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Antileukemic Activity and Mechanism of Action of Cordycepin against Terminal Deoxynucleotidyl Transferase-Positive (TdT^+) Leukemic Cells

Eiichi N. Kodama, Ronald P. McCaffrey,† Keisuke Yusa* and Hiroaki Mitsuya*‡§*

*Experimental Retrovirology Section, Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.; †Martin H. Semler Laboratory, Section of Medical Oncology, Evans Memorial Department of Clinical Research, Boston University Medical Center, Boston, MA 02118, U.S.A.; and ‡Department of Internal Medicine II, Kumamoto University School of Medicine, Kumamoto 860, Japan

ABSTRACT. The nucleoside analogue cordycepin (3′-deoxyadenosine, 3′-dA) is substantially more cytotoxic to terminal deoxynucleotidyl transferase positive (TdT^+) leukemic cells than to TdT^- leukemic cells *in vitro* in the presence of an adenosine deaminase inhibitor, deoxycoformycin (dCF), and has been considered as a therapeutic agent for TdT^+ leukemia. The intracellular metabolism of 3′-dA was examined with HPLC, and the mechanism of its anti-TdT^+ leukemic activity was analyzed. In the presence of dCF (2.5 μM), TdT^+ leukemic cells (N = 5) were sensitive to the cytotoxic effect of 3′-dA, whereas TdT^- (N = 6) cells were not. A high level of 3′-dA-5′-triphosphate (3′-dATP) formation was detected in TdT^+ NALM-6 cells (67 pmol/10^6 cells) and TdT^- K562 cells (49 pmol/10^6 cells) when cultured with 1 μM [3′-^{3}H]-labeled 3′-dA. A substantial level of 3′-dATP was detected in TdT^- HUT-102 cells (27 pmol/10^6 cells), whereas the level of 3′-dATP in TdT^+ MOLT-4 cells was low (0.3 pmol/10^6 cells). The mean IC_{50} values of 3′-dA against phytohemagglutinin (PHA)-activated and resting peripheral blood mononuclear cells (PBM) (N = 5) were 8 and 32 μM, respectively. There was a modest level of 3′-dATP (7 pmol/10^6 cells) in PHA-PBM, whereas a lower level of 3′-dATP was detected in resting PBM (2.5 pmol/10^6 cells). These data suggest that the presence of 3′-dATP is not sufficient for the antileukemic effect of 3′-dA, but that TdT positivity is essential, and that PBM are significantly less sensitive to the cytotoxicity of 3′-dA *in vitro*. Further development of 3′-dA as a potential antileukemic agent to treat patients with TdT^+ leukemia is warranted. BIOCHEM PHARMACOL **59**;3:273–281, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. cordycepin; adenosine deaminase; antileukemic agent; terminal deoxynucleotidyl transferase

TdT‖ (EC 2.7.7.31), which polymerizes a single-stranded deoxynucleotidyl sequence without need of templates [1], is expressed in pre-T cells, thymocytes, and early pre-B cells [1–4], and is considered to play an important role during immunoglobulin and T cell receptor gene rearrangements, thereby increasing the diversity of immunoglobulin and T cell receptor molecules [5–8]. Clinically, over 90% of leukemic cells in acute lymphocytic leukemia and approximately 30% of leukemic cells in the chronic myelogenous leukemia crisis exhibit elevated TdT activity, and the TdT activity of such leukemic cells is associated with a poor prognosis on chemotherapy and survival time [9–11], although what role TdT plays in the outcome of leukemias is not well defined.

The nucleoside analogue cordycepin (3′-dA) has been shown to inhibit the growth of various tumor cells [12–15]. Such inhibition of cellular growth is linked to the inhibition of RNA synthesis mediated by RNA polymerases, especially poly(A) tail-RNA polymerases [13, 16]. Recently 3′-dA also was shown to interact with DNA primase at least *in vitro* [17]. Although these studies demonstrated that 3′-dA inhibits RNA synthesis at 7–70 μM, an interest in 3′-dA as an antineoplastic agent has been renewed due to other recent studies showing that 3′-dA inhibits the activity of TdT and is cytotoxic against TdT^+ cells *in vitro* in the presence of ADA inhibitors (e.g. dCF), but not to TdT^- cells [18, 19].

Based on these *in vitro* data, the combination of 3′-dA and dCF is undergoing Phase I clinical trials in several medical centers in the United States. Open questions on

§ Corresponding author: Dr. Hiroaki Mitsuya, Experimental Retrovirology Section, Medicine Branch, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892. Tel. (301) 402-3631; FAX: (301) 402-0709; E-mail: hmitsuya@helix.nih.gov

‖ *Abbreviations*: TdT, terminal deoxynucleotidyl transferase; 3′-dA, 3′-deoxyadenosine, cordycepin; 3′-dI, 3′-deoxyinosine; dCF, deoxycoformycin; ADA, adenosine deaminase; AK, adenosine kinase; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; and RT-PCR, reverse transcription coupled with polymerase chain reaction.

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the cytotoxicity of 3′-dA include: (i) whether the triphosphate of 3′-dA (3′-dATP) is an intracellular active moiety; (ii) whether 3′-dA is preferentially phosphorylated in TdT^+ cells in comparison with TdT^- cells; (iii) whether the cytotoxicity of 3′-dA is affected by other cellular factors or components; and (iv) how the presence of ADA inhibitors affects the ultimate intracellular metabolism of 3′-dA. In this study, we tested the antileukemic activity of 3′-dA combined with dCF against TdT^+ and TdT^- leukemic cells and PHA-activated or resting PBM, examined the intracellular pharmacology of 3′-dA and its deaminated analog 3′-dI, and attempted to elucidate the mechanism of antileukemic action of 3′-dA.

