Enhanced Metabolism of 1-β-D-Arabinofuranosylcytosine in Down Syndrome Cells: A Contributing Factor to the Superior Event Free Survival of Down Syndrome Children With Acute Myeloid Leukemia

By Jeffrey W. Taub, Larry H. Matherly, Mark L. Stout, Steven A. Buck, James G. Gurney, and Yaddanapudi Ravindranath

Down syndrome (DS) children with acute myeloid leukemia (AML) have significantly higher event-free survival (EFS) rates compared with non-DS children when treated with protocols containing 1-β-D-arabinofuranosylcytosine (ara-C). Sensitivity and metabolism of ara-C was examined in myeloblasts from DS and non-DS patients with AML, DS infants with the transient myeloproliferative disorder, and Epstein-Barr Virus (EBV) transformed lymphoblastoid cell lines with and without trisomy 21. DS myeloblasts were approximately 10-fold more sensitive to ara-C (measured by the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) colorimetric sensitivity assay), compared with non-DS myeloblasts, following exposure to ara-C for 72 hours. Mean levels of 1-β-D-arabinofuranosylcytosine 5′-triphosphate (ara-CTP) were significantly higher in DS myeloblasts compared with non-DS myeloblasts after incubation with 5 μmol/L ara-C (621.4 *v* 228.4 pmol/mg protein). DS cell lines also generated higher levels of ara-CTP compared with cell lines with diploid chromosome numbers (66.5 *v* 13.6 pmol/mg protein and 137.6 *v* 41.7 pmol/mg protein at 1 and 5 μmol/L ara-C, respectively). Elevated ara-CTP levels in the DS cells were accompanied by slightly lower levels of endogenous deoxycytidine triphosphate (dCTP) pools, slightly greater extent of ara-C incorporation into DNA, and increased relative numbers of double strand DNA strand breaks. There were no significant differences in the cell cycle distributions of DS and non-DS cells. These in vitro studies support our hypothesis that enhanced metabolism of ara-C in DS cells may be a contributing factor to the superior survival rate of DS children with AML and is possibly based on a gene dosage effect of genes localized to chromosome 21 including cystathionine-β-synthase. Further study of the mechanisms (ie, alterations in dCTP pools and DNA methylation) involved may lead to improvements in the treatment of all AML patients.

DOWN SYNDROME (DS) children have an increased disposition to developing both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), the risk ranging from 10 to 20 times greater than that of non-DS children.[1,2] DS children with ALL have comparable response rates to current therapy compared with non-DS children,[3] although they commonly have pronounced toxicity associated with methotrexate.[4] This excessive methotrexate toxicity has been linked to imbalances in folate metabolism in DS cells and may be due to the increased gene dosage effect of purine synthetic enzymes and/or cystathionine-β-synthase now localized to chromosome 21.[4-6] AML in DS children is characterized by a young age of onset (predominantly under 2 years of age), a low white blood cell count, and a high frequency of megakaryocytic leukemia (M_7).[7] An interesting association is the transient myeloproliferative disorder (TMD) observed in newborn DS children that resembles megakaryocytic leukemia and resolves spontaneously, though 20% to 30% of affected babies subsequently develop AML.[8,9]

An analysis of the childhood cancer survival data from the Surveillance, Epidemiology, and End Results (SEER) registry reported that the 5-year relative survival rates for AML have only increased slightly from 20.4% for the 1973 to 1977 time period to 26.4% for the 1983 to 1987 time period.[10] In striking contrast, DS children with AML have recently been reported to have the highest cure rate of any group of AML patients (Table 1).[7,11-15] The Pediatric Oncology Group (POG) reported an event-free survival (EFS) of 100% for 12 DS children with AML treated on the POG 8498 compared with a 28% EFS at 3 years for non-DS children.[7] A larger cohort of DS children treated on the subsequent POG 8821 protocol had an EFS rate of 68% compared with 35% for non-DS children at a mean follow-up of 39 months (Table 1). More recently, the Children's Cancer Group (CCG) and pediatric treatment groups in Scandinavia, Japan, Canada, and Germany have reported an approximate 75% EFS rate and leukemia relapse rates of less than 15% for DS children with follow-up periods ranging from 4 to 89 months[11-17] (Table 1). These results suggest that further intensification of therapy is not necessary for the treatment of DS children with AML and that decreasing the intensity of therapy would reduce treatment-related toxicity but maintain the very high EFS rates.

A common component of all these AML treatment protocols is 1-β-D-arabinofuranosylcytosine (ara-C), the most active agent in AML therapy. We previously proposed that the enhanced sensitivity of DS cells to ara-C may involve the generation of higher levels of the active intracellular metabolite 1-β-D-arabinofuranosylcytosine 5′-triphosphate (ara-CTP) and lower endogenous deoxycytidine triphosphate

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Table 1. Treatment Response of DS Children With AML

	DS					Non-DS		
Protocol	n	M_7 (%)	EFS (%)	Follow-Up	Rx Deaths (%)	n	EFS (%)	Reference
POG 8498	12	42	100	63-96+ mo	0	285	28	7
POG 8821	34	44	68	23-76+ mo	5 (15)	614	35	(unpublished POG data)
CCG-213	39	NA	75*	5 yr	NA	591	31	11, 16
NSPHO	17	35	76	5-89+ mo	3 (18)	70	39	12, 17
Japan	9	100	78	4-68+ mo	1 (11)	NA	NA	13
Toronto	15	100	73	12-82+ mo	NA	NA	NA	14
Germany	14	29	55†	5 yr	(21)	NA	NA	15

Abbreviations: POG, Pediatric Oncology Group; CCG, Children's Cancer Group; NSPHO, Nordic Society of Pediatric Hematology/Oncology; n, sample size; M_7, megakaryocyte phenotype; EFS, event free survival; NA, not available.

