CHARACTERIZATION OF DEPRESSED URIDINE KINASE IN RAT THYMUS FOLLOWING 5-AZACYTIDINE ADMINISTRATION

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The administration of 5-azacytidine to rats results in the depression of the activity of uridine kinase in the thymus. The thymic enzyme has been isolated and partially characterized. It does not differ from the control with respect to the K_m constant as well as the inhibition by cytidine 5'-triphosphate, and its elution profile on a Sephadex G-200 column is the same as of the corresponding enzyme from untreated animals.

In normal mice the effects of 5-azacytidine are directed not only against the haemopoietic but also against the lymphatic system[1]. The drug causes the thymic depletion that may be partially alleviated by the simultaneous administration of uridine or cytidine[2]; furthermore, a considerable inhibition of RNA and DNA synthesis has been observed in the thymus[1]. More recently we have described that following 5-azacytidine treatment *in vivo* liver uridine kinase in adult rats is stimulated while in the thymus its activity is diminished[3]. The administration of the drug to pregnant rats results in embryonic liver in the depression of uridine kinase in 17-day-old foetuses[4]. At that time about 60% of the liver is composed of proliferating and vascular tissue, but the percentage falls rapidly at the time of delivery[5]. The reaction towards 5-azacytidine of thymus uridine kinase isolated from the adult rat is thus similar to that of rat embryonic liver enzyme.

In the present report we have investigated the depression of uridine kinase in rat thymus following 5-azacytidine administration more in detail. Our interest has also been directed towards the purification and partial characterization of the isolated enzyme.

EXPERIMENTAL

Reagents and Animals

6-Azauridine-[4,5-^{14}C] (80 mCi/mmol), uridine-[U-^{3}H] (2800 mCi/mmol) and thymidine-[U-^{3}H] (6800 mCi/mmol) were delivered by the Institute for Research, Production and Uses of Radioisotopes, Prague. Adenosine 5'-triphosphate was purchased from Calbiochem, Los Angeles.

Streptomycin sulfate was delivered by Jenapharma, Jena. Sephadex G-200 was a product of Pharmacia, Uppsala. Ammonium sulphate was of analytical grade, 3 times recrystallized. Wistar male rats (170 to 180 g) kept under standard conditions were used throughout the experiments. The animals were injected intraperitoneally at 8 to 9 A. M., while the controls received the same volume of 0·9% NaCl solution. Adrenalectomized animals were kept 4 days before starting the experiments with food and 1% KCl in their drinking water *ad libitum*.

Uridine Kinase Activity

Uridine kinase activity was determined in a standard reaction mixture (0·3 ml) containing 50 mM-Tris-HCl buffer, pH 7·5, 4 mM adenosine 5'-triphosphate, 2 mM $MgCl_2$, 0·1 mM 6-azauridine-[4,5-^{14}C] and 0·1 ml of enzyme preparation obtained by homogenizing freshly exstirpated thymus glands in a glass homogenizer with a tight-fitting Teflon pestle in 9 volumes of 0·01M-Tris-HCl buffer, pH 7·5, containing 0·25M sucrose followed by centrifugation (20000 *g*, 4°C, 60 min). The supernatant fraction was used as a source of enzyme preparation. After a 10 min of incubation at 37°C the mixture was heated in a boiling water-bath for 1 min and analysed by descending chromatography on a Whatman No 1 paper in n-butanol–acetic acid–water (10 : 1 : 3). The radioactive zones were cut out by comparison with standards and their radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer. The rate of phosporylation was linear over the period studied.

Purification of Uridine Kinase

The thymus glands were homogenized in a glass homogenizer with a motor-driven Teflon pestle in 7 volumes of 0·01M-Tris-HCl buffer, pH 7·5, containig 0·25M sucrose. The homogenate was centrifuged at 20000 *g*, 4°C, 60 min and the resulting supernatant was adjusted to 4·5 to 5·5 mg of protein per milliliter with 0·01M-Tris-HCl buffer, pH 7·5, containing 0·1 mM dithiothreitol (Fraction I). To Fraction I streptomycin sulfate was added to the final concentration of 1%, the suspension was stored overnight at 4°C and centrifuged at 20000 *g* for 50 min. The resulting supernatant (Fraction II) was adjusted to 29% saturation with ammonium sulfate. After centrifugation the supernatant fraction was adjusted to 38% saturation with ammonium sulfate and the sediment was dissolved in 5 ml of 0·01M-Tris-HCl buffer, containing 0·1 mM dithiothreitol (Fraction III). Protein content was determined according to Lowry and coworkers[6].