MATERIALS AND METHODS

Cells and Chemicals

Five TdT^+ cell lines [NALM-6 (a human pre-B-cell leukemic cell line), P388D1 (a murine lymphoid neoplasm cell line), S49.1 (a murine thymoma cell line), CEM, and MOLT-4 (a human early T-cell leukemia cell line)] and six TdT^- cell lines [HUT-102, MT-2 (a human T-cell leukemia virus-transformed leukemic T-cell line), Raji (a human Burkitt lymphoma cell line), HL-60 (a human promyelocytic leukemia cell line), PM-1 (a human cutaneous T-cell leukemia cell line), and K562 (a human erythroid leukemic cell line)] were employed. The TdT status of these cell lines has been described by McCaffrey *et al.* [20]. All cell lines were maintained in RPMI 1640 (HyClone Laboratories Inc.) supplemented with 10% fetal bovine serum, 50 μg/mL of streptomycin, 50 U/mL of penicillin, and 2 mM L-glutamine. PBM were prepared from five different normal healthy donors with Ficoll–Conrey as previously described [21]. Freshly isolated PBM were stimulated with PHA (10 μg/mL, Sigma Chemical Co.), and were cultured for 72 hr in interleukin-2-containing culture medium; they served as PHA-activated, dividing PBM (PHA-PBM). Unstimulated, resting PBM (R-PBM) isolated from the same individual also were employed.

3′-dA and MTT were purchased from Sigma. [3′-^{3}H]-Labeled 3′-dA (specific activity 5.51 Ci/mmol) was obtained from Research Triangle Institute. dCF, an ADA inhibitor, was provided by Dr. Varma Ravi K. (Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis, and Centers, National Cancer Institute). ADA and TdT were purchased from Boehringer Mannheim and United States Biochemical, respectively. Bio-Rad protein assay kits [22] were purchased from Bio-Rad.

Cytotoxicity Assays

3′-dA was examined at 10-fold serial concentrations (0.01 to 100 μM) for its cytotoxicity in 96-well microtiter culture plates (Costar). Cells growing in the exponential phase were plated at a final concentration of 2×10^5 cells/mL for long-term cultured cell lines and 10^6 cells/mL for R-PBM and PHA-PBM. In certain experiments, cells were preincubated with dCF for 30 min and treated with 3′-dA or 3′-dI. Cell viability was measured by the MTT method as previously described [23, 24].

HPLC Analysis of 3′-dA Metabolites

Cells were exposed to [3′-^{3}H]-3′-dA (1 μM) for 3 hr with or without prior exposure to dCF, washed three times with cold PBS, pelleted, and subjected to extraction of nucleotides with 60% methanol as previously described [25]. Then the extracts were heated for 2 min at 95° and analyzed by HPLC using an ion-exchange column (Partisil 10-SAX; Whatman Inc.). The samples containing 3′-dA nucleotides were collected, and their radioactivities were determined in a liquid scintillation counter.

ADA-Mediated Deamination of 3′-dA

ADA-mediated deamination of three forms of 3′-dA (3′-dA, 3′-dAMP, and 3′-dATP) was monitored at 37° by measuring optical density with a spectrophotometer (UV1601, Shimadzu). Briefly, deamination was initiated by the addition of ADA (10 μg) to 1 mL of a reaction mixture containing 20 mM Tris–HCl, 25 mM KH_2PO_4, pH 7.4, and either 3′-dA, 3′-dAMP, or 3′-dATP. Changes of absorbance (265 to 245 nm) were considered to indicate that deamination occurred, as previously described by Battistuzzi *et al.* [26]. 3′-dI, which was produced through deamination of 3′-dA, served as a reagent and a marker in HPLC analyses and also was employed in the cytotoxicity assay.

Determination of Enzymatic Activity of ADA in Leukemic Cells

ADA activity was determined using the method described by Battistuzzi *et al.* [26] and Daddona [27] with slight modifications. Briefly, cells were harvested, washed three times with PBS, resuspended at a final concentration of 10^7 cells/mL in 25 mM Tris–HCl at pH 7.4, and disrupted by sonication. The cell lysates were clarified by centrifugation at 20,000 *g* for 30 min at 4°. The ADA activity assay was conducted in 1 mL of reaction buffer containing 20 mM Tris–HCl, 25 mM KH_2PO_4, pH 7.4, 50 μL of the cell extract supernatant, and 50 μM adenosine at 37°. Conversion of adenosine to inosine was monitored by a spectrophotometer as a peak shift at 265 nm.

