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Nucleoside Transport and Cytosine Arabinoside (araC) Metabolism in Human T Lymphoblasts Resistant to araC, Thymidine and 6-Methylmercaptopurine Riboside*

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Abstract—*Alteration in cellular nucleoside transport characteristics, deoxynucleoside triphosphate (dNTP) pools and araCTP formation from cytosine arabinoside (araC) were determined for human T lymphoblasts (CCRF-CEM) resistant to araC, thymidine or 6-methylmercaptopurine riboside (6-MMPR). AraC resistance was accompanied by reduced activities of deoxycytidine and thymidine kinases, lowered cellular deoxycytidine triphosphate concentration and markedly reduced formation of araCTP from araC. The ID_{50} values for araC, araCTP formation and dNTP levels of thymidine-resistant lymphoblasts, which exhibited reduced thymidine kinase activity, and 6-MMPR-resistant cells, with reduced adenosine kinase activity, were similar to the corresponding parameters of wild type lymphoblasts. The nucleoside transport sites density determined by nitrobenzylmercaptopurine riboside binding was comparable for araC-resistant, wild type and thymidine- or 6-MMPR-resistant lymphoblasts.*

INTRODUCTION

CYTOSINE arabinoside (araC) is one of the more effective agents in the treatment of acute non-lymphocytic leukemia [1, 2] and has been advocated for the control of aggressive lymphomas such as T lymphoblastic lymphoma [3, 4]. A critical event for cell death is araC phosphorylation [5] and a prerequisite for this is cellular influx of araC via specific nucleoside receptors prior to its phosphorylation to araCTP [6, 7]. A continuing clinical problem with araC therapy is refractory disease. Mechanisms for this resistance include reduced levels of deoxycytidine kinase [8], the activity required for initial araC phosphorylation [5] or increased araC degradation by augmented cytidine deaminase activity [9]. Another potential limiting factor for araC effectiveness may be the rate of its membrane transport and the number of cell nucleoside transport sites measured by equilibrium binding of [^{3}H]nitrobenzylmercaptopurine riboside (NBMPR) have been shown to correlate closely with araC influx and, indeed, clinical responsiveness to araC therapy [6, 7].

To determine alteration in araC metabolism, including araC influx associated with acquired araC resistance, mutant human T lymphoblasts have been derived which are highly resistant to either the cytotoxic effect of araC or the other nucleosides, thymidine and 6-methylmercapto-

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Abbreviations: araC: cytosine arabinoside; 1-β-D: arabinofuranosylcytosine; dNTP: deoxynucleoside triphosphate; araCTP: cytosine arabinoside; dATP: deoxyadenosine, dCTP: deoxycytidine, dGTP: deoxyguanosine, dTTP: thymidine triphosphate; ID_{50} concentration inhibiting cell growth by 50%; NBMPR: nitrobenzylmercaptopurine riboside; 6-MMPR: 6-methylmercaptopurine riboside.

purine riboside. The present study has examined in these mutant cells araC cytotoxicity, cellular araCTP accumulation and changes in nucleoside transport parameters.

MATERIALS AND METHODS

Materials

Non-radioactive nucleosides, nucleotides, poly(d[A-T]), poly(d[I-C]), *Micrococcus lysodeikticus* (*M. luteus*), DNA polymerase and all other chemicals were obtained from Sigma (St. Louis, MO). Radioactive compounds were from the Radiochemical Centre (Amersham, U.K.). PEI cellulose plates were supplied by Merck (Dramstadt, F.R.G.). Di-*n*-butylphthalate (British Drug Houses, Cole, U.K.) and di-*n*-octylphthalate (Ajax Chemical Limited, Australia) were blended 4:1 (v/v), yielding an oil mixutre of density 1.032. NBMPR was synthesized as described by Paul *et al.* [10] and stirred vigorously with imidazole-buffered saline to yield a saturated solution of approximately 20 μM. [G-^{3}H]NBMPR was from Morevek Biochemicals (Brea, CA).