* Disease free survival.

† Six of seven patients (86%) treated with 1 to 2 courses of high-dose ara-C remain in continuous complete remission. CCG and NSPHO results include all patients treated on study. Toronto cases include megakaryocytic leukemia and myelodysplastic syndrome.

(dCTP) pools.[7,18] These effects could reflect changes in intracellular folate pools and/or metabolism and the presence of three copies of chromosome 21, as described above (Fig 1). For the present study, we explore these possibilities in myeloblasts from DS and non-DS patients with AML or TMD and Epstein-Barr Virus (EBV) transformed lymphoblastoid cell lines with trisomy 21 and diploid chromosomes.

MATERIALS AND METHODS

Chemical Reagents

DNA Polymerase I and the deoxynucleotide standards were purchased from Promega Corp, Madison, WI. [5-^{3}H]-cytosine-β-D-arabinofuranoside (25 Ci/mmol) and [2,8-^{3}H]-2′-deoxyadenosine 5′-triphosphate (35 Ci/mmol) were obtained from Moravek Biochemicals, Brea, CA. Calf thymus DNA, 1-β-D-arabinofuranosylcytosine, 1-β-D-arabinofuranosyl 5′-monophosphate, 1-β-D-arabinofuranosyl 5′-triphosphate, dipyridamole, tetrabutylammoniumhydrogen sulphate, tetrahydrofuran, and MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium-bromide) were obtained from Sigma Chemical Co, St Louis, MO.

Patient Specimens

Leukemia cells were obtained from newly diagnosed AML patients and DS infants with TMD (Table 2). Heparinized bone marrow or peripheral blood (when blast counts were sufficiently high) was diluted with RPMI 1640 with ITS solution (insulin-5 μg/mL, transferrin-5 μg/mL, selenite-5 ng/mL)[19] and maintained overnight at room temperature. The mononuclear cells were isolated on a Ficoll-Hypaque gradient and washed twice with phosphate-buffered saline (PBS) [8.1 g NaCl, 0.22 g KCl, 1.14 g Na_2HPO_4, 0.27 g KH_2PO_4/L H_2O, pH 7.4] before incubation with ara-C or extraction of deoxynucleotide pools.

Cell Culture

EBV-transformed lymphoblastoid cell lines were obtained from the Coriell Institute for Medical Research, Camden, NJ (GM 1921 and GM 4927 were derived from DS individuals; GM 3715, GM 3798, and GM 5398 were derived from non-DS individuals). One EBV-transformed lymphoblastoid cell line (DET 1577) was established at Children's Hospital of Michigan, Detroit, MI from a 2.5-year old girl with DS (patient TH) who had a history of TMD and megakaryocytic leukemia and was off therapy for 6 months (Table 2). The cells were maintained in RPMI 1640 medium supplemented with 15% fetal calf serum (Hyclone, Logan, UT), penicillin (100 U/mL), streptomycin (100 μg/mL), and ITS solution. For in vitro ara-C incubation experiments, the cell lines were expanded in 15% dialyzed fetal calf serum for at least two generations. The GM 4927 and GM 1921 cell lines have previously been used in experiments examining DNA damage produced in DS cells exposed to ionizing radiation,[20] and the GM 5398 cell line has been used as a diploid control to study the gene dosage effect of carbonyl reductase localized to 21q22.1.[21] Chromosome 21 copy numbers of the lymphoblastoid cell lines were confirmed by fluorescent in situ hybridization (FISH) with whole chromosome painting probes for chromosome 21 on metaphase spreads.[22]

Cell Cycle Determination

Cells (1×10^6) were washed with PBS, fixed in 95% ethanol, incubated for 30 minutes with RNase A (Sigma), and stained with propidium iodide before cell cycle analysis.[23] Cell cycle analysis was performed with the Multicycle software (Phoenix Flow Systems, Inc, San Diego, CA) on a Model 90-2 Coulter EPICS flow cytometer (Coulter, Miami, FL).

MTT Drug Sensitivity Assay

The MTT drug sensitivity assay was based on the method of Pieters et al[19] Briefly, leukemia cells (2×10^4) were suspended in triplicate in 80 μL of RPMI 1640/15% dialyzed fetal calf serum, supplemented with ITS solution and 20% supernatant of the 5637 bladder cell line (as a source of granulocyte-macrophage colony-stimulating factor [GM-CSF])[24,25] in the presence of varying concentrations of ara-C, or no drug (controls). After 72 hours at 37°C, MTT was added to a final concentration of 1 mmol/L. After 6 hours, the colored formazan crystals (resulting from the reduction of MTT by viable cells) were dissolved with the addition of 100 μL acidified isopropranol and the optical densities were read by a microplate reader at 540 nmol/L.

The absorbances were corrected for blank readings. Absorbance (A) at 540 nm directly correlated with cell number; the percentage cell survival was calculated as: A(treated)/A(untreated control) $\times$ 100. Data was plotted as ara-C concentration versus percent cell survival and the IC_{50}'s were determined by the MINSQ (Micromath Inc, Salt Lake City, UT) curve fitting program, corresponding to the ara-C concentration, which decreased cell survival by 50%.