Elution of Uridine Kinase from a Sephadex G-200 Column

A portion of Fraction III (6—7 mg of protein) was layered on a Sephadex G-200 column (2 × 30 cm) equilibrated with 0·01M-Tris-HCl buffer, pH 7·5, containing 0·1 mM dithiothreitol. The elution was carried out at 4°C with the same buffer. The flow rate was 15—20 ml per hour and fractions of 2·5 ml were collected. The optical density was recorded at 280 nm and fractions were assayed for uridine kinase activity.

Autoradiography

Groups of 3 animals were given *i.p.* 5-azacytidine (12 mg per kg) and 2 hours before killing (22 hours after the administration of the drug) they received thymidine-[^{3}H] (250 μCi/0·5 μmol) or uridine-[^{3}H] (250 μCi/μmol). The differential counts of thymus cells were done on imprints. Autoradiographs were coated with a Kodak AR. 10 stripping film. Exposition at 4°C lasted 12

days. Staining of the preparations was performed according to May-Grünwald-Giemsa. The number of grains was evaluated per 300 cells. In each individual animal within one group the results agreed to ±10%.

TABLE I

Depression of Uridine Kinase Activity in the Thymus of 5-Azacytidine-Treated Intact and Adrenalectomized Rats

Groups of 4–6 male intact and adrenalectomized rats were given *i.p.* 5-azacytidine (12 mg/kg) 24 hours before killing. The activity of uridine kinase was assayed during a 10-min incubation period at 37°C in a total volume of 0·3 ml with 0·1 ml of cell-free thymus extract. The enzyme activity is expressed as μmol ± S.E. of 6-azauridine 5'-monophosphate formed during 1 h of incubation in the presence of cell-free extract corresponding to 1 g of the thymus.

Conditions	Intact		Adrenalectomized	
	thymus (w.w.) mg ± S.E.	uridine kinase μmol/h per g ± S.E.	thymus (w.w.) mg ± S.E.	uridine kinase μmol/h per g ± S.E.
Control	480 ± 20	1·80 ± 0·03	380 ± 39	1·15 ± 0·09
5-Azacytidine	330 ± 23	0·92 ± 0·06	375 ± 40	1·16 ± 0·10

TABLE II

DNA and RNA Synthesis in Thymus Cells following the Administration of 5-Azacytidine *in vivo*

Groups of 3 rats were given *i.p.* 5-azacytidine (12 mg per kg) and 2 hours before killing (22 hours after the administration of the drug) they received thymidine-[^{3}H] (250 μCi/0·5 μmol) or uridine-[^{3}H] (250 μCi/ μmol). In each instance 300 cells were evaluated.

Characteristic	Small thymocytes		Large thymocytes	
	control	5-azacytidine	control	5-azacytidine
Thymidine-[^{3}H] *pulse*				
Labelled cells (%)	8·1	1·4	54·0	11·1
Labelling index[a]	33·2	6·3	181·2	31·7
Uridine-[^{3}H] *pulse*				
Labelled cells (%)	24·0	5·1	84·0	27·6
Labelling index[a]	95·8	17·4	825·5	130·4

[a] Percentage of labelled cells multiplied by the number of grains per one average labelled cell.

TABLE III

Partial Purification of Thymus Uridine Kinase

Groups of 10 male rats (165—175 g) were given *i.p.* 5-azacytidine (15 mg/kg) or saline 24 hours before killing.

Fraction	Protein mg/ml	Activity m µmol/ml	Total activity m µmol/fraction (%)		Specific activity m µmol/mg (increase)	
Control						
I Cell-free extract	5·50	9·3	270	(100)	1·69	1·00
II Streptomycin	5·40	9·7	266	(98)	1·80	1·07
III Ammonium sulfate	1·50	38·4	192	(71)	25·6	15·10
5-Azacytidine-treated						
I Cell-free extract	4·85	5·3	154	(100)	1·10	1·00
II Streptomycin	4·20	5·1	148	(96)	1·20	1·09
III Ammonium sulfate	1·56	25·2	126	(82)	16·20	14·70

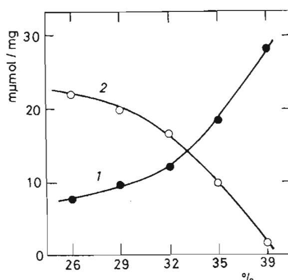

FIG. 1

Precipitation of Uridine Kinase from Normal Rat Thymus at Different Concentrations of Ammonium Sulfate

To 20 ml of Fraction II isolated from the normal rat thymus ammonium sulfate was added at indicated concentrations (%). After standing for 1 h at 4°C the samples were centrifuged (20 000 *g*, 30 min), sediments dissolved (4 ml of 0·01M-Tris-HCl buffer, pH 7·5, with 0·1 mM dithiothreitol), and used as a source of uridine kinase activity, which was expressed as m µmol of 6-azauridine 5′-phosphate formed per mg of protein after a 10-min incubation period (*1*). The corresponding supernatant fractions (*2*) were saturated with ammonium sulfate at indicated concentrations and the further procedure was similar as for the sediments.

RESULTS

Depression of Uridine Kinase Activity in Rat Thymus in vivo

The administration of 5-azacytidine to adult rats results in the decrease of thymus uridine kinase (Table I). The drug affects not only the enzyme activity but also leads to the decrease of the thymus weight. The experiments performed in adrenalectomized rats indicate that the effect of 5-azacytidine on uridine kinase is mediated through adrenals since in adrenalectomized group of animals no depression of uridine kinase activity in the thymus following the administration of 5-azacytidine has been noted. This finding is in contrast to the effect of the drug on the enhancement of liver uridine kinase which does not depend on adrenal secretion[3].

The autoradiographic analysis of thymus cells revealed that following 5-azacytidine administration the synthesis of DNA and RNA in thymocytes was strongly decreased (Table II). This depression is even more marked in large thymocytes as the amount of the radioactivity in these cells (expressed as labelling index) following 5-azacytidine administration is decreased more considerably than in small thymocytes that are regarded as responsible for the immune response of the organism[7].

Characterization of Partially Purified Uridine Kinase from the Thymus of 5-Azacytidine-Treated Rats

Salting out of the enzyme molecules from the thymus had a similar course both in control preparations and in those isolated from the animals that have received 5-azacytidine. It could be established that uridine kinase was precipitated at 29 to 38% saturation with ammonium sulfate (Fig. 1). Using streptomycin followed with ammonium sulfate the enzyme from both sources was purified about 15-fold (Table III) and the loss of the total enzyme activity did not surpass 30%. The partially purified enzyme preparations were applied to a Sephadex G-200 column (Fig. 2). Both enzymes were eluted in a single peak centred around the same fraction although the activity of the control enzyme preparation was considerably higher.

The pooled fractions with highest specific activity from both preparations showed the same value of K_m constant (0·16 mM) when plotted with respect to the phosphate acceptor. V_{max} values were 125 and 62·5 μmol of 6-azauridine 5′-phosphate for the control and the treated enzyme preparation, respectively (Fig. 3). In contrast to uridine kinase from mouse leukemic liver partially purified by Sephadex G-200 chromatography[8] the thymus enzyme was considerably less stable when kept at −25°C than its counterpart from leukemic liver. Furthermore, the inhibition of the enzyme from control and 5-azacytidine-treated thymuses by varying amounts of cytidine 5′-triphosphate indicated an analogous inhibition of both enzyme preparations by this allosteric inhibitor of uridine kinase[9].

DISCUSSION

Uridine kinase is a salvage enzyme which is especially active in rapidly proliferating tissues, and its content is considered to represent the relative efficiency of the tissue to utilize preformed pyrimidine precursors[10]. The comparison of uridine kinase in different organs revealed a correlation between the enzyme activity and the growth rate[10]. Various tissues and organisms have been used for the studies on uridine kinase. The activity in rat kidney, spleen, thymus and especially regenerating liver is considerable[11,12]. The enzyme has been partially purified for the first time from Ehrlich ascites tumour cells[13] and it was clearly established that it differs from thymidine and deoxycytidine kinase[14,15].

Uridine kinase has attracted interest because of its pharmacological importance in experimental cancer chemotherapy with pyrimidine analogues that are converted into biologically active 5'-phosphates[16,17]. During the development of resistance towards 5-azacytidine in AKR mouse lymphatic leukemia uridine kinase activity in leukemic liver is partially deleted and this impairment has been considered as the metabolic deviation underlying the change to resistance[18]. It was therefore of interest to follow the reaction of normal lymphatic tissue, the rat thymus, towards 5-azacytidine administered in a single dose.

Table I shows the depression of uridine kinase in rat thymus following 5-azacytidine which does not occur in adrenalectomized animals indicating that the effect of the

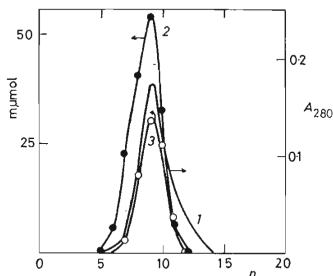

FIG. 2

Sephadex G-200 Column Chromatography of Uridine Kinase from Normal (*2*) and 5-Azacytidine-Treated (*3*) Rat Thymus

The optical density was recorded at 280 nm (*1*) and individual fractions (*n*) were assayed for uridine kinase activity, which was expressed as m μmol of 6-azauridine 5'-phosphate formed per fraction in a 10-min incubation period.

drug is dependent on adrenal secretion. Consequently the specificity of its effect on the thymus uridine kinase would require further clarification. At any rate, it is known that 5-azacytidine is especially active in experimental neoplasms of lymphatic origin[19-21] which is in agreement with the finding that it considerably affects the synthesis of DNA and RNA in the thymus (Table II).

It has been demonstrated by Sköld[22] that uridine kinase from Ehrlich ascites tumour made resistant to 5-fluorouracil is precipitated by ammonium sulfate under conditions different from those of its counterpart from the parental cell line. A similar phenomenon has been described in uridine kinase isolated from rat embryonic liver which was precipitated at a higher concentration of ammonium sulfate than the corresponding enzyme from the adult animal[4]. However, thymic uridine kinase from both control and 5-azacytidine-treated animals was salted out at the same ammonium sulfate concentrations with the implication that the stability of uridine kinase from both sources was the same. In agreement with this finding is the observation (Table III) that following streptomycin and ammonium sulfate treatment no difference has been found between the degree of purification of the thymus uridine kinase isolated from 5-azacytidine-treated and control rats. Equally, the chromatography on a Sephadex G-200 column did not reveal differences in molecular weight of both enzyme preparations; also the profile of protein elution was identical (Fig. 2).

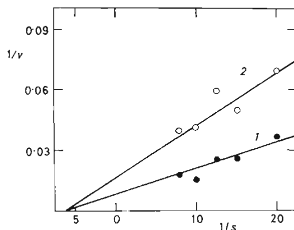

FIG. 3

Lineweaver–Burk Test for Partially Purified Uridine Kinase Isolated from Normal (*1*) and 5-Azacytidine-Treated (*2*) Rat Thymus

Uridine kinase activity was assayed in a reaction mixture (0·3 ml) containing adenosine 5'-triphosphate 40times in excess of 6-azauridine-[4,5-^{14}C], 50 mM-Tris-HCl buffer, pH 7·5, 2 mM-$MgCl_2$ and 20 μg of enzyme protein obtained from the fractions with highest specific activities following Sephadex G-200 chromatography. Incubation was carried out at 37°C for 10 min. s, Concentration of 6-azauridine in mM; *v*, uridine kinase activity expressed in m μmol of 6-azauridine 5'-phosphate formed in a 10-min incubation period per mg of protein.

The Lineweaver–Burk plot with respect to 6-azauridine (Fig. 3) indicate unchanged Michaelis K_m constants; however, the apparent maximum velocity V_{max} is decreased some 50% in case of uridine kinase from the thymus of 5-azacytidine-treated rats. Since also cytidine 5'-triphosphate feedback inhibition has been found unchanged following 5-azacytidine administration the situation reminds the modifications of liver uridine kinase observed in lymphatic leukemia of AKR mice made resistant towards this drug[8]. In general it is thought that mutation and selection constitute the mechanism leading to resistance that develops more or less in a stepwise fashion, and is maintained in the absence of the drug. The mouse leukemic liver during the terminal stage of the disease represents essentially the lymphatic tissue since about 99% of the cells present are leukemic lymphoblasts. The partial deletion of uridine kinase occuring in these cells takes place during the course of 3–4 transplant generations in the presence of 5-azacytidine; mutants are thought to be selected under the pressure of the drug regardless whether they have existed independently or whether they have arisen under its effect[23].

The depression of uridine kinase in rat thymus following the administration of 5-azacytidine is observed after a single injection. It is likely that the impairment of the enzyme activity is accounted for at least partially by the inhibition of its synthesis. Study of the possible relation between the depression of uridine kinase in normal thymus following the administration of a single dose of 5-azacytidine and the partial deletion of the enzyme in mouse leukemic lymphoblasts during successive treatment with this antimetabolite associated with the development of resistance towards 5-azacytidine would undoubtedly be rewarding.

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