Cell lines and culture

The parent cell line referred to as wild type CEM is the CCRF-CEM human T cell line [11]. Mutant cell lines were selected in mass culture from non-mutagenized cells for resistance to araC 71 μM (RCH-74b^9); 6-methylmercaptopurine riboside (6MMPR) 20 μM (RCH-81b^6); and thymidine 500 μM (RCH-1020). Cells were maintained in RPMI 1640 medium with 10% heat-inactivated horse serum. For cell growth inhibitory studies cultures were initiated in duplicate at 4-5 × 10^5 cells/ml in RPMI 1640 medium containing 10% heat-inactivated horse serum and varying concentrations of araC. Cell counts were performed at 72 hr with a hemocytometer using tryptan blue exclusion as an index of viability. ID_{50} values were defined as the concentration of araC to inhibit growth by 50%.

Enzyme assays

Lymphoblast extracts were prepared as described [12] and used to assay for adenosine kinase activity with [^{14}C]adenosine 5 μM [13], thymidine kinase with [^{14}C]thymidine 25 μM [14] and deoxycytidine kinase with [^{3}H]deoxycytidine 50 μM [12] as the respective radiolabelled substrates.

AraC phosphorylation and deamination by intact cells

Phosphorylation of araC to araCTP by intact cells was assayed by the method of Harris *et al.* [15]. Briefly, cells suspended in 0.1 ml of RPMI 1640 (1 × 10^6/ml) were incubated with 1 and 50 μM of [^{3}H-araC]. At fixed intervals cells were pelleted by centrifugation and suspended in 5% ice-cold trichloroacetic acid (25 μl) and 5 μl of araCTP (2 μg/ml), freeze-thawed in liquid nitrogen, neutralized with 1.5 M Tris-acetate buffer, pH 8.5, and clarified by centrifugation. Aliquots (10 μl) were spotted on PEI cellulose plates together with unlabelled araC and araCTP markers. The plates were developed with ascending chromatography, with 0.5 M ammonium sulphate as solvent. AraCTP was identified under ultraviolet light and radioactivity determined. To express cellular araCTP as a function of intracellular water content with each araCTP formation experiment, the intracellular water content was determined using tritiated water and [^{14}C]polyethylene glycol [16]. Results are expressed as μmol of araCTP formed/l of intracellular water.

The rate of araC deamination was measured by quantitation of formation of ara-uridine by intact cells as described previously [15].

Cellular deoxynucleoside triphosphate levels

Deoxynucleotides were extracted from cell pellets containing 5 × 10^6 lymphoblasts with 1 ml of cold 60% aqueous methanol. Samples were stored at -20°C overnight, centrifuged and the supernatants evaporated to dryness. Residues were suspended in 0.25 ml of distilled water and assayed for dATP and TTP, using poly(d[A-T]) as primer, and dCTP and dGTP, using poly(d[I-C]) as primer [17, 18].

Measurement of [^{3}H]*NBMPR binding*

[^{3}H]NMBPR binding to cells was determined over a range of ligand concentrations (0.2-8 nM) as previously described [6]. Total binding was corrected for non-specific uptake of [^{3}H]NBMPR, which was measured in parallel incubations of cell suspensions, containing both labelled and unlabelled NBMPR (3 μM). Another correlation was made for [^{3}]NBMPR trapped in the extracellular space of the cell pellet which was measured in each experiment as the [^{14}C]polyethylene glycol space and which was always <5% of the total pellet counts.

The specific binding of [^{3}H]NBMPR was analyzed by Scatchard plots and the maximum number of binding sites per cell was obtained by extrapolation of the regression line to the abscissa. The dissociation constant (K_d) was the reciprocal of the association constant derived from the slope of the regression line.