Detection of TdT mRNA Expression

Expression of TdT mRNA was examined with RT-PCR as described previously [28] with some modifications. Briefly, total RNA was extracted from cells using TRIZOL LS reagent (GIBCO-BRL), and the RNA obtained (1 μg) was reverse transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). The cDNA

TABLE 1. Sensitivity to 3′-dA of various cell lines and their cellular ADA and TdT activity

TdT status	Cell line	Origin	IC_{50} of 3′-dA (μM) Without dCF	IC_{50} of 3′-dA (μM) With dCF (2.5 μM)	ADA activity* nmol/min/10^7 cells	TdT activity† nmol/hr/10^7 cells
+	NALM-6	Human ALL	50.4 ± 11.51	0.16 ± 0.054	18.8 ± 2.5	6.31 ± 1.92
+	MOLT-4	Human ALL	> 100	0.92 ± 0.23	74.4 ± 9.3	0.89 ± 0.19
+	CEM	Human ALL	ND	0.18 ± 0.08	ND	ND
+	P388D1	Murine lymphoma	ND	0.07 ± 0.01	ND	ND
+	S49.1	Murine thymoma	ND	0.03 ± 0.005	ND	ND
					$P < 0.0087$	
−	HUT-102	Human ALL	83.2 ± 15.8	30.8 ± 7.6	0.400 ± 0.06	< 0.3
−	K562	Human CML	> 100	13.8 ± 5.0	4.71 ± 0.52	< 0.3
−	Raji	Human lymphoma	ND	1.4 ± 0.4	ND	ND
−	HL-60	Human ANLL	ND	0.5 ± 0.16	ND	ND
−	MT-2	Human ALL	ND	2.2 ± 0.5	ND	ND
−	PM-1	Human ALL	ND	3.4 ± 0.2	ND	ND
−	R-PBM		> 100	31.8 ± 12.8	3.9 ±1.9	ND
−	PHA-PBM		> 100	8.2 ± 4.16	14.6 ± 8.4	< 0.3

Values are means ± one SD of triplicate determinations. ND, not determined.

* The enzymatic activity of ADA was determined based on the conversion of adenosine to inosine monitored by a spectrophotometer as described in Materials and Methods.

† TdT activity was determined using cell extracts.

was used as a template in the subsequent PCR. The primers used for amplification of TdT cDNA were 5′-GTC ACC CAC ATT GTA GCA GAG-3′ and 5′-ATG ATA CCC TTC ACC TTG GAC-3′ [28]. A human β-globin cDNA segment also was subjected to PCR and served as an internal control. The primers used for amplification of β-globin cDNA were 5′-ACA CAA CTG TGT TCA CTA GC-3′ and 5′-CAA CTT CAT CCA CGT TCA CC-3′ [29]. PCR was performed with a DNA thermal cycler (Perkin Elmer Corp.) for 30 cycles under the following conditions: 30 sec at 94° for denaturing, 30 sec at 55° for annealing, and 60 sec at 72° for extension. The amplified products were electrophoresed in 2% agarose gels and visualized with the addition of ethidium bromide.

Determination of Enzymatic Activity of TdT

The enzymatic activity of TdT was determined as previously described by Sasaki and colleagues [28, 30, 31] with some modifications. Briefly, cells were washed three times with PBS, resuspended (10^8 cells/mL) in a solution containing 25 mM KH_2PO_4, pH 7.4, and lysed by sonication. The cell lysates were clarified by centrifugation at 20,000 *g* for 30 min at 4°, followed by a 30-min incubation of a reaction mixture (100 μL) containing 200 mM sodium cacodylate at pH 7.4, 0.5 μM [^{3}H]dGTP (100 dpm/pmol), 0.6 mM $MnCl_2$, 10 μM poly$(dA)_{40}$, 1 mM dithiothreitol, and the cell extract supernatant (25 μL). Then the reaction mixture (50 μL) was spotted on GF/C filters (Whatman Inc.), and the radioactivity of [^{3}H]dGTP incorporated into poly$(dA)_{40}$ was determined in a liquid scintillation counter.

RESULTS

Activity of 3′-dA against Leukemic Cells and PBM

We first evaluated the cytotoxic activity of 3′-dA against various leukemic cell lines and PBM in the absence of dCF. The IC_{50} values of 3′-dA alone for TdT^+ NALM-6 cells and TdT^- HUT-102 cells were 50.4 and 83.2 μM, respectively. Those for TdT^+ MOLT-4 cells, TdT^- K562 cells, R-PBM, and PHA-PBM were all > 100 μM (Table 1). We subsequently determined the toxicity of 3′-dA in the presence of dCF. Since significant toxicity was observed when the cells were cultured in the presence of dCF alone at concentrations up to 10 μM (data not shown), a clinically achievable concentration of dCF, 2.5 μM [32], was used throughout the subsequent experiments. The cytotoxicity of 3′-dA was potentiated significantly when 3′-dA was combined with dCF (Table 1). The IC_{50} values of 3′-dA against five TdT^+ cell lines were significantly lower than those against the six TdT^- cell lines ($P < 0.0087$; Wilcoxon rank-sum test), in agreement with previously published data by Koc and McCaffrey [18]. The mean IC_{50} values of 3′-dA with dCF against R-PBM and PHA-PBM obtained from five different donors were also much greater [31.8 and 8.2 μM, respectively (Table 1 and Fig. 1)] than those against the five TdT^+ cell lines. There was no significant variation observed in the sensitivity to 3′-dA among the five different PBM preparations in the presence or absence of dCF (Fig. 1).