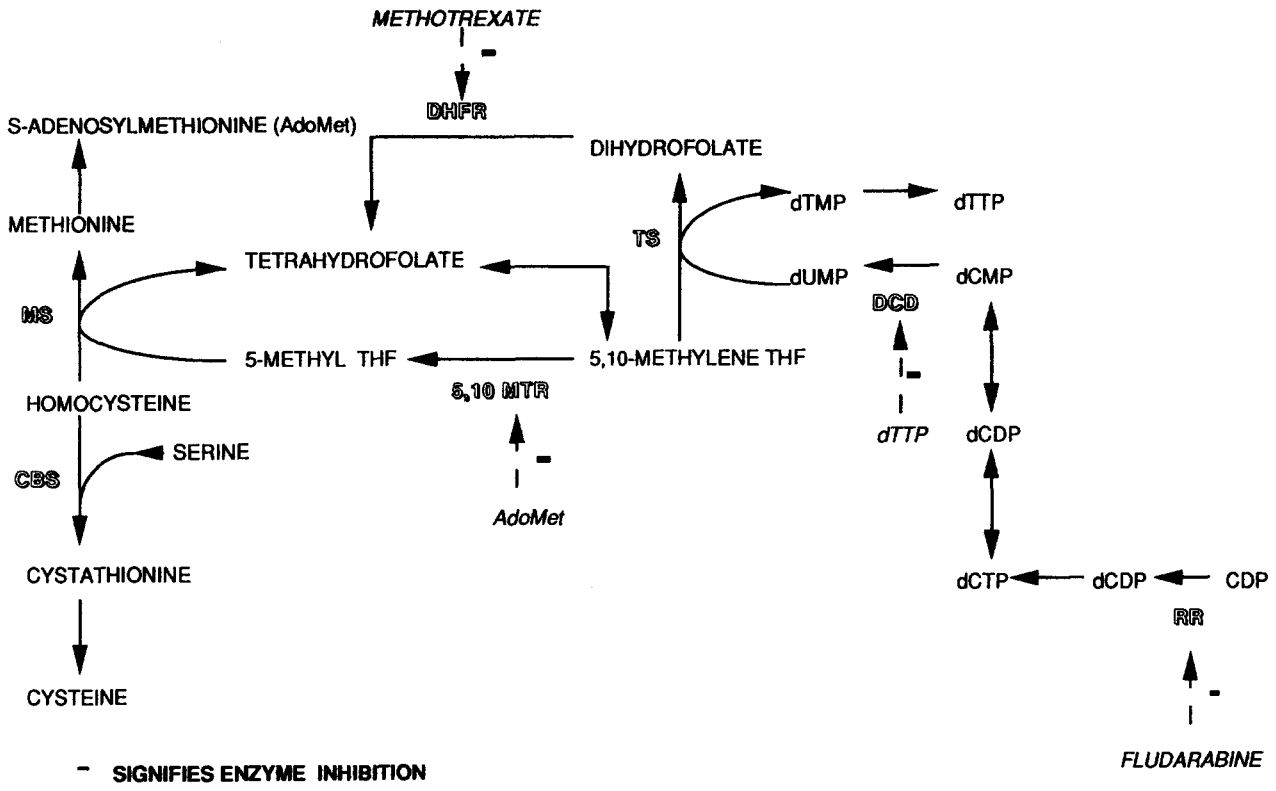

Fig 1. Relationship of deoxynucleotide pools, reduced intracellular folate pools, and homocysteine metabolism. In the DS model, increased cystathionine-β-synthase activity depletes homocysteine pools resulting in (1) decreased S-adenosylmethionine levels and "trapping" of 5-methyl tetrahydrofolate (THF); (2) diversion of 5,10-methylene THF to 5-methyl THF secondary to allosteric enzyme release of 5,10-methylene THF reductase; (3) decreased dTTP pools secondary to 5,10-methylene THF diversion; and (4) decreased dCTP pools secondary to allosteric enzyme release of deoxycytidylate reductase. CBS, cystathionine-β-synthase; MS, methionine synthase; TS: thymidylate synthase; DCD, deoxycytidylate deaminase; 5,10 MTR, 5,10-methylene THF reductase; RR, ribonucleotide reductase; DHFR, dihydrofolate reductase.

Ara-C Incubations and Measurement of ara-CTP

Cells (5×10^6/mL) were suspended in Hank's balanced salt solution[26] and incubated in the presence of 1 μmol/L or 5 μmol/L ^{3}H-ara-C (1250 μCi/μmol and 250 μCi/μmol, respectively) at 37°C for 3 hours in specially-designed flasks stirred with Teflon paddles under an atmosphere of 95% O_2/5% CO_2. The incubations were stopped with the addition of ice-cold PBS (10× volume) containing 20 μmol/L dipyridamole (to inhibit nucleoside transport) and centrifuged (5 minutes, 1,500 rpm) at 4°C.

After two additional washes with PBS (10× volume), the cells were aliquotted and processed as follows: (1) one sample was solubilized with 0.5 N NaOH and assayed for radioactivity and protein content (representing total intracellular accumulation of ara-C and metabolites) and (2) one sample was treated with 0.5 N ice-cold perchloric acid (PCA).[27] After centrifugation (5 minutes, 12,000 rpm), the supernatant (acid-soluble fraction) was neutralized with 5 N KOH and 1 mol/L KH_2PO_4. The PCA insoluble precipitate was solubilized with 0.5 N NaOH and the radioactivity measured (representing ara-C incorporation into macromolecules including DNA).