Statistical analysis

The mean values ± standard error of the mean of respective data are given unless otherwise stated. The significance of differences between means was analyzed by Student's *t* test. Regression lines were fitted by the method of least squares.

RESULTS

The adenosine, deoxycytidine and thymidine kinase activities of wild type CEM cells and the respective mutant cell lines are shown in Table 1. The deoxycytidine and thymidine kinase levels of araC-resistant cells (RCH-72B[9]) were respectively less than 10 and 5% of the mean activity of wild type cells, while the level of adenosine kinase was not significantly different to that exhibited by control cells. The levels of either thymidine or adenosine kinase were significantly reduced in the thymidine (RCH-1020)- or 6-MMPR (RCH-81b[6])-resistant cells and in respective mutant cells these reduced activities were not accompanied by significant changes in the levels of the other two kinases (Table 1). The araC-resistant cell lines were also resistant to the cytotoxic effect of thymidine (500 μM).

Determination of cellular deoxynucleoside triphosphate (dNTP) pools of wild type lymphoblasts and cells resistant to araC, thymidine or 6-MMPR showed that the dCTP concentration of araC-resistant cells was significantly reduced compared to dCTP levels of the wild type, or thymidine- or 6-MMPR-resistant cells. The dNTP levels for the latter resistant cells were not significantly altered from values exhibited by wild type lymphoblasts (Table 2).

Determination of the ID_{50} concentration for araC cytotoxicity together with cellular araCTP formation by wild type and mutant cells showed that cells exhibiting either reduced adenosine or thymidine kinase activities had ID_{50} values for araC of the same order as that of wild type cells (Table 3) and araC cytotoxicity was paralleled by cellular araCTP formation. In the deoxycytidine kinase-deficient cells araCTP accumulation was markedly reduced at both 1 and 50 μM of araC, and this considerably reduced araCTP formation was observable for up to 100 min (Fig. 1). In data not shown the rate of ara-uridine formation from araC by wild type cells was negligible and not augmented in the mutant cell lines.

The [^{3}H]NBMPR binding characteristics of wild type and mutant cells are given in Table 4 and a representative finding for Scatchard analysis in Fig. 2. These data show that resistance to either araC or the nucleosides thymidine or 6-MMPR was not accompanied by significant alteration in maximum NBMPR binding sites or the affinity characteristics for NBMPR binding.

DISCUSSION

The cytotoxicity of araC is mediated through the nucleoside triphosphate, araCTP, which inhibits DNA polymerase [19, 20] and leads to araC incorporation into DNA [21, 22]. The initial metabolism of araC to the monophosphate is via deoxycytidine kinase and resistance to araC *in vitro* in cell lines, or *in vivo* in myeloblasts of leukemic patients refractory to araC therapy, is accompanied by considerably reduced levels of deoxycytidine kinase or rates of araC phosphory-

Table 1. Activities of nucleoside kinases in human T-CEM lymphoblasts and mutant cell lines

Cell line	Resistant to:	Deoxycytidine kinase nmol/hr/mg protein*	Thymidine kinase	Adenosine kinase
CCRF-CEM	wild type	4.0 ± 0.4	15 ± 2	116 ± 14
RCH-72b[9]	araC (71 μM)	0.3 ± 0.1†	0.5 ± 0.1†	144 ± 21
RCH-1020	thymidine (500 μM)	3.7 ± 0.9	1.3 ± 0.3†	68 ± 9
RCH-81b[6]	6-MMPR (20 μM)	4.4 ± 0.5	27 ± 2	2 ± 0.2†

*All values are shown as mean ± S.E. for >5 determinations.
†$P < 0.001$.