Anabolic Phosphorylation of 3′-dA

3′-dA is a nucleoside analogue, and its anabolically triphosphorylated form is presumably an active moiety that selectively kills TdT^+ cells. Therefore, we examined the phosphorylation profile of 3′-dA in various cell lines. As shown

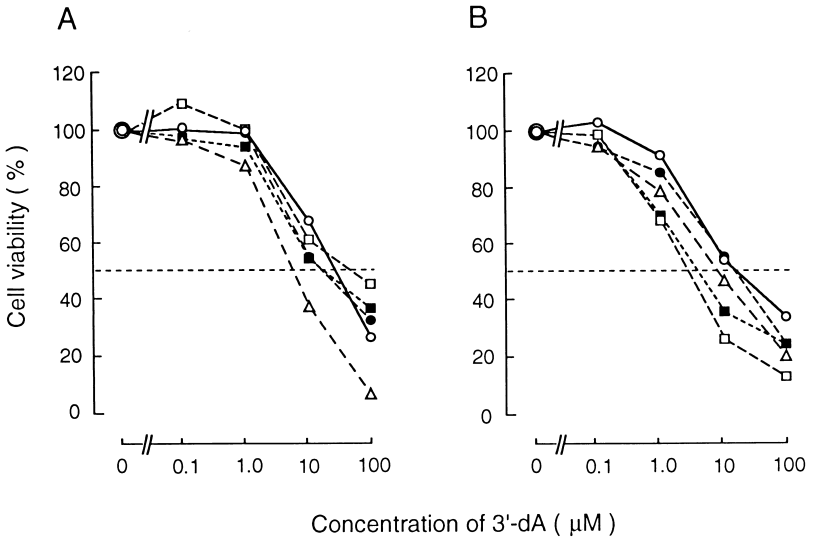

FIG. 1. Cytotoxic profile of 3′-dA with 2.5 μM dCF against R-PBM and PHA-PBM. The cytotoxic effect of 3′-dA with dCF against R-PBM (panel A) and PHA-PBM (panel B) was determined with the MTT method as described in Materials and Methods. Cell viability shown represents the mean of triplicate determinations expressed as percent viable cell numbers in a drug-free control culture. The same symbols in panels A and B represent identical donors. The mean IC_{50} values of 3′-dA were 31.8 and 8.2 μM in panels A and B, respectively. A broken line denotes the concentration of 3′-dA that brought about 50% reduction of cell viability (IC_{50}).

in representative profiles in Fig. 2, it was found that 3′-dA was phosphorylated and converted to mono-, di-, and triphosphates in both leukemic cell lines and PBM when incubated with dCF. The amount of 3′-dATP formed in TdT^+ NALM-6 cells after 3 hr of incubation with [3′-^{3}H]-3′-dA was the greatest (67 ± 7.2 pmol/10^6 cells) among the cell lines examined (Fig. 3). In contrast, no significant amount of 3′-dATP was detected in TdT^+ MOLT-4 cells (0.3 ± 0.03 pmol/10^6 cells) when examined under the same conditions. Since MOLT-4 cells were sensitive to 3′-dA in a 48-hr cytotoxicity assay (Table 1), these cells were incubated for an additional 7 hr, resulting in the formation of a detectable amount of 3′-dATP (15.9 ± 1.9 pmol/10^6 cells) (Fig. 3). By 24 hr in culture, under the inverted microscope we did not observe significant killing of MOLT-4 cells, particularly in the absence of dCF. We reason that the absence of MOLT-4 killing was due to a high level of ADA activity in the cytoplasm of MOLT-4 cells. In the two TdT^- cell lines, HUT-102 and K562, substantial amounts of 3′-dATP were formed (27 ± 5 and 49 ± 18 pmol/10^6 cells, respectively). In R-PBM, relatively low levels of 3′-dATP were found (2.5 ± 0.7 pmol/10^6