All samples were assayed for radioactivity, as described below; proteins were assayed according to a modification of the Lowry assay with bovine serum albumin used as the standard.[28]

The neutralized acid soluble fractions were analyzed for ara-C metabolites by an ion-pairing high performance liquid chromatography (HPLC) method on an Isco liquid chromatograph equipped with a 5 μm C-18 octadecylsilane column with a C-18 precolumn.[29] The flow rate was 1 mL/min for the first 19 fractions and 2 mL/min for the remaining 41 fractions; sixty 0.5-mL fractions were collected and determined for radioactivity as described below. The mobile phase consisted of 90 mmol/L potassium phosphate buffer (pH 6.0), 10 mmol/L tetrabutylammoniumhydrogen sulphate, and 0.35% tetrahydrofuran. Samples were reconstituted in equal volumes of mobile phase buffer, and 100 μL was injected for each analysis. The elution positions of the standard unlabeled ara-C metabolites (ara-C, ara-CMP, ara-CTP) were monitored at 280 nm and correlated with those of the radioactive metabolites. Radioactivity was measured with an LKB 1209 liquid scintillation counter using Ready Value scintillation mixture (Beckman, Irving, CA).

The levels of intracellular metabolites (expressed in pmol/mg protein) were calculated from the total intracellular radiolabel (in pmol/mg), the fraction comprising the PCA soluble pool, and the percentage of each compound from chromatographic analysis.

In several cases, frozen myeloblasts that were cryopreserved in RPMI 1640/20% fetal calf serum with 10% dimethyl sulfoxide at −196°C were used for ara-C incubation experiments after the cells were thawed and maintained in culture medium for 4 hours. Equivalent ara-C incubation results were obtained with freshly obtained marrow specimens and cryopreserved bone marrow cells.

dCTP Pool Assay

A modification of the method of Skoog et al[30] was used to determine dCTP pools. The cells were washed two times with PBS and extracted with PCA as for the total ara-C metabolites. After 20 minutes at 0°C, the supernatant was collected after centrifugation (5

Table 2. Characteristics of Patient Samples and Lymphoblastoid Cell Lines

Patient	Age (yr)	Diagnosis	DS	S-Phase %	dCTP	Karyotype
KT	newborn	M_7-TMD	+	7.8	5.1 ± 0.4	46,XX/47,XX,+21 (mosaic)
	11 mo	M_7-AML	+	5.8	ND	46,XX/47,XX,+21 (mosaic)
JB	newborn	M_7-TMD	+	5.9	ND	47,XY,+21
SS	newborn	M_7-TMD	+	ND	5.9 ± 0.6	47,XX,+21
TH	1.5	M_7	+	ND	ND	47,XX,+21/46,XX,−8,+21
CO*	17 mo	M_7	+	7.7	4.7 ± 1.5	47,XY,+21/47,XY,del 5,del 16,+21
YJ*	5 mo	M_7	+	9.8	5.5 ± 0.3	47,XX,+21/47,XX,+19,+21,+22
RH*	22 mo	M_7	+	3.2	5.3 ± 0.8	47,XY,+21/47,XY,+8,+21
AB	1.5	M_7	−	13.5	10.2 ± 0.7	46,XX/46,XX,del 7,del 1
AH	14	M_3	−	ND	8.4 ± 0.5	46,XX,46,XX,t(15;17)
JP	9	M_4	−	ND	ND	46,XY
KG	14	M_1	−	17.6	7.2 ± 1.3	46,XX/47,XX,+8
BB	12	M_4	−	2	ND	46,XY
JL	8 mo	M_{5a}	−	5.8	ND	46,XY/46,XY,t(5;10)
TR	9	M_{5a}	−	7.4	ND	46,XX/46,XX,t(10;11)
DM*	9	M_2	−	ND	ND	46,XY/46,XY,t(8;21)
CE*	15	M_4	−	2.0	ND	46,XY/46,XY,t(6;11),del(11q23)
KI*	3	M_2	−	7.2	ND	46,XX
CW*	8	M_6	−	7.9	ND	46,XX
PB*	14	M_{5b}	−	10.7	ND	NA
BC*	17 mo	M_5	−	8.6	ND	NA
TN	17	M_7 (release)	−	ND	10 ± 0.4	46,XY
Cell line						
GM 1921	23	−	+	25.2-32.1	12.8 ± 0.4	47,XY,+21,inv(9),t(8;14)
GM 4927	27	−	+	34.1-37.8	11.8 ± 0.8	47,XX,+21
DET 1577	3	−	+	17.4-25.1	9.8 ± 0.4	47,XX,+21
GM 3715	12	−	−	28.5-40.6	13.1 ± 0.5	46,XX (12% random chromosome loss)
GM 3798	10	−	−	24.3-34.9	15.5 ± 0.6	46,XY (10% random chromosome loss)
GM 5398	44	−	−	34.5-54.8	14.4 ± 1.0	46,XY (6% random chromosome loss)

Patients SS and TN are not included in ara-CTP analysis.

Abbreviations: NA, not available; ND, not done.

* Included in MTT data, dCTP ± SE of 3 to 6 experiments.

minutes, 12,000 rpm) and neutralized with 5 N KOH and 1 mol/L KH_2PO_4 (pH of 7.0 to 7.5). The potassium perchlorate precipitate was removed by centrifugation. The recoveries of deoxynucleotides were >90%. All supernatants were stored at −20°C.

Aliquots of the supernatants were incubated in a total volume of 100 μL consisting of 50 μL of assay buffer (200 mmol/L Hepes, 20 mmol/L $MgCl_2$, 5 mmol/L mercaptoethanol, pH 7.3), 200 pmol deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and 200 pmol ^{3}H-deoxyadenosine triphosphate (dATP; 3400 μCi/μmol), with calf thymus DNA (5 mg/assay) as the template. The incubations were performed at 37°C for 30 minutes with the reactions initiated by the addition of DNA polymerase I (0.2 U/assay).