Table 2. Deoxynucleoside triphosphate (dNTP) pools of wild type and mutant T lymphoblasts

Cell line	Resistant to:	dNTP (pmol/10[6] cells)* dATP	dGTP	dTTP	dCTP
CCRF-CEM	wild type	27 ± 8	17 ± 11	25 ± 13	11 ± 5
RCH-72b[9]	araC	34 ± 7	12 ± 9	16 ± 5	6 ± 2†
RCH-1020	thymidine	39 ± 13	17 ± 5	34 ± 18	13 ± 6
RCH-81b[6]	6-MMPR	24 ± 13	12 ± 9	15 ± 5	9 ± 3

*Value is mean ± S.D. of >10 separate determinations.
†$P < 0.01$.

Table 3. ID_{50} araC values and araCTP formation of human T-CEM lymphoblasts and mutant cell lines

Cell line	Kinase deficiency	araC ID_{50}* (M)	araCTP formation -† (μmol/l cell water/15 min) 1×10^{-6} M araC	5×10^{-5} M araC
CCRF				
Wild type	—	15×10^{-9}	105 ± 14	217 ± 19
RCH-72b[9]	deoxycytidine and thymidine	1×10^{-4}	0.2 ± 0.3†	0.5 ± 0.2‡
RCH-1020	thymidine	35×10^{-9}	70 ± 8	220 ± 21
RCH-81b[6]	adenosine	20×10^{-9}	91 ± 32	204 ± 29

*Value given is mean of 4 separate determinations.
†Value given is mean ± S.E. of >5 separate determinations.
‡$P < 0.001$.

lation [8, 23-26]. However, reduced deoxycytidine kinase is not a universal finding. Momparler *et al.* [27] failed to find significant differences for araC phosphorylation between araC-resistant and -sensitive murine cells, but did find an expanded cellular deoxycytidine triphosphate (dCTP) pool.

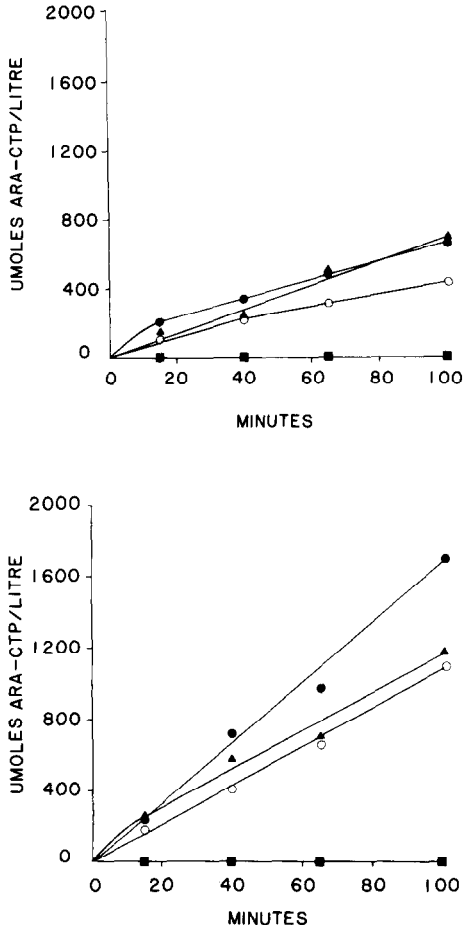

Fig. 1. Time course of intracellular araCTP formation by human wild type and mutant T lymphoblasts at concentrations of araC of 1 μM (A) and 50 μM (B). Cell lines are wild type (▲), araC (■)-, thymidine (○)- and 6-MMPR-resistant (●) human T lymphoblasts.

This may contribute to araC resistance by feedback inhibition of deoxycytidine kinase and competition with araCTP for insertion into DNA.

In the present study araC resistance in human T lymphoblasts is accompanied by reduced cellular dCTP levels, decreased araCTP formation from araC and significantly reduced deoxycytidine and thymidine kinase activities. In araC-resistant cells reduction in thymidine kinase activity appears not to be of central importance for this resistance, as mutant cells resistant to thymidine alone and with comparable thymidine kinase activities exhibit an araC ID_{50} value and rate for araCTP formation indistinguishable from wild type or adenosine kinase-deficient lymphoblasts. Reduced thymidine kinase activity accompanying araC resistance has, to our knowledge, not been previously described. de Saint Vincent and Buttin have reported for mutagenized hamster fibroblasts two patterns for araC resistance: resistance to low araC concentration accompanied by joint thymidine resistance, while with resistance to high araC concentration thymidine sensitivity was retained; for these cell lines changes in thymidine kinase activities were not determined [28]. In our laboratory resistance to low concentration of araC in human T lymphoblasts is accompanied by normal thymidine kinase activity while increased araC concentration resistance is accompanied by reduced thymidine kinase levels [Young and Van der Weyden, unpublished observations]. The potential mechanisms for this phenomenon require elucidation, but may be related to changes in cellular araCTP pools.

AraC is deaminated by cytidine deaminase to the inactive metabolite arauridine. Alteration in this activity has been suggested to play a role in either *de novo* or acquired araC resistance [9] but the clinical relevance of these data has been questioned [15, 29]. In our study there was

Table 4. Nucleoside transport characteristics of human T-CEM lymphoblasts and mutant cell lines

Cell line	Kinase deficiency	Maximium NBMPR binding sites/cell	Affinity of binding at equilibrium (K_d)	Intracellular water $\mu l/10^7$ cells
CCRF-CEM ($n = 5$)*	wild type	48730 ± 21800†	0.28 ± 0.1†	4.40 ± 1.0†
RCH 72b[9] ($n = 2$)	deoxycytidine and thymidine	29,000	0.48	3.72
RCH 1020 ($n = 1$)	thymidine	58,800 49,200	0.30 0.37	2.98 5.68
RCH 84b[6] ($n = 2$)	adenosine	40,000 39,600	0.19 0.32	4.45 4.80

*No. of determinations.
†Values given are mean ± S.D.

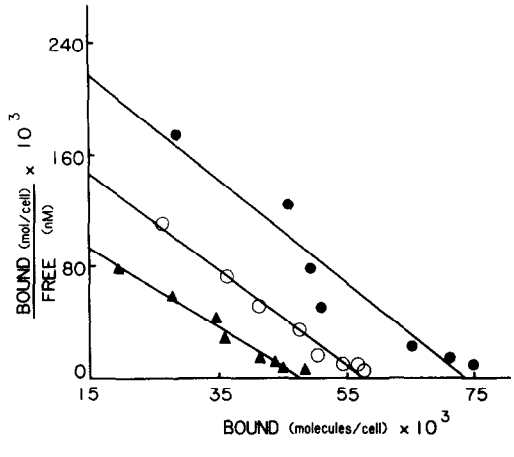

Fig. 2. Scatchard analysis of specific NBMPR binding to human wild type and mutant T lymphoblasts. Regression lines have been fitted by the method of least squares. The intersection the × axis estimates the maximum number of binding sites. Cell lines are wild type (●), thymidine (○)- and araC (▲)-resistant human thymoblasts.

negligible araC deamination by human T lymphoblasts incubated with concentrations of araC attained by standard dose therapy and acquisition of araC resistance was not accompanied by augmentation of araC deamination.

AraC enters leukemic blast cells by a facilitated diffusion pathway and the influx correlates closely with the density of nucleoside transport sites as determined by NBMPR binding [6, 7]. Low transport rates for araC was associated with poor clinical response [6]. Alteration in araC transport is one mechanism for acquired araC resistance but in the present study lymphoblasts resistant to a variety of nucleosides, including araC, thymidine and MMPR, all exhibited no significant changes in nucleoside transport characteristics. Others have isolated murine lymphoma cells with defective transport of either purine or pyrimidine nucleosides [30, 31], which suggests that such a mechanism may be operative in some instances of cellular araC resistance. The frequency of this mechanism for acquired *in vivo* araC resistance remains to be determined.

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