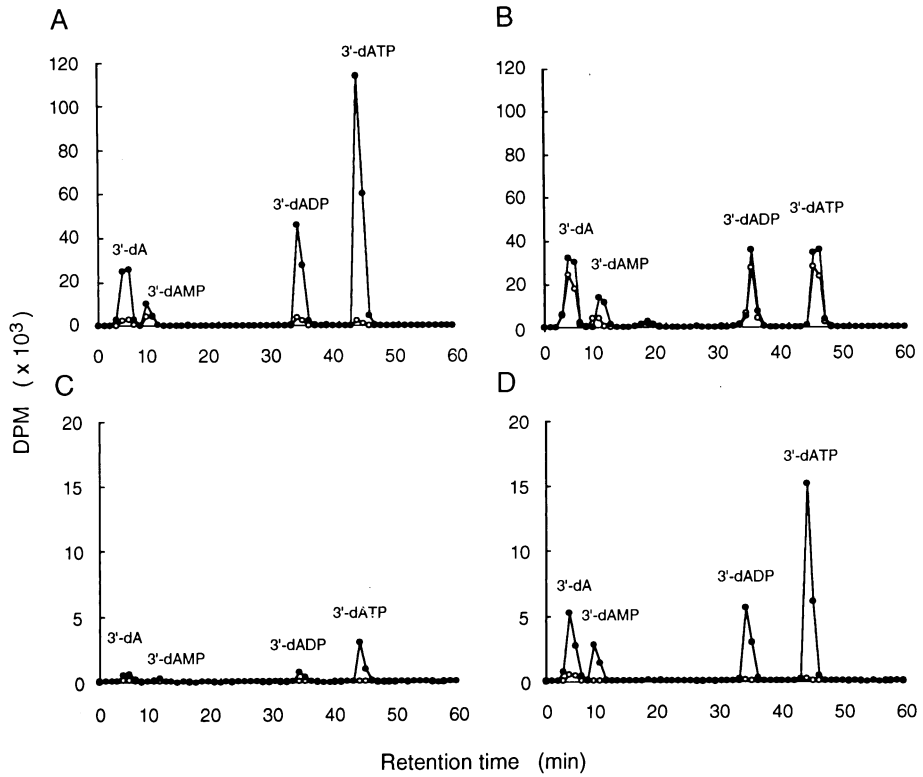

FIG. 2. HPLC elution profiles of intracellular metabolites of 3′-dA in NALM-6 cells, HUT-102 cells, R-PBM, and PHA-PBM. NALM-6 cells (panel A), HUT-102 cells (panel B), R-PBM (panel C), and PHA-PBM (panel D) were incubated with 1 μM [3′-^{3}H]-3′-dA in the presence (solid circles) or absence (open circles) of 2.5 μM dCF for 3 hr. Intracellular nucleoside metabolites were extracted and analyzed with HPLC as described in Materials and Methods. Shown are representative data obtained from at least three independent experiments in each cell line.

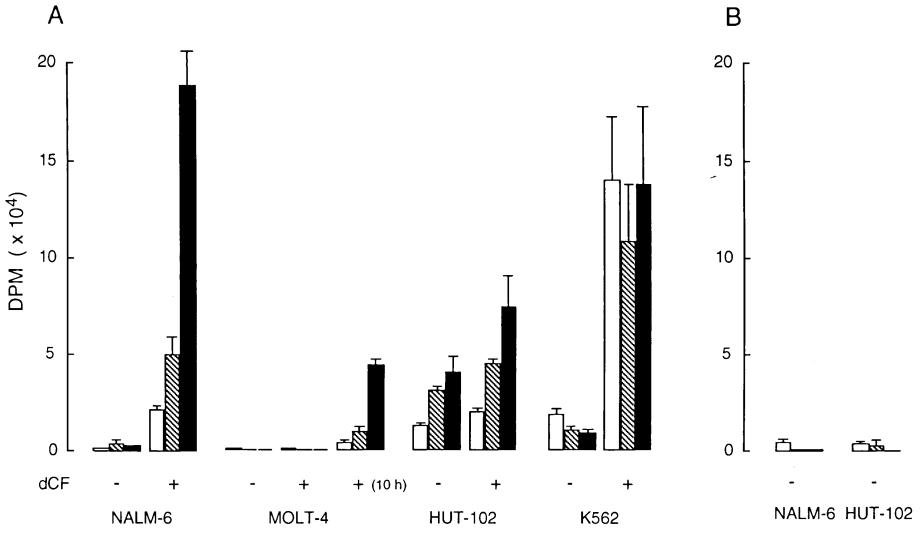

FIG. 3. Anabolic phosphorylation of 3′-dA (panel A) and 3′-dI (panel B). Data represent the means of triplicate determinations ± one SD. The levels of 3′-dAMP (open bars), 3′-dADP (hatched bars), and 3′-dATP (solid bars) are shown. The amounts of 3′-dATP after 3 hr of incubation in NALM-6, MOLT-4, HUT-102, and K562 cells were 67, 0.3, 27, and 49 pmol/10^6 cells, respectively. A phosphorylation profile of 3′-dA with dCF in MOLT-4 cells after 10 hr of incubation is also shown (panel A). Note that 3′-dI was phosphorylated less efficiently in NALM-6 and HUT-102 cells compared with 3′-dA (panel B).

cells), whereas about 3-fold higher levels of 3′-dATP were detected in PHA-PBM (6.8 ± 0.9 pmol/10^6 cells) (Figs. 2 and 4). There was no significant variation in the level of 3′-dATP when R-PBM and PHA-PBM from five different individuals were examined (Fig. 4). When incubated with [3′-^{3}H]-3′-dA in the absence of dCF (Fig. 3), as expected, substantially lower amounts of 3′-dATP were detected in NALM-6 and K562 cells, whereas there was only ~40% reduction in the amount of 3′-dATP in HUT-102 cells when incubated in the absence of dCF (Fig. 3). These data strongly suggested that both NALM-6 and K562 cells express substantial amounts of ADA, whereas HUT-102 cells express a low amount of the enzyme. This was confirmed when ADA activity was determined in these cell lines (*vide infra*).

Lack of Cytotoxicity of 3′-dI, the Deaminated Product of 3′-dA

Since 3′-dA was active against TdT$^+$ leukemic cells only in the presence of dCF (Table 1), we asked whether 3′-dA was deaminated to 3′-dI by purified ADA and whether dCF sufficiently blocked the deamination. The absorbance peak of 3′-dA as examined by spectrophotometer was located at 265 nm, which rapidly shifted to 245 nm following the addition of ADA, clearly indicating that conversion of 3′-dA to 3′-dI occurred. This absorbance shift was blocked completely by 2.5 μM dCF (data not shown). On the other hand, the absorbance peaks of 3′-dAMP and 3′-dATP showed no significant shift after incubation with ADA for up to 2 hr as examined under the same conditions (data not shown), suggesting that 3′-dAMP and 3′-dATP were poor substrates for ADA.