Aliquots (20 μL) were spotted onto Whatman DE81 ion exchange paper (Whatman, Hillsboro, OR) at the completion of the incubation. The papers were dried and washed with 5% Na_2HPO_4 (3 × 10 minutes). The filter papers were washed once with distilled water (5 mL) and 95% ethanol (5 mL). The papers were air-dried, transferred to liquid scintillation vials, and assayed for radioactivity as described above. Standard curves for dCTP were established for each assay and were linear from 0 to 80 pmoles.

Fluorometric DNA Strand Break Assay

Detection of DNA strand breaks after in vitro incubation of the cell lines with ara-C was based on the fluorometric analysis of DNA unwinding in alkali.[31,32] This assay measures the rate of unwinding of double-stranded DNA in alkali that is proportional to the length of DNA and increases with the number of DNA strand breaks. The percentage of double-stranded DNA remaining after exposure to alkaline conditions is inversely proportional to the number of strand breaks present.

Cells were incubated in the presence and absence of 25 μmol/L ara-C for 48 hours in RPMI 1640/15% dialyzed fetal calf serum and antibiotics at 37°C. After 48 hours, the cells were processed as in the ^{3}H-ara-C incubation method above to stop the incubation reaction. The cells were solubilized in urea and the lysates exposed to alkaline conditions for 30 minutes at 0°C followed by incubation for 30 minutes at 20°C. The amount of remaining double-stranded DNA was quantitated after staining with ethidium bromide (6.7 μg/mL) on a Gilford Fluoro IV fluorescence spectrophotometer (Oberlin, OH; excitation 520 nm; emission 590 nm). The substitution of 0.17 N NaOH for 0.20 N NaOH in our assay resulted in a greater percentage of double stranded DNA remaining after alkali exposure.[33]

Statistical Analysis

Statistical comparisons were performed to test the null hypothesis that ara-C metabolism did not differ between patients with and without DS. The nonparametric Mann-Whitney two-sample test was used to conduct these analyses.[34] For the cell lines, the mean values of up to three assays were used to compare cells with trisomy 21 versus cells without trisomy 21 using the Mann-Whitney two-sample statistic.

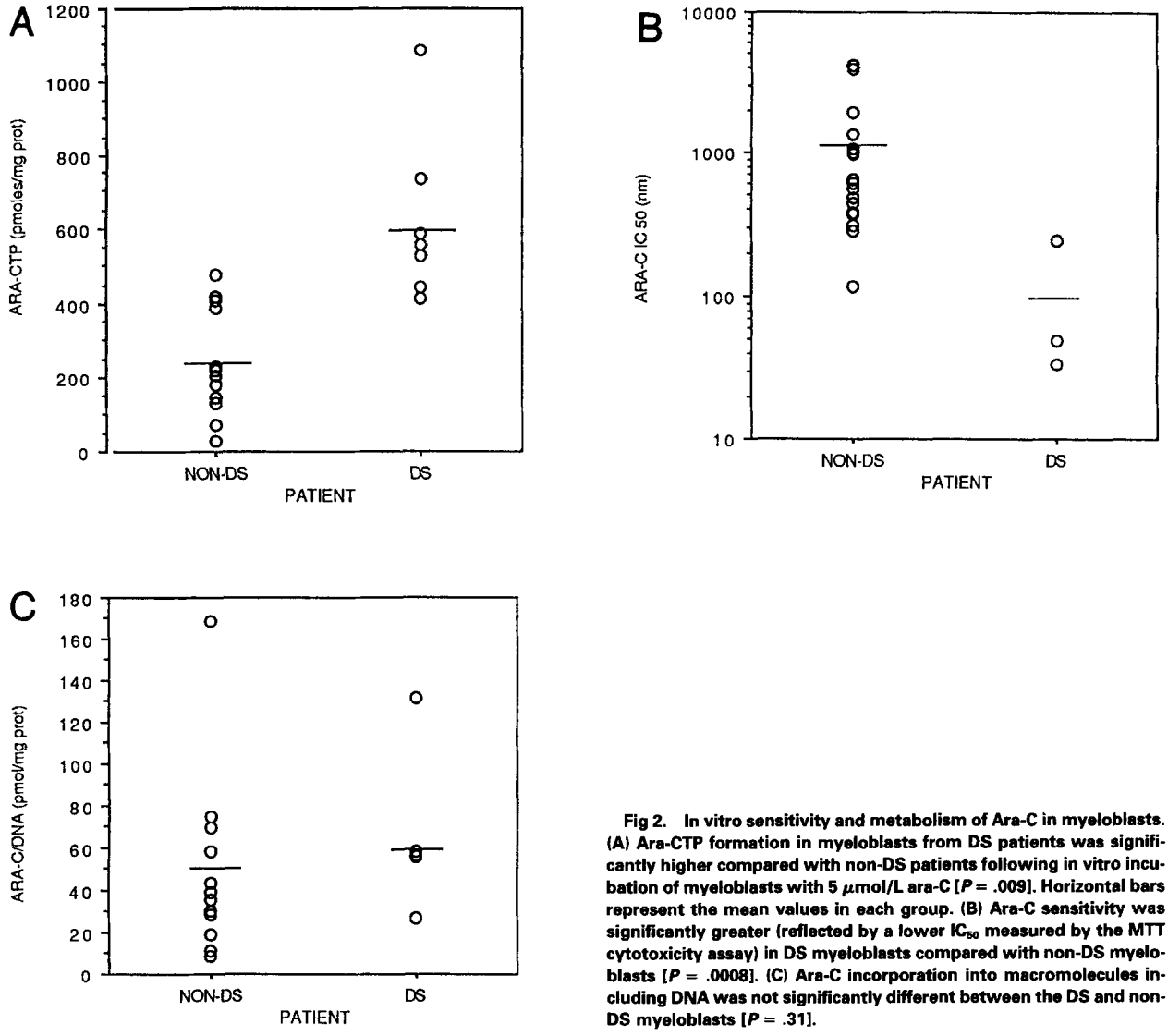

Fig 2. In vitro sensitivity and metabolism of Ara-C in myeloblasts. (A) Ara-CTP formation in myeloblasts from DS patients was significantly higher compared with non-DS patients following in vitro incubation of myeloblasts with 5 μmol/L ara-C [$P = .009$]. Horizontal bars represent the mean values in each group. (B) Ara-C sensitivity was significantly greater (reflected by a lower IC_{50} measured by the MTT cytotoxicity assay) in DS myeloblasts compared with non-DS myeloblasts [$P = .0008$]. (C) Ara-C incorporation into macromolecules including DNA was not significantly different between the DS and non-DS myeloblasts [$P = .31$].

RESULTS

Ara-C Sensitivity of Leukemic Myeloblasts

Ara-C cytotoxicities were determined by the MTT assay of myeloblasts from newly diagnosed AML patients with DS (n = 3) and non-DS (n = 18). The DS myeloblasts were approximately 10-fold more sensitive to ara-C than the non-DS myeloblasts after 72 hours of drug exposure (IC_{50}'s of 108.3 ± 66.8 nm and 1040.8 ± 272.9 nm, respectively) (Fig 2). Addition of the 5637 supernatant as a source of growth factors including GM-CSF,[24,25] improved the viabilities and proliferation of the cells for the assay.

Ara-C Metabolism and DNA Incorporation in Human Leukemia Cells and Lymphoblastoid Cell Lines

In vitro incubations with ^{3}H-ara-C (1 μmol/L and 5 μmol/L) were performed with DS and non-DS myeloblasts and with EBV-transformed lymphoblastoid cell lines established from both DS and non-DS individuals. The ara-C concentrations used correspond to plasma ara-C levels achievable with clinical treatment protocols.[35,36] Although the cell lines were not malignant, they have been previously used for a variety of biologic studies of chromosome 21-localized genes (ie, carbonyl reductase).

At 5 μmol/L ara-C, the DS patient myeloblasts (KT, TH, JB, CC, YJ, RH) accumulated significantly higher levels of ara-CTP than the non-DS patients; the differences between these groups 621.4 ± 42.3 versus 228.4 ± 86.6 pmol/mg protein) were statistically significant (Fig 2). Qualitatively similar results were obtained at 1 μmol/L for one DS patient (KT) compared with 3 non-DS patients (AB, JP, and BB). In general, non-ara-CTP metabolites represented less than 50% of the total cellular drug forms. There were no differences in the low levels of other ara-C metabolites (mostly ara-CMP) between the groups (mean, 47 ± 11 and 29.6 ± 9.2 pmol/mg protein, $P = .14$). The significantly increased levels of unmetabolized ara-C in DS patients (131.6 ± 24.6 pmol/mg protein) compared with non-DS patients (57.7 ±

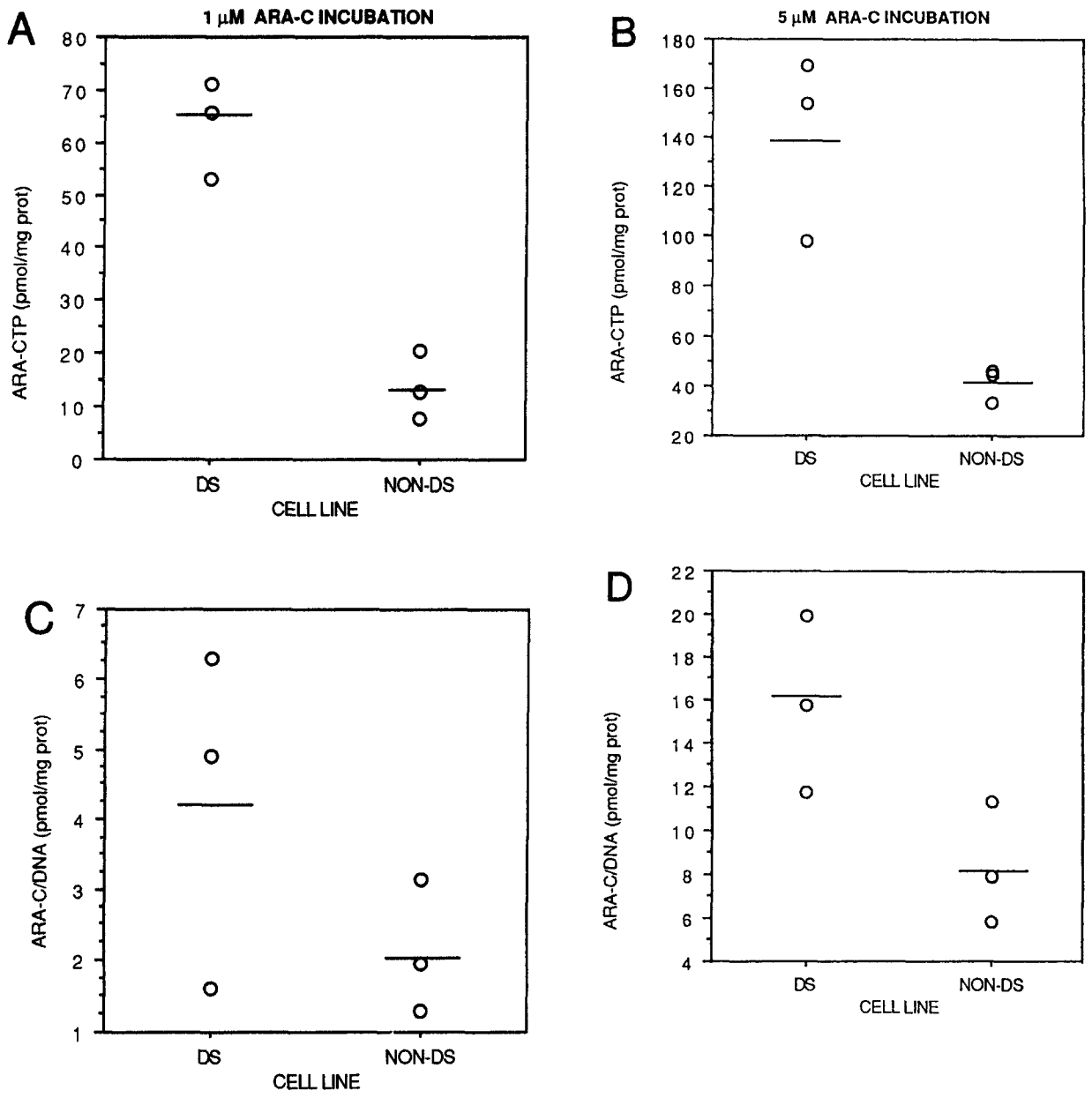

Fig 3. In vitro metabolism of Ara-C in EBV-transformed lymphoblastoid cell lines. Trisomy 21 cell lines generated significantly higher levels of ara-CTP compared with diploid chromosome cell lines after incubation in vitro with 1 μmol/L (A) and 5 μmol/L ara-C (B) [P = .049 and .049, respectively]. The values represent the means of two to three individual experiments for each cell line except for DET 1557 (n = 1). Ara-C incorporation into DNA was not significantly different between trisomy 21 and diploid chromosome cell lines incubated with 1 μmol/L ara-C (C) but was significantly greater in the trisomy 21 cells after incubation with 5 μmol/L ara-C (D) [P = .28 and .049, respectively].

20.1 pmol/mg protein), may reflect differences in ara-C transport between the two groups (Fig 2).

Similar results were obtained with trisomy 21 (GM 4927, DET 1577, and GM 1921) and diploid (GM 5398, GM 3715, and GM 3798) lymphoblastoid cell lines. Hence, for both 1 and 5 μmol/L ara-C, the DS cell lines accumulated appreciably higher levels of ara-CTP compared with the non-DS cell lines (62.8 ± 5.4 *v* 13.6 ± 3.7 pmol/mg protein at 1 μmol/L and 140.3 ± 21.6 *v* 41.7 ± 4.2 pmol/mg protein at 5 μmol/L; see Fig 3).

For both the cultured cells and patient myeloblasts, increases in ara-CTP synthesis over 3 hours were accompanied by no or only slightly increased incorporation of ^{3}H-ara-C into PCA soluble macromolecules, including DNA (Figs 2 and 3) This suggests that this parameter may not adequately reflect the adverse effects of ara-C on DNA structure.

Despite this anomaly, a 1.7-fold increase in the relative levels of double-stranded DNA breaks were recorded for the DET 1577 line (45.9% ± 9.9% strand breaks [control] and 82.3% ± 2.6% [ara-C treated]; P = .049); conversely, no

differences over untreated controls were measured for the non-DS GM 3798 line (47.1% ± 2.7% [control] and 55.2% ± 13.1% [ara-C treated]; $P = .51$, n = 3 separate experiments).

Relationship Between Endogenous Deoxycytidine Triphosphate Pools and Increased In Vitro ara-CTP Generation

The relationship between DS and ara-CTP synthesis, may partly reflect alterations in endogenous dCTP levels, an indirect result of changes in intracellular folates resulting from increased gene dosage effect of cystathionine-β-synthase (CBS) or glycinamide ribonucleotide transformylase (GART).[18] Low dCTP pools may result in increased generation of ara-CTP by release of the feedback inhibition of deoxycytidine kinase (dCk).[35] For both the DS patients and in trisomy 21 lymphoblastoid cell lines (GM 1921, DET 1577, GM 4927), endogenous dCTP pools were decreased only slightly relative to corresponding non-DS samples (Table 2; P values of .01 and .049 for patients and cell lines, respectively).

S-Phase Determination of Leukemia Cells and Cell Lines

Differences in the extent of ara-C metabolism between DS and non-DS cells may also be related to the percentage of cells in S-phase, ara-C being an S-phase specific agent.[35] However, no significant differences in the percentage of cells in S-phase (determined before incubation studies) were found between the DS and non-DS groups with either patient myeloblasts (mean, 6.7% ± 0.9% and 8.3% ± 1.5%, respectively, $P = .55$) or the lymphoblastoid cell lines (mean, 28.6% ± 4.2% and 36.9% ± 4.5%, respectively, $P = .28$).

DISCUSSION

The significantly superior EFS rate of DS children with AML compared with non-DS children has been reported by a number of pediatric oncology groups throughout the world,[7,11-15] and may be based on a gene dosage effect for critical genes localized to chromosome 21; this may enhance the sensitivity of DS AML cells to selected chemotherapeutic agents. This increased sensitivity of DS children to current therapy is also reflected in the very low leukemia relapse rates, suggesting that significant leukemia cell kill occurs before the development of drug resistance.

The studies described herein strongly implicate an increased sensitivity of DS cells to ara-C as a contributing factor in the improved survival of DS children with AML. Ara-C was significantly more cytotoxic toward DS myeloblasts than non-DS myeloblasts as measured in vitro with the MTT assay. In both myeloblasts from DS patients and trisomy 21 lymphoblastoid cell lines, ara-CTP synthesis was significantly increased over non-DS cells, suggesting a causal relationship to drug activity. Our finding that qualitatively identical results were obtained in patient specimens and EBV-transformed diploid and trisomy 21 lymphoblastoid cell lines, establish the generality of this observation and strongly supports the conclusion that chromosome 21-localized genes contribute to this enhanced ara-C metabolism.

A total of eight DS myeloblast specimens were analyzed for the studies. DS patient KT was studied twice, initially as a neonate with TMD and 1 year later when she developed AML. The lower ara-CTP levels in the myeloblasts at diagnosis (416 pmol/mg protein) compared with the TMD state (1,085 pmol/mg protein) likely reflects the differences in percent blasts between the samples (50% and 95%, respectively) because malignant blast cells generate ara-CTP levels two to 20-fold higher than normal lymphocytes.[37] These results demonstrate the difficulties encountered in studying the biochemical pharmacology of AML in DS patients and provide further rationale for our inclusion of the lymphoblastoid cell lines in this study. The DS and non-DS leukemia samples used in this study had mean blast counts of 74% ± 6.5% and 86% ± 2.2%, respectively.

Differences in the extent of ara-C metabolism between cell populations may reflect variations in the number of S-phase cells and, consequently, dCk activity between DS and non-DS cells. However, our results suggest no significant difference in the cell cycle profiles between the groups.

Changes in the extent of ara-C metabolism may also reflect alterations in dCTP pools which (1) result in decreased feed-back inhibition of dCk, the rate-limiting step in the conversion of ara-C to ara-CTP and/or (2) decreased competitive binding with ara-CTP to DNA polymerase.[35] Decreased dCTP pools in DS may arise from the increased expression of CBS localized to chromosome 21[38] and the consequent depletion of homocysteine[39] and likely, S-adenosylmethionine (AdoMet)[40] (Fig 1). The decreased AdoMet releases the allosteric inhibition on 5,10-methylene tetrahydrofolate reductase, resulting in an increased diversion of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate.[41] At the same time, the 5-methyl tetrahydrofolate is effectively "trapped" because of the lower homocysteine levels[42] (Fig 1). The net effect of reduced 5,10-methylene tetrahydrofolate pools is the decreased synthesis of dTMP and lower dTTP pools, and ultimately, lowered dCTP pools due to the diminished allosteric inhibition of deoxycytidylate deaminase by dTTP[43] (Fig 1). The purine biosynthetic enzyme GART is also localized on chromosome 21 and has been suggested to lower dCTP pools in an analogous fashion.[7] However, GART activity is not increased in DS,[44] and it is not apparent how changes in GART could sequester tetrahydrofolates; inhibition of GART by 5,10-dideaza-5,6,7,8-tetrahydrofolate does not result in lower dCTP pools.[45]

Increased remission rates for AML and myelodysplastic syndrome patients have been observed from combined therapy with ara-C and fludarabine (a ribonucleotide reductase inhibitor which decreases dCTP pools),[46] suggesting a modulatory role for dCTP in ara-C activity. However, our studies in DS and non-DS myeloblasts and lymphoblastoid cell lines, demonstrate only slight differences in dCTP pools between the groups. While it is unclear to what extent dCTP pools must vary to manifest an effect on ara-CTP formation; measurement of total nucleotide pools may not reflect the intracellular compartmentation of dCTP pools such that significant localized changes are not detected.[47] In any case, these data suggest other explanations for the increased accumulation of ara-CTP in DS cells must be considered.

An intriguing possibility is that low AdoMet levels may contribute to the enhanced metabolism of ara-C to ara-CTP in DS cells through the effects on dCk gene expression via hypomethylation.[18] Indeed, 5-azacytidine (5-aza-C), a DNA methylation inhibitor, sensitizes ara-C resistant cell lines to ara-C[48] and dCK activity has been shown to be increased in relapsed leukemia patients treated with 5-aza-C and ara-C.[49] An alternative mechanism of action of ara-C, in which ara-C inhibits cell membrane phospholipid synthesis leading to cell lysis,[50,51] may also be enhanced in DS cells leading to greater cytotoxicity.

It is of interest that all of the DS leukemia samples in this study were of the M_7 megakaryocytic (M_7) phenotype, which accounted for the highest AML phenotype of DS patients in the studies summarized in Table 1. It has been estimated that the incidence of AML-M_7 in DS children is up to 600-fold higher than for non-DS children,[14] while it accounts for approximately 2% of all cases of childhood AML.[11] Whether the enhanced ara-CTP formation in DS cells is related to the M_7 phenotype is unclear, though previous reports of non-DS children with M_7 have noted a poor prognosis.[52,53]

In conclusion, DS children represent an unique group of patients in which the underlying genetic abnormality increases both the risk of leukemia and also enhances the response to chemotherapy of individuals who develop leukemia. The enhanced ara-C sensitivity of DS myeloblasts in cytotoxicity assays, the elevated levels of ara-CTP in both DS myeloblasts and trisomy 21 EBV-transformed lymphoblastoid lines, and the slightly lower endogenous dCTP pools in this study strongly suggest an unique role of chromosome 21-localized genes in ara-C metabolism. It should be recognized, however, that the significantly superior EFS of DS children with AML is likely multifactorial and may include effects on the activity of other agents used in combination with ara-C (ie, daunorubicin) or other chromosome 21 enzymes (carbonyl reductase, superoxide dismutase).[18] In any case, the better delineation of the mechanisms of chemotherapy sensitivity in DS patients may lead to improvements in the treatment of AML patients overall, by using biochemical modulation of chemotherapy drugs to mimic the DS model.

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