a cell-free extract of AKR/s and AKR/r leukemic cells. The decreased effect of 5-azacytidine on the incorporation of orotic acid and on the excretion of orotate in AKR/r leukemic mice suggested an examination of orotate

TABLE 1. INHIBITION OF OROTIC ACID INCORPORATION BY 5-AZACYTIDINE IN LEUKEMIC MICE SENSITIVE AND RESISTANT TO 5-AZACYTIDINE*

Leukemic mice	Incorporation into liver RNA (cpm/μmole UMP)		Inhibition (%)
	Control	5-Azacytidine (1 μmole/mouse)	
AKR/s	2226 ± 143	1014 ± 56	54·5
AKR/r	1886 ± 77	1220 ± 55	35·4

* Orotic acid-6-^{14}C (1 μc/μmole/mouse) was administered to groups of 6 animals on the sixth day after inoculation together with 5-azacytidine 2 hr before killing the animals.

TABLE 2. URINARY EXCRETION OF OROTIC ACID AND OROTIDINE AFTER ADMINISTRATION OF 5-AZACYTIDINE*

Leukemic mice	5-Azacytidine (μmole/mouse)	Orotic acid (μmole/mouse)	Increase (%)	Orotidine (μmole/mouse)	Increase (%)
AKR/s	0	0·26–0·34	100	0·17–0·25	100
AKR/s	3	3·42–3·97	1345	2·34–2·81	1282
AKR/r	0	0·26–0·37	100	0·15–0·27	100
AKR/r	3	2·25–2·70	766	1·98–2·14	943

* After application of 5-azacytidine, the animals were left for 24 hr without food but with water *ad libitum*. Each group included 4 leukemic female mice 5 days after inoculation. The minimum and maximum values of excreted compounds per 24 hr are given. Increase (%) is calculated on the basis of average values.

phosphoribosyltransferase and orotidylic acid decarboxylase activities. Both of these enzymes, however, did not reveal any differences when cell-free extracts from leukemic cells of both types were used.

It has been found that the administration of 5-azacytidine to mice brings about a pronounced inhibition of orotidylic acid decarboxylase in their liver extracts (Fig. 2). This inhibition is probably caused by the newly formed 5-azacytidine 5′-phosphate. However, this nucleotide has not yet been prepared in sufficient quantities; for this reason its effect on orotidylic acid decarboxylase could not be tested *in vitro*. Fig. 2 also shows that the inhibitory effect of 5-azacytidine in mutant cells is lower than in AKR/s cells, but this difference is not significant. The time course of decarboxylation of orotidine 5′-phosphate after treatment *in vivo* with 5-azacytidine is compared with controls in Fig. 3.

Anabolic conversion of 5-azacytidine in livers of AKR/s and AKR/r leukemic mice. In order to determine the cause of the decreased effect of 5-azacytidine an orotate metabolism in AKR/r cells, we applied 5-azacytidine-4-^{14}C to leukemic AKR/s and AKR/r mice and followed its conversion to 5-azacytidine-4-^{14}C 5′-phosphate. It follows from the data obtained (Table 3) that the formation of the phosphorylated derivative in the mutant cells is 59–72 per cent lower than in their AKR/s counterparts.

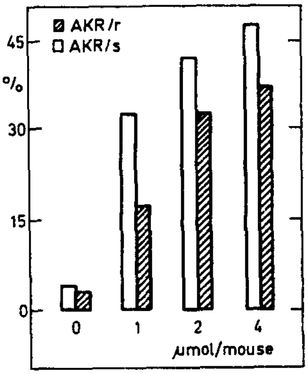

FIG. 2. Decarboxylation of orotidine 5′-phosphate by a cell-free liver extract from leukemic mice sensitive and resistant to 5-azacytidine 1 hr after the administration of 5-azacytidine *in vivo*. 5-Azacytidine (μmole/mouse) was applied to groups of leukemic mice on the sixth day after inoculation, 1 hr before killing the mice. Nonreacted orotidine 5′-phosphate after 5 min of incubation at 37°, %.

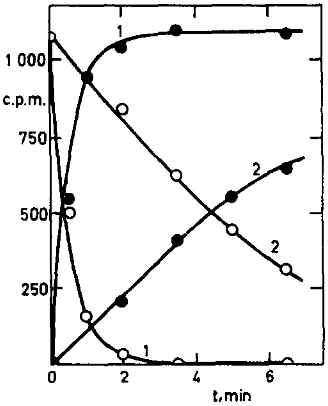

FIG. 3. Time course of orotidylic acid decarboxylase after administration of 5-azacytidine to leukemic mice *in vivo*. Radioactivity (cpm) of nonreacted orotidine 5′-phosphate, ○—○; of formed uridine 5′-phosphate, uridine, and uracil, ●—●; incubation in min, t. Liver extract from control mice,1 liver extract from mice after 1 hr of treatment with 2 μmole 5-azacytidine, 2.

TABLE 3. *In vivo* PHOSPHORYLATION OF 5-AZACYTIDINE IN THE LIVER OF LEUKEMIC MICE SENSITIVE AND RESISTANT TO 5-AZACYTIDINE*

Expt. No.	Leukemic mice	Phosphorylation (%)	5-Azacytidine 5′-phosphate (mμmole/g liver)	Depression (%)
1	AKR/s	37·3	18·56	
	AKR/r	18·1	5·19	72·3
2	AKR/s	42·0	22·30	
	AKR/r	19·8	6·78	59·3

* 5-Azacytidine-4-^{14}C (1 μc/μmole/mouse) was administered to 2 leukemic mice of both groups on the sixth day after inoculation and 1 hr before killing the animals.

DISCUSSION

The sensitivity of experimental tumours and tissues to various antimetabolites of nucleic acids depends to a considerable extent on the character of their metabolism. The synthesis of pyrimidines *de novo* and hence a substantial incorporation of orotic acid into ribonucleic acids is pronounced, especially in the liver.[13, 14] Progressive infiltration of this organ by leukemic cells results in the depression of pyrimidine synthesis *de novo* (Fig. 1), since lymphocytes possess a capacity to utilize efficiently the preformed precursors of nucleic acids.[15] This would account for the relatively low sensitivity of lymphoid leukemia of AKR mice to inhibitors affecting pyrimidine synthesis *de novo*, such as 6-azauridine, 6-azacytidine, 5-azauracil and 5-azaorotate (J. Veselý, unpublished observations). An exception to this rule is represented by 5-azacytidine, which in addition to inhibiting pyrimidine synthesis *de novo* also is incorporated into nucleic acids.[4, 5]

Since the biological effect of 5-azacytidine consists both in an inhibition of orotidylic acid decarboxylase and in an incorporation into various types of nucleic acids, it was of interest to establish whether the development of resistance is associated with a single mechanism for both types of effect. The decreased effect of 5-azacytidine on the incorporation of orotic acid and on oroturia in AKR/r leukemic mice was related to the extent of its phosphorylation, which was impaired in 5-azacytidine-resistant leukemic cells. This assumption was confirmed (Table 3) and the decreased phosphorylation of 5-azacytidine *in vivo* is in agreement with its depressed transformation *in vitro* in a cell-free system from mutant cells.[1]

Thus it appears that in AKR/r leukemic cells the depressed anabolic transformation of 5-azacytidine to the biologically active form underlies the impairment of both mechanisms of its action, i.e. inhibition of orotidylic acid decarboxylase and incorporation into nucleic acids. According to these findings, the possible cause of resistance should be sought in a decreased activity of uridine kinase,[1] which is responsible for the formation of 5-azacytidine 5′-phosphate.

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