When the cells were incubated with [3′-^{3}H]-3′-dA and the intracellular nucleoside metabolites were subjected to HPLC analyses, four peaks representing 3′-dA, 3′-dAMP, 3′-dADP, and 3′-dATP were identified, but no additional peaks representing 3′-dI, 3′-dIMP, 3′-dIDP, or 3′-dITP were seen regardless of the presence or absence of dCF (Fig. 2). It was possible that the elution times for 3′-dI and its metabolites were similar to or the same as those for 3′-dA

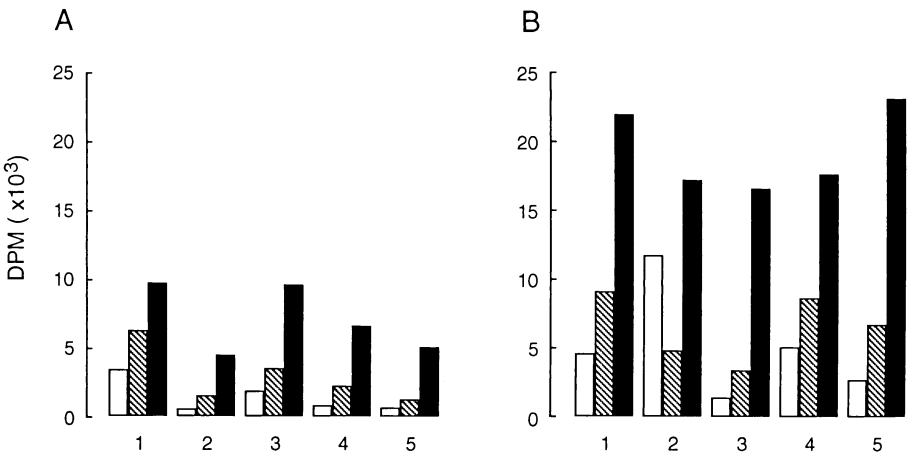

FIG. 4. Phosphorylation profiles of 3′-dA with dCF in R-PBM and PHA-PBM. R-PBM (panel A) and PHA-PBM (panel B) were incubated with 1 μM [^{3}H]3′-dA in the presence of 2.5 μM dCF for 3 hr, and intracellular nucleoside metabolites were analyzed with HPLC: 3′-dAMP (open bars); 3′-dADP (hatched bars); and 3′-dATP (solid bars). The mean amounts of 3′-dATP in R- and PHA-PBM were 2.5 and 7 pmol/10^6 cells, respectively. The same numbers in panels A and B represent identical donors.

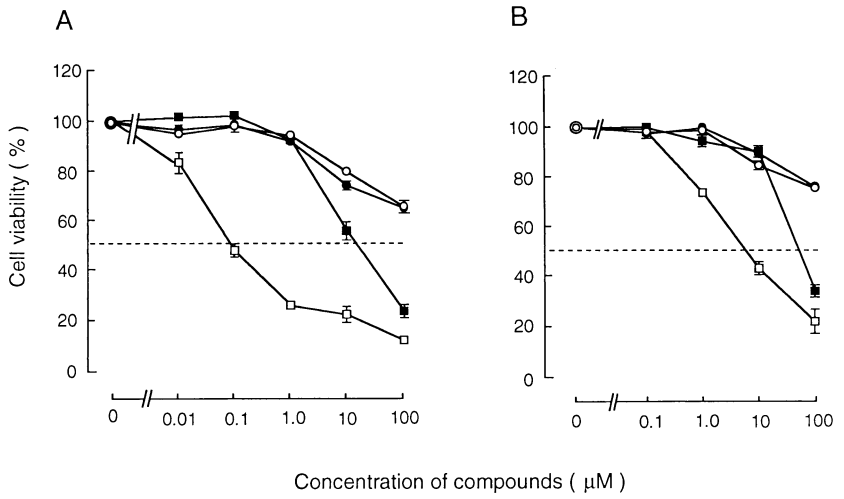

FIG. 5. Cytotoxicity of 3′-dA and 3′-dI against TdT$^+$ NALM-6 and TdT$^-$ HUT-102. NALM-6 (panel A) and HUT-102 (panel B) cells were exposed to 3′-dA alone (solid squares); 3′-dA with dCF (2.5 μM) (open squares); 3′-dI alone (solid circles); and 3′-dI with dCF (2.5 μM) (open circles). The viability of the cells shown represents the mean of triplicate determinations ± one SD, expressed as percent of the value for no drug control.

and its metabolites. It was also possible that the phosphorylation of 3′-dI was so poor that its metabolites were not distinguishable under the HPLC conditions used. Therefore, we asked whether 3′-dI metabolites were formed intracellularly, using [3′-^{3}H]-3′-dI. HPLC analyses revealed that the phosphorylation of 3′-dI was extremely low (by about 1/100) as compared with that of 3′-dA both in TdT$^+$ NALM-6 cells and TdT$^-$ HUT102 cells, and only marginal amounts of mono- and diphosphates of 3′-dA were detected in both cell lines (Fig. 3B). It also was noted that the elution time of these molecules was similar to that of 3′-dA (data not shown). These data were in agreement with our observation that 3′-dI exerted a marginal cytotoxic effect or no cytotoxic effect against TdT$^+$ NALM-6 even at an extremely high concentration, 100 μM, regardless of the presence of 2.5 μM dCF (Fig. 5).

ADA Activity in Leukemic Cells

We also asked if the intracellular level of ADA was a determinant of cellular sensitivity to 3′-dA-induced cytotoxicity. Two TdT$^+$ cell lines, NALM-6 and MOLT-4, had the highest ADA activity, and two TdT$^-$ cell lines, HUT-102 and K562, had a low level of ADA activity, as shown in Table 1. The ADA activity in cell lysates of all these four cell lines was blocked by > 95% by dCF at concentrations as low as 10 nM and was blocked completely at 2.5 μM. It was further confirmed that the intracellular ADA activity of MOLT-4 cells, which contained the highest amount of ADA (74 nmol/min/10^7 cells), essentially was abrogated completely (>98%) when these cells were incubated with 2.5 μM dCF for 10 hr.

These results strongly suggested that the level of ADA activity in cell lines examined in this study did not affect the 3′-dA-mediated antileukemic activity as long as 2.5 μM dCF was present in the culture.

TdT Activity and Its Inhibition by 3′-dA

We finally asked whether the sensitivity of the cell lines examined in this study to 3′-dA-mediated antileukemic activity was associated with the level of intracellular TdT activity. TdT activity was readily detectable in cell lysates of the NALM-6 and MOLT-4 cell lines (Table 1), but not in HUT-102 cells, K562 cells, or PHA-PBM. We further examined the expression of TdT mRNA in TdT$^+$ and TdT$^-$ cells and PBM, using the RT-PCR assay. TdT-encoding gene-specific PCR products were recognized abundantly in NALM-6 and MOLT-4 cell lines, but no signals were identified in HUT-102, K562, R-PBM, or PHA-PBM (Fig. 6).

Although 3′-dATP has been shown to inhibit calf thymus TdT activity [33], it remains to be tested whether 3′-dATP blocks human TdT activity. We, therefore, asked whether 3′-dATP inhibited the enzymatic activity of TdT isolated from NALM-6 cells. The cell extract of NALM-6 had the highest level of TdT activity (6.31 nmol/hr/10^7 cells) (Table 1), which was inhibited by 3′-dATP with an IC_{50} value of 21.3 ± 3.0 μM when examined as described in Materials and Methods. This relatively high IC_{50} value is presumably due to the experimental condition requiring a high level of dGTP (0.5 mM) as a substrate for TdT, with which a high concentration of 3′-dATP was needed to compete.

DISCUSSION

In this study we demonstrated that 3′-dA was anabolically phosphorylated to its triphosphate form, 3′-dATP, inhibited TdT activity intracellularly, and exerted potent antileukemic activity against human TdT$^+$ cells *in vitro*. In TdT$^+$ NALM-6 cells, 3′-dATP was formed rapidly up to a level of 67 pmol/10^6 cells in 3 hr in the presence of dCF, and a high level of cytotoxicity was observed. In TdT$^+$ MOLT-4 cells, the magnitude of phosphorylation of 3′-dA

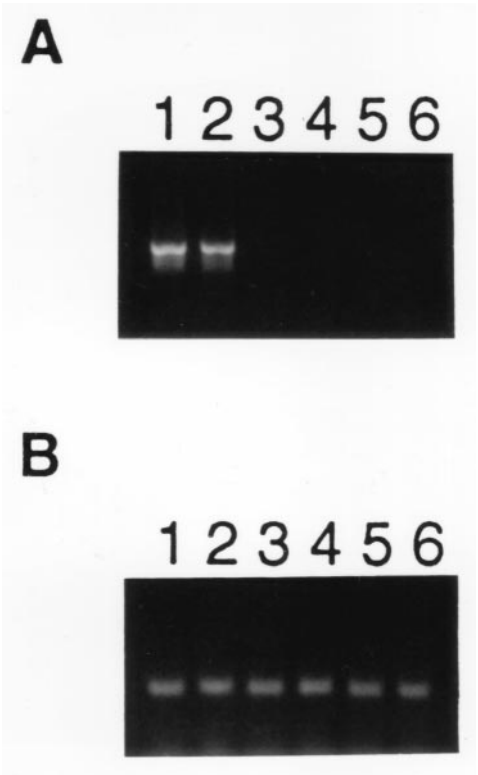

FIG. 6. TdT expression in various cell populations as assessed by RT-PCR. TdT expression (panel A) was examined with RT-PCR as described in Materials and Methods. Note that TdT mRNA was detected by PCR products (443 bp) in only the two TdT^+ cell lines, NALM-6 (lane 1) and MOLT-4 (lane 2), and not in the two TdT^- cell lines, HUT-102 (lane 3) and K562 (lane 4), R-PBM (lane 5), or PHA-PBM (lane 6). The control mRNA for β-globin was detected (panel B: PCR product size, 110 bp) in all the cell populations examined.

to 3′-dATP was quite low under the same conditions; however, these cells were highly sensitive to 3′-dA combined with dCF. In contrast, two TdT^- cell lines examined in this study, HUT-102 and K562, were both insensitive to the 3′-dA-induced cytotoxicity despite forming a substantial amount of 3′-dATP intracellularly. These results strongly suggested that 3′-dA phosphorylation was required for, but not necessarily directly associated with, the cytotoxicity of 3′-dA, and that TdT positivity was essential for 3′-dA to exert its cytotoxicity (Table 1).

It should also be noted that whereas TdT^+ cells were more sensitive to 3′-dA plus dCF than their TdT^- counterparts, this did not appear to be universally the case. Indeed, TdT^- HL-60 cells were more sensitive than TdT^+ MOLT-4 cells (Table 1), indicating that TdT positivity could not be the sole determinant of sensitivity. In this regard, even TdT^- cells were damaged by high concentrations of 3′-dA, presumably because 3′-dATP also inhibits RNA polymerases, thus resulting in cytotoxicity against TdT^- cells. 3′-dA, however, effectively inhibits the enzymatic activity of TdT; hence, TdT^+ cells are as a whole more sensitive to the toxic effect of the compound. One could argue that measurement of incorporation of 3′-dA into RNA might yield critical insights into this issue. In fact, inhibition of RNA synthesis by 3′-dA has been reported by some other groups [13, 16].

The ADA inhibitor dCF was required for the antileukemic activity of 3′-dA as assessed in cell culture assays. However, it is possible that if the target cells have a high level of ADA activity, the 3′-dA-mediated antileukemic activity might be compromised. In our study, TdT^+ MOLT-4 cells had the highest level of ADA activity, followed by TdT^+ NALM-6 cells, whereas the two TdT^- cell lines, HUT-102 and K562, had little ADA activity (Table 1), in agreement with data published by others, indicating that ADA activity is generally higher in TdT^+ cells than in TdT^- cells [9, 26]. In the absence of dCF, most 3′-dA was readily deaminated by ADA and converted to 3′-dI, which had only a weak antileukemic effect or no antileukemic effect against both TdT^+ and TdT^- cells (Table 1, Fig. 5). We confirmed, however, that 2.5 μM dCF abrogated virtually all the intracellular ADA activity, and 3′-dA exerted potent cytotoxic activity in NALM-6 and MOLT-4 cells, strongly suggesting that as long as 2.5 μM dCF is present, levels of ADA do not affect the 3′-dA-mediated cytotoxicity.

In this study, TdT expression was detected readily in NALM-6 and MOLT-4 cells by both enzymatic assay and RT-PCR assay (Table 1 and Fig. 6). In contrast, no TdT activity or expression was identified in HUT-102 cells, K562 cells, or PHA-PBM, all of which were much less sensitive to 3′-dA-mediated cytotoxicity (Table 1, Fig. 6). Indeed, much higher concentrations of 3′-dA were required for detectable cytotoxic effects on those 3′-dA-insensitive cells, with IC_{50} values reaching 8.2 to 31.8 μM (Table 1). The mechanism of this toxicity exerted by high 3′-dA concentrations is due presumably to the fact that 3′-dATP is recognized as a substrate by RNA polymerase and incorporated into the growing RNA chain, and causes RNA-chain termination of RNA transcription, leading to cellular damages [34].

It is noteworthy that the IC_{50} values of 3′-dA against R-PBM and PHA-PBM were 31.8 and 8.2 μM in the presence of dCF, higher than those against NALM-6 cells by 202- and 52-fold, respectively. These results imply that resting cells may be relatively resistant to the toxic effect of 3′-dA, whereas activated and dividing cells are more vulnerable to 3′-dA-mediated toxicity during possible antileukemic therapy. In this regard, over 95% of circulating cells are reportedly in the resting state in humans [35, 36], suggesting that most hematologic and immunologic TdT^- cells may resist the toxic effect of 3′-dA with dCF. Needless to say, caution should be used, since TdT^+, activated, dividing cells in tissues such as bone marrow or thymus, may be highly sensitive to the toxic effect of 3′-dA *in vivo*. Indeed, a toxicological study of 3′-dA combined with dCF recently revealed bone marrow and gastrointestinal toxicity as major adverse effects in beagle dogs [37]. It should be noted that the experimental setting for PBM assays is different from that for assays using cell lines (i.e. the use of

PHA for PBM assays). It should be considered to employ fresh TdT^+ tumor cells from patients with TdT^+ leukemia.

As shown in Fig. 4, once 3′-dA was deaminated, only a small amount of 3′-dIMP (or 3′-dAMP) was formed, and no significant amount of 3′-dIDP (or 3′-dADP) or 3′-dITP (or 3′-dATP) was produced. This observation suggests that 3′-dI was only poorly phosphorylated to 3′-dIMP. 3′-IMP perhaps did not get converted to 3′-dAMP, nor did it follow the pathway for ATP synthesis (Fig. 3B). This is in contrast with 2′,3′-dideoxyinosine (ddI), which is monophosphorylated efficiently by phosphotransferase (5′-nucleotidase), follows a pathway utilized for ATP synthesis with adenylosuccinate synthetase and adenylosuccinate lyase, and is converted to ddAMP and ultimately to ddATP [38]. Thus, the presence of dCF seems to be essential for the TdT-specific antileukemic activity of 3′-dA.

The major enzyme responsible for monophosphorylation of 3′-dA has been thought to be the ubiquitous AK, based on the observations that AK-deficient cells are insensitive to 3′-dA-mediated cytotoxicity [39, 40]. In this regard, we recently have generated variant NALM-6 cells, which remain TdT^+, are highly resistant to the toxicity of 3′-dA, have a variant AK with one amino acid substitution, and are capable of producing AMP but not 3′-dAMP (Kodama *et al.*, unpublished observation), supporting the concept that AK is a major enzyme for the initial phosphorylation of 3′-dA.

Taken together, the present data suggest that the antileukemic activity of 3′-dA combined with dCF requires TdT positivity of the cells and that the blockade of ADA-mediated deamination of 3′-dA is essential for its antileukemic activity. Further development of 3′-dA as a potential antileukemic agent for therapy of TdT^+ leukemia is warranted.

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