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Research Article

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Ecto-Adenosine Triphosphatase Deficