Differences in deoxycytidine metabolism in mouse and rat

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Bone-marrow macrophages from both rat and mouse release deoxycytidine derived from phagocytosed nuclei. Mouse plasma contains no detectable deoxycytidine (less than $0.1\mu\text{M}$), whereas the concentration in rat plasma is $18\mu\text{M}$. Enzyme assays of tissue extracts show that both mouse and rat spleen contain high deoxycytidine kinase activity. Mouse organs, including kidney, liver and lung, also have deoxycytidine deaminase activity. In contrast, rat tissues have virtually no deoxycytidine deaminase activity. Lack of deaminase provides an explanation for the presence of deoxycytidine in rat plasma. Cytotoxicity assays show that cultured mouse lymphoid cells grown in undialysed rat serum are more resistant to cytotoxic effects of deoxyadenosine than are those cells grown in dialysed rat serum. The results suggest that a major difference in deoxycytidine metabolism between mouse and rat may account for discrepancies in the pharmacological response of the two animals to certain nucleoside compounds.

The bone marrow is a major site of nucleic acid turnover in adult mammals. At the final stage of erythrocyte differentiation, nuclei are expelled from the erythroblasts and phagocytosed by surrounding macrophages (Skutelsky & Danon, 1970). In mouse peritoneal macrophages, the products of DNA digestion are uric acid, thymidine and deoxycytidine (Chan, 1979; Chan & Lakhchaura, 1982). Furthermore, when adenosine deaminase in the cells is inhibited by deoxycytidine, deoxyadenosine production increases, while uric acid production decreases (Chan, 1979). The products thus may be determined by the set of purine-salvage enzymes present in the macrophages.

The fate of purines and pyrimidines after their release is not entirely clear. Probably thymidine is taken by proliferating cells in bone marrow and then phosphorylated and reutilized. As a result, peripheral blood of mammals contains no thymidine (Hartwick *et al.*, 1978). An analogous situation may occur in patients with inherited deficiency of adenosine deaminase. The bone-marrow macrophages of the patients release deoxyadenosine. These cells are shown to contain high concentrations of deoxyadenosine triphosphate (Donofrio *et al.*, 1978).

Regarding the metabolic fate of deoxycytidine, we speculated that it too was reutilized by proliferating cells in bone marrow. Consistent with this prediction, we found that mouse plasma contained very little deoxycytidine. Surprisingly, the concentration

of deoxycytidine in rat plasma was $18\mu\text{M}$. We present data in this paper that demonstrate the basis for a high plasma concentration of deoxycytidine in rat is the almost total absence of deoxycytidine deaminase activity in its tissues. This study also shows that the difference in deoxycytidine metabolism could account for discrepancies in the pharmacological responses of the two animals to some nucleoside compounds and their analogues.

Materials and methods

Chemicals

Tissue-culture supplies were purchased from Gibco (Long Island, NY, U.S.A.). [$5\text{-}^3\text{H}$]Deoxycytidine (27 Ci/mmol) was obtained from ICN, Irvine, CA, U.S.A. Rabbit anti-(chicken erythrocyte) serum was a product of Cappel, Cochranville, PA, U.S.A. Fertilized chicken eggs were supplied by Texas A & M University, College Station, TX, U.S.A. Tetrahydrouridine and deoxycytidine were gifts from Dr. J. Dauros of the National Cancer Institute, Bethesda, MD, U.S.A. Purines and pyrimidines were purchased from Sigma, St. Louis, MO, U.S.A. All other chemicals were of analytical grade.

Culture of adherent bone-marrow cells

Bone-marrow cells were flushed from the femora of C57/BL mice and cultured by the method of Klimetzek & Remold (1980). The cells were grown in Dulbecco-modified Eagle's medium containing

10% foetal bovine serum and 5% horse serum, supplemented with 1% non-essential amino acids, 2% essential amino acids, 200 mM L-glutamine and 10% L-cell-conditioned medium. The cell density was 10^7 per 60 mm Petri dish. The cultures were maintained in air/CO₂ (9:1) at 37°C for 7–10 days. A uniform monolayer containing over 90% macrophages was routinely obtained at this stage.

L-cell-conditioned medium was collected from 5-day-old cultures of mouse L cells grown in Dulbecco's medium containing 10% foetal bovine serum. The conditioned medium was centrifuged at 830 g for 10 min, filtered with 0.22 µm Nalgene filters, and stored at -20°C.

Phagocytosis experiments

Chicken erythrocytes (17–19 days old) were coated with rabbit anti-(chicken erythrocyte) serum (Chan, 1979), and then added to bone-marrow macrophage cultures at 5–10 cells per macrophage. The mixtures were then cultured in Phenol-Red-free Eagle's minimum essential medium (Flow Laboratories, Rockville, MD, U.S.A.) containing 15% dialysed foetal bovine serum. The medium was harvested after 2 days incubation.

Plasma collection

Animal blood was collected in heparin (10 units/ml) and tetrahydrouridine (10 µM), an inhibitor of deoxycytidine deaminase activity (Neil *et al.*, 1970). After removal of blood cells, plasma was stored at -20°C. The sources of animals were: C57/BL mouse (Texas A & M strain) and Sprague-Dawley rat from Timco, Houston; New Zealand rabbit and guinea pig from Rich-Glo, El Campo, TX, U.S.A.

High-pressure liquid chromatography

Reversed-phase chromatography was performed as described by Hartwick & Brown (1976), with a Spectra-Physics model 8700 instrument. The eluate was monitored by its A_{254} . Absorbance peaks were quantified with a Spectra-Physics computing integrator model 4100. The ion-pair reversed-phase chromatography techniques of Chan & Lakhchaura (1982) were followed.

Cell extract preparations

Monolayers of macrophages were washed twice with phosphate-buffered saline (Dulbecco & Vogt, 1954). Cells were scraped off the plates, transferred to a centrifuge tube, and disrupted by sonication (Branson sonifier 185; 10 s at energy level 2). Mouse or rat organs were cut into 2–3 mm pieces. They were homogenized by Dounce homogenization and sonication as above. The sonicated cells or tissue homogenates were centrifuged at 20000 g for 15 min. The supernatant fluids were dialysed

against phosphate-buffered saline containing 0.1 mM dithiothreitol. They were divided into batches and stored at -70°C until used.

Enzyme assays

The assay methods for deoxycytidine kinase (EC 2.7.1.74) and deoxycytidine deaminase (EC 3.5.4.5) are described by Chan *et al.* (1975). Tetrahydrouridine (Neil *et al.*, 1970) (0.4 mM) was included in the reaction mixture.

Cytotoxicity assays

The method described by Chan (1978) was followed for determining the inhibition of growth of S49 lymphoma cells in the presence of cytotoxic nucleosides.

Results

Production and release of deoxycytidine by bone-marrow macrophages

The HClO₄ extracts of media harvested from mouse bone-marrow macrophage cultures were analysed by ion-pair reversed-phase chromatography. The results agreed with those reported for mouse peritoneal macrophage cultures (Chan & Lakhchaura, 1982). Three forms of pyrimidine were detected: uracil, thymidine and deoxycytidine. In addition to the markers previously tested, deoxycytidine also did not co-chromatograph with any of the following: cytosine, pseudouridine, 5-methylcytidine, 5-methyldeoxycytidine and 5-methylcytosine. We thus are confident that our identification of deoxycytidine is correct.

Similar studies were carried out with rat bone-marrow macrophages. These cells also released deoxycytidine as well as thymidine.

Concentration of deoxycytidine in rat plasma

To gain insight into the metabolic fate of deoxycytidine, we investigated the concentration of deoxycytidine in mouse and rat plasma. The mouse plasma contained no detectable deoxycytidine. In contrast, there was a substantial amount of deoxycytidine in rat plasma (Fig. 1). Its concentration was determined to be 18.7 µM. No thymidine was detectable in mouse or rat plasma. The results suggest a major difference in deoxycytidine metabolism between mouse and rat.

Table 1 shows the plasma concentrations of deoxycytidine in a number of mammals. A wide variation is evident, ranging from its total absence in some animals (mouse, man, horse and calf) to micromolar concentrations in others. In comparison, none of these animal plasmas contained detectable thymidine (i.e. below 0.1 µM).

Fig. 1. Identification of deoxycytidine (dC) in rat plasma. The HClO_4 extracts ($20\mu\text{l}$) of rat plasma were assayed by ion-pair reversed-phase chromatography. The abscissa indicates retention time. The eluted compounds were monitored by the A_{254} , shown on the ordinate. The full scale is 0.01 absorbance unit.

Deoxycytidine in tissue-culture media

We also studied the release of deoxycytidine by cultured mutant cell lines. The findings should aid in determining the enzymic basis for release of this compound. The mouse fibroblast line 3T6 did not release any deoxycytidine (Table 2), and this is consistent with data from our plasma studies.

The 3T6 cells contain both deoxycytidine kinase and deoxycytidine deaminase (Chan *et al.*, 1975). A mutant mouse line, CA88, lacking deoxycytidine kinase (Chan *et al.*, 1974, 1975), released some deoxycytidine. Another mutant line, BC11E, lacking both deoxycytidine kinase and deoxycytidine deaminase (Chan *et al.*, 1975), released substantially more deoxycytidine than did CA88 cells. Conditioned media of Walker's rat carcinoma (WRC) cell culture from Fischer rat contained a large quantity of deoxycytidine. Enzyme assays of cell extracts showed that the rat cells contain

Table 1. Plasma concentrations of deoxycytidine in mammals

The concentrations of deoxycytidine in plasmas were determined by ion-pair reversed-phase chromatography as described in the Materials and methods section. The areas under peaks were integrated with a Spectra-Physics computing integrator SP4100, and converted into concentrations by comparing with the standard. Values are means of three measurements.

Species	Deoxycytidine concn. (μM)
Rat	18.7
Dog	4.3
Rabbit	2.2
Guinea pig	0.6
Cat	Trace
Mouse, man, horse, calf	0

Table 2. Concentrations of deoxycytidine in media harvested from cell cultures

Media were collected from near-confluent cultures after an incubation period of 2 days. Deoxycytidine contents were determined as described in Table 1. Means of three experiments are shown. Abbreviations: dCK, deoxycytidine kinase; dCD, deoxycytidine deaminase.

Cell line	Species (phenotype)	Deoxycytidine concn. (μM)
3T6	Mouse (fibroblast)	0
CA88	Mouse (fibroblast) (dCK ⁻)	6.9
BC11E	Mouse (fibroblast) (dCK ⁻ dCD ⁻)	20.3
WRC	Rat (epithelial) (dCD ⁻)	8.3

virtually no deoxycytidine deaminase, but their deoxycytidine kinase specific activity is 27% of that in 3T6 cells. These results suggest that a deficiency in either deoxycytidine deaminase and/or deoxycytidine kinase would lead to the release of deoxycytidine by cultured cells into culture media.

Deoxycytidine kinase and deoxycytidine deaminase activities in mouse and rat tissues

The most important enzymes for deoxycytidine metabolism in mammals are its kinase and deaminase. The third enzyme, deoxycytidine phosphorylase, was not found in most organisms, including mammals (Henderson & Paterson, 1973). The activities of deoxycytidine kinase and deoxycytidine deaminase in mouse and rat tissues are shown in Table 3. The mouse tissues contained both enzyme activities. Mouse spleen had the highest deoxycytidine kinase activity, with liver and kidney having lesser activities. Mouse kidney contained the highest deoxycytidine deaminase activity, although

Table 3. *Deoxycytidine kinase and deoxycytidine deaminase activities in mouse and rat organs*

Mouse or rat tissues were homogenized by Dounce homogenization and sonication. The enzyme activities in the 20000g supernatants were assayed. Protein concentrations in extracts were determined by the Bio-Rad method. Abbreviation: N.A., not assayed.

Species	Organ	Specific enzyme activity (pmol/min per mg)	
		Deoxycytidine kinase	Deoxycytidine deaminase
Mouse	Spleen	0.28	N.A.
	Liver	0.069	0.012
	Kidney	0.050	0.132
Rat	Spleen	0.091	N.A.
	Liver	0.032	0
	Kidney	N.A.	<0.001

Fig. 2. *Cytotoxic effects of deoxyadenosine on S49 mouse lymphoma cells*

Lymphoma cells were seeded in wells of multi-titre plates, at a density of 4×10^4 cells/well in 0.28 ml culture medium containing 10% horse serum. Deoxyadenosine was added into each well at increasing concentrations, as indicated on the abscissa. Deoxycytidine ($2 \mu\text{M}$) or undialysed or dialysed rat serum (final concn. 10%) was added in some wells. All wells contained $1 \mu\text{g}$ of deoxycytidine/ml. After 3 days of growth, the cells in each well were counted and numbers were expressed as percentages of control, to which no deoxyadenosine was added. $\square$, Deoxyadenosine; $\bullet$, deoxyadenosine plus $2 \mu\text{M}$ -deoxycytidine; $\circ$, deoxyadenosine plus 10% undialysed rat serum; $\blacksquare$, deoxyadenosine plus 10% dialysed rat serum. Results are means $\pm$ S.E.M. ($n = 8$).

the other organs also had this enzyme. Significantly, the rat tissues were almost totally devoid of deoxycytidine deaminase activity. The specific ac-

tivity of deoxycytidine kinase in rat tissue was approximately one-third of that in corresponding mouse tissues. The basis for the presence of deoxycytidine in rat plasma, therefore, is the virtual absence of deoxycytidine deaminase in rat tissues.

Effects of rat serum on cytotoxicity of deoxyadenosine

On the basis of the plasma differences in deoxycytidine content of animals, we predict that the pharmacological responses of rat to deoxycytidine analogues, such as cytosine arabinoside, will be different from the responses in man or mouse. Also, cytotoxic agents that exert their effects via a mechanism of deoxycytidine depletion may be less toxic to the rat than to the mouse. We tested the latter possibility by assaying the toxicity of deoxyadenosine in the presence of rat serum, because deoxyadenosine toxicity is partially preventable by deoxycytidine. We grew S49 mouse lymphoma cells and assayed them for the lethal effects of deoxyadenosine in horse serum, dialysed rat serum or undialysed rat serum (Fig. 2). Deoxyadenosine was highly toxic to S49 cells; its ID_{50} (the concentration at which growth is inhibited by 50%) in horse serum was $4 \mu\text{M}$. Deoxycytidine increased the ID_{50} to $11 \mu\text{M}$, indicating partial antagonism. Undialysed rat serum had a similar antagonistic effect, whereas dialysed rat serum did not. We confirmed by chromatography that deoxycytidine was present in undialysed, but not in dialysed, rat sera (results not shown). We conclude that a diffusible substance in rat serum, most likely deoxycytidine, can partially prevent lethal effects of deoxyadenosine.

Discussion

Data presented in this paper support the finding by Schneider (1955) that deoxycytidine is present in rat blood. We show the biochemical basis for this to be the absence of deoxycytidine deaminase from rat tissues. Rat tissues contain deoxycytidine kinase, which converts deoxycytidine into dCMP. The kinase activity in bone-marrow cells may not be sufficient to phosphorylate all of the deoxycytidine that is generated in the bone marrow. Some deoxycytidine is spilled into blood as a result. The peripheral organs are expected to take up and utilize deoxycytidine. At steady state, when influx of deoxycytidine into blood equals its efflux, the plasma concentration of deoxycytidine is $18 \mu\text{M}$.

The physiological role of deoxycytidine deaminase is not clear. The enzyme may be needed for eliminating deoxycytidine or for generating deoxyuridine. Three of the four nucleosides that can be generated from DNA by enzymic degradation are deoxyadenosine, deoxyguanosine and thymidine, which are toxic to mammalian cells (Klenow, 1962;

Meuth & Green, 1974). Deoxycytidine, however, is not toxic even at concentrations as high as 0.1mM. The cells do not have to eliminate deoxycytidine, and deoxyuridine can be generated by way of alternative pathways. Deoxycytidine deaminase activity therefore should not be an essential function for survival of an animal. Indeed, the rat thrives without this enzyme. In contrast, a deficiency in adenosine deaminase, which has a role in detoxifying deoxyadenosine, would be lethal. Clinical evidence shows that patients lacking adenosine deaminase suffer from a severe combined immunodeficiency disease (Giblett *et al.*, 1972).

Animals whose plasma contains high deoxycytidine concentrations might respond differently to certain nucleosides or nucleoside analogues than do those with no deoxycytidine in plasma. An example is partial resistance to deoxyadenosine killing exhibited by cells bathed in rat serum (Fig. 2). We propose that experiments involving whole rats should be subjected to the same interpretation and that the rat model is not suitable for studying human diseases related to deoxycytidine metabolism.

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