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Pyrimidine Pathways Enzymes in Human Tumors of Brain and Associated Tissues: Potentialities for the Therapeutic Use of *N*-(Phosphonacetyl-L-aspartate and 1-β-D-arabinofuranosylcytosine

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Abstract—*The activities of aspartate transcarbamylase* (de novo *pyrimidine biosynthesis pathway*) *and of deoxycytidine kinase as well as deoxycytidine deaminase* (*salvage pyrimidine biosynthesis pathway*) *were determined in extracts prepared from* 40 *brain tumors of different types in comparison with extracts from normal nervous tissues. Aspartate transcarbamylase, which is undetectable in normal brain tissue, is present in all tumor samples and in some cases rises to very high activities. Deoxycytidine kinase activity is present in all tissues but its level is generally higher in tumors. Deoxycytidine deaminase is present in all the tissues which were analyzed, although its activity is lower in some of the tumor samples.* 1-β-D-*Arabinofuranosylcytosine is a substrate for both deoxycytidine kinase and deaminase in all the samples used except one. These results suggest some potential for the utilization of* 1-β-D-*arabinofuranosylcytosine and* N-(*phosphonacetyl*)-L-*aspartate in the treatment of brain tumors.*

INTRODUCTION

THE ENZYMES of the pyrimidine pathways play an important role in cell metabolism and proliferation by providing the precursors for the biosynthesis of nucleic acids. There are several lines of evidence which indicate that the intracellular pools of nucleotides interfere with the regulation of DNA replication and consequently with cell division [1, 2]. Two metabolic pathways contribute to the biosynthesis of the pyrimidine triphosphates: the *de novo* pathway which ensures the complete synthesis of the pyrimidine nucleoside, and the salvage pathway which allows the re-utilization of preformed bases or nucleosides provided by either the growth medium or the degradation of nucleic acids [1, 2]. Therefore, it is not surprising that some of the more efficient and recently discovered antitumor agents are either pyrimidine analogs or have some of the pyrimidine pathways enzymes as targets [3, 4]. Although these two pathways were demonstrated to exist in the central nervous system, very little information is available concerning the particular enzymes. However, there are indications that the *de novo* pathway does not participate significantly in the production of pyrimidine nucleotides in the mature adult brain [5, 6].

Previous reports suggest that several antitumor agents acting at the level of the pyrimidine pathways might be of interest in the treatment of some brain tumors. This is the case for *N*-(phosphonacetyl)-L-aspartate (PALA) and of 1-β-D-arabinofuranosylcytosine (Ara-C).

PALA is a bisubstrate analog of aspartate transcarbamylase (ATCase), the seond enzyme of the *de novo* pathway, and consequently is a potent inhibitor of the activity of this enzyme [7]. It has been used in clinical investigations [8–10]. It appears that both human and rodent tumors differ greatly in their responsiveness to PALA and this phenomenon is not due to any intrinsic property of ATCase [11, 12]. When PALA is administered by intra-

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venous injection, this compound penetrates efficiently in the cerebrospinal fluid and accumulates into brain tumors, its concentration decreasing as the distance from the center of the tumor inreases [13].

Ara-C is extensively used in the treatment of acute myeloid leukemia [14–16]. Although its mode of action is not entirely elucidated, it is commonly considered that it acts mainly through its incorporation into DNA after phosphorylation into its triphosphate derivative [17] by deoxycytidine kinase. Ara-C could be used successfully in the treatment of brain tumors and meningeal leukemia [18–20], in particular because it can be injected directly into the cerebrospinal fluid [18–21].

Since the efficiency of these two compounds will depend on the intracellular levels and activities of aspartate transcarbamylase and of the deoxycytidine kinase, the activity of these enzymes was examined in 40 samples of human tumors of brain and associated tissues in comparison with samples of normal brain tissue obtained from autopsy. Concerning the phosphorylation of Ara-C, it has been reported that mammalian cells contain two molecular forms of dCyt kinase: one which is cytoplasmic and can use cytidine and Ara-C as well as deoxycytidine as substrates, and another one which is mitochondrial and uses exclusively deoxycytidine. Consequently, the phosphorylation of Ara-C by the kinase present in the extracts coming from the tumoral and normal tissues was also investigated in comparison with the phosphorylation of deoxycytidine. Deoxycytidine deaminase activity interferes with the mode of action of Ara-C by catalyzing its deamination into Ara-uracil which does not have any therapeutic effect. Since the relative utilization of Ara-C by the dCyt kinase and dCyt deaminase will determine the amount of Ara-C which will be available for its incorporation into DNA, the effectiveness of its utilization by these two enzymes were compared in most of the samples studied.

MATERIALS AND METHODS

Chemicals

ATP, carbamylphosphate, phosphoenolpyruvate, pyruvate kinase, deoxycytidine and Ara-C were purchased from Sigma Chemical Co. (St. Louis, MO); L-aspartate and β-mercaptoethanol were from Fluka (Buchs, Switzerland); Coomassie Brillant Blue G250 was from Serva (Heidelberg, F.R.G.); AG 50W-X8 (200–400 mesh) was from Bio-Rad (Richmond, CA); DEAE-cellulose filters (DE 81) were from Whatman (Maidstone, U.K.); Aquasol-2 was from New England (Boston, MA); L-[U-^{14}C]aspartate (200 mCi/mmol) was from CEN (Saclay, France); [5-^{3}H]deoxycytidine (19 Ci/mmol) and [5-^{3}H]Ara-C (11 Ci/mmol) were from Amersham International PLC (Amersham, U.K.). Other chemicals were from Merck (Darmstadt, F.R.G.).

Collection and preparation of samples

Brain tumors obtained at surgery were immediately frozen in liquid nitrogen and kept at −30°C until utilization. Samples of normal brain tissue obtained at autopsy were kept under the same conditions. For the preparation of extracts, the tissue samples were cut into small pieces and homogenized using a Thomas potter (AA) in a volume of buffer approximately equal to the volume of the sample (0.5–1 ml). This extraction buffer contained Tris-acetate 50 mM pH 7.5, β-mercaptoethanol 5 mM and glycerol 10% (v/v). The cellular extracts were then disrupted by sonication three times for 30 s using an Ultra-cell 10 W microsonicator from Sonics and Materials (Danbury, CT). After centrifugation for 5 min at 12,000 ***g***, these extracts were dialyzed twice against 100 times their volume of the same buffer in order to eliminate metabolites and nucleotides which might interfere with the enzymatic assays. All these operations were carried out at 0°C. These samples were used immediately for the enzymatic determinations and were kept at −30°C for further controls.

Enzymatic assays

ATCase. The ATCase activity was determined as previously described [11] using [^{14}C]L-aspartate as substrate. ATCase is a very stable enzyme and it was shown that its activity is not decreased after months of storage under the conditions described above.

Deoxycytidine kinase. The deoxycytidine kinase (dCyt kinase) activity was measured as in Ref. [22] under the following conditions: 50–150 μl samples of extracts (0.5–2.5 mg/ml of protein) were incubated 1 h at 37°C in the presence of Tris–HCl 50 mM pH 8, $MgCl_2$ 10 mM, β-mercaptoethanol 10 mM, [^{3}H]deoxycytidine or [^{3}H]Ara-C 1 mM (0.6 mCi/mM), phosphoenol pyruvate 2 mM, pyruvate kinase 1 unit, ATP 10 mM and NaF 15 mM in a total volume of 200 μl. The reaction was stopped by immersing the test tubes into boiling water for 2 min. After cooling in ice, the samples were centrifuged for 5 min at 12,000 ***g***. Fifty microliters of the supernatants were then deposed on DE 81 Whatman filters (dia. 2.3 cm). After drying, these filters were washed twice for 2 min in water (30 ml per filter) and placed into scintillation vials containing 1 ml of NaCl 0.5 M in HCl 0.1 M. After 30 min swirling, 8 ml of Aquasol-2 were added and the radioactivity was determined using an Intertechnique SL32 scintillation counter.

Deoxycytidine deaminase. The deoxycytidine deaminase (dCyt deaminase) activity was determined as in Ref. [23] under the following conditions: 10–50 μl of extracts (0.1–0.5 mg of protein) were incubated 10 min at 37°C in the presence of Tris-acetate 50 mM pH 7.5 and [^{3}H]deoxycytidine or [^{3}H]Ara-C 1 mM (0.6 mCi/mM) in a total volume of 300 μl. At the end of the reaction, 250 μl samples were taken and pipetted rapidly into 1.6 ml of 0.2 N acetic acid previously placed on top of a 0.7 × 7 cm column of the H^+ form of AG 50W-X8 (200–400 mesh). The eluate was collected directly into a scintillation vial. After rinsing the column three times with 1 ml of 0.2 N acetic acid, 8 ml of Aquasol-2 were added and the radioactivity was determined using an Intertechnique SL32 scintillation counter.

The protein content of the extracts was determined either as in Ref. [24] or Ref. [25] using bovine serum albumin as standard. For each of these enzymatic activity determinations, it was verified that the reaction rate was linear over the incubation time and that it was proportional to the amount of extract used. All the specific enzymatic activities are expressed in nmol/h/mg of protein. The standard deviation was about 15%.

RESULTS

1. *Pyrimine pathways enzymes in normal and tumoral brain and meningeal tissues*

The cell-free extracts from 40 brain tumors of different types and of related tissues were compared for their content of enzymes of the *de novo* pyrimidine pathways (ATCase) and in those of the salvage pathway (dCyt kinase and dCyt deaminase) at the same time as the extracts derived from normal brain samples obtained from autopsy. Among the enzymes of the salvage pathway, dCyt kinase and dCyt deaminase were chosen because of their particular implication in the mode of action of Ara-C. The results obtained are presented in Table 1. These results confirm the absence of detectable ATCase activity in the samples of normal tissues. These samples come from 10 different locations in the brain. In all the tumors except one, one observed the reappearance of ATCase activity, which is especially high in meningiomas and in a glioblastoma obtained from a child; the highest value was obtained from a medulloblastoma from an adult patient. Three meningiomas out of 12 showed low or undetectable ATCase activity but these three samples are of a special nature or location: pterion (sp.act. = 61), falx cerebri (sp.act. = 82), jugum and clenoides (sp.act. ND). In all other tumor types the specific ATCase activity varies widely from one tumor to another. dCyt kinase and dCyt deaminase activities are present in all normal cells confirming that the pyrimidine biosynthesis in adult central nervous system occurs virtually only on the salvage pathway [5, 26]. The dCyt kinase activity increases significantly in meningiomas (6.45 ± 2.6 compared to 2.93 ± 0.97 on average) and its level varies from an individual to another in the other types of tumors. dCyt deaminase activity is not significantly different in normal tissues and meningiomas, although it tends to decrease in most of the other types of tumors.

2. 1-β-D-*Arabinofuranosylcytosine is a substrate for both dCyt kinase and dCyt deminase*

Since there are indications that Ara-C might be successfully used in the treatment of brain tumors and meningeal leukemia [18], it was of interest to determine to which extent this pyrimidine analog can be used as a substrate by the dCyt kinase and the dCyt deaminase present in these types of tissues. Ara-C phosphorylation by dCyt kinase is important for the efficiency of its use since it is likely that its main mode of action is through its incorporation into DNA by DNA polymerase [17].

It appears that in all the extracts coming from all the normal and tumor tissues except one (adenocarcinoma from the orbitory cavity) dCyt kinase is able to use Ara-C as substrate (Table 2). The ratio of the activities using deoxycytidine and Ara-C is constant (2.5 ± 0.6 in normal tissues), all the values except one being within the standard deviation. There is no difference in this ratio between the normal tissues and the different types of tumors.

As far as the ratio of Ara-C deaminase/Ara-C kinase activities are concerned, the results reported in Table 2 show that it is highly variable from one tumor to another (1–70), a result which might be of importance regarding the therapeutic use of this compound.

DISCUSSION

The results of the enzymatic determinations reported here indicate that the enzymatic equipment of the tumors which were examined is related to their histological origin (meningeal, glial or connective tissue). These results also confirm that there is virtually no involvement of the *de novo* pyrimidine pathway in the adult normal brain tissues [5, 6] since ATCase activity is not detectable in the extracts prepared from these samples. In all 40 extracts from different brain tumors except one, this ATCase activity is present and can reach very high values. This phenomenon is particularly remarkable in the case of the meningiomas. Although dCyt kinase activity can be easily measured in the extracts coming from normal brain, its level increases by about a factor of two in the tumors. dCyt deaminase activity tends to decrease in some of the tumors. It

Table 1. AtCase, dCyt kinase and dCyt deaminase activities in brain tumors

	ATCase			dCyt kinase			dCyt deaminase		
Normal tissue	ND	ND	ND	2.6	1.9	2.1	27	21	22
	ND	ND	ND	2.7	1.8	4.0	26	41	31
	ND	ND	ND	3.7	2.2	3.6	33	15	33
	ND			3.9			33		
Meningioma	220	162	150	8.2	4.5	16.8	19	73	22
	265	290	282	2.3	4.4	5.1	31	10	15
	314	205	145	6.2	8.0	7.1	7	22	12
	82*	61*	ND*	3.3	7.8	0.9	21	10	1
Medulloblastoma	20	673*	54	1.8	9.2	3.0	8	6.2	7
	296			3.6			8		
Glioblastoma	113	52	52	2.8	9.5	1.2	15	35	20
	10	86	153	0.4	11.3	2.5	49	12	16
	457*			4.0			10		
Adenocarcinoma	236	146	35	5.4	7.9	—	9	19	28
Angiomyoma	182	185		3.6	1.8		41	13	
Adenoma	80			4.5			12		
Neurinoma	12	102	15	—	3.4	0.8	4	15	38
	22			0.9			9		
Craniopharyngioma	77	21		3.9	1.2		30	9	
Ependymoblastoma	112	64		1.9	2.2		2	61	
Astrocytoma	42			0.4			—		
Glioma	126			4.8			9		

These enzymatic activities were determined in the cell-free extracts of brain tumors or normal tissues as described in Materials and Methods. The specific activities are expressed in nmol/h/mg of protein.
*Indicates particular samples whose nature is discussed in the text.
ND means not detectable. — Means not determined.
Each value corresponds to an individual, and is the result of at least two determinations.

has been reported that in human leukemic cells, kinase activities fall and deaminase activities rise with cell maturity [27]. As in the case of ATCase, the results obtained here show the reversal of this phenomenon in tumors. The reappearance of ATCase in brain tumors is in favor of the use of PALA, a specific inhibitor of this enzyme, for the treatment of these tumors. The fact that it has been shown that intrathecally injected PALA accumulates preferentially in the tumoral brain tissues rather than in the normal one reinforces this conclusion [13].

As far as the therapeutic use of Ara-C is concerned, the results obtained here are also in favor of its utilization. Interestingly these results show that both dCyt kinase and dCyt deaminase present in the nervous central system can use this nucleoside analog as substrate. Although the ratio of these two enzymatic activities varies widely from one tumor to another it is always in favor of the deaminase activity. This observation would suggest that Ara-C could be rapidly deaminated when injected in the cerebrospinal fluid. Some physiological observations indicated, however, that the deamination of this compound is much slower in the cerebrospinal fluid than in the plasma [20]. In accordance with the hypothesis that a high level of Ara-C deaminase would impair the efficiency of Ara-C treatment, it has been reported that the utilization of tetrahydrouridine, an inhibitor of the deaminase, in combination with Ara-C, leads to a significant decrease in the Ara-uracil production [21].

There is evidence that mammalian cells possess two dCyt kinases: one located in the cytoplasm which is able to phosphorylate cytidine and Ara-C as well as dCyt and another located in the mitochondria and which is strictly specific for dCyt [28]. All the samples tested in this work should contain these two enzymes in similar proportions since the ratio of utilization of the two substrates is constant. However, one sample from an adenocarcinoma showed a high level of dCyt kinase activity toward deoxycytidine but none with Ara-C. Unfortunately, the amount of material obtained did not allow one to determine if this pattern was due to the absence of the cytoplasmic enzyme.

Table 2. Utilization of Ara-C by dCyt kinase and dCyt deaminase

Origin	Specific activity (nmol/h/mg of protein) Deaminase activity			Kinase activity			Deaminase/kinase ratio			Mean value of ratio
Normal tissue	12	17	18	1.0	0.6	1.1	12	28	16	
	21	32	11	0.8	0.9	2.2	26	36	5	16.8 + 10.0
	15	13	21	1.5	0.8	2.2	10	16	10	
	14			1.5			9			
Meningioma	19	103	8	2.4	4.8	6.9	8	21	1	
	15	10	17	1.3	1.3	1.3	12	8	13	10.6 + 9.9
	7	19	22	3.0	4.0	3.2	2	5	7	
	14	14	22	1.7	2.9	0.6	8	5	37	
Medulloblastoma	8	11	6	1.3	2.2	1.5	6	5	4	5.7 + 1.7
	16			2.0			8			
Glioblastoma	12	15	13	0.7	2.7	0.4	17	6	32	
	65	—	8	0.5	—	0.8	130*	—	10	14 + 11
	8			1.7			5			
Adenocarcinoma	11	—	—	3.8	ND	—	3	—	—	
Angiomyoma	—	—		—	—		—	—		
Adenoma	13			1.6			8			
Neurinoma	—	9	14	—	1.3	—	—	7	—	
	—			—			—			
Craniopharyngioma	26	14		1.4	0.2		19	70		
Ependymoblastoma	—	18		—	1.0		—	18		
Astrocytoma	—			—			—			
Glioma	8			1.8			4			

These enzymatic activities were determined in the cell-free extracts of brain tumors or normal tissues as described in Materials and Methods. The specific activities are expressed in nmol/h/mg of protein.

*Indicates one particular sample whose nature is discussed in the text.

ND means not detectable. — Means not determined.

Each value corresponds to an individual, and is the result of at least two determinations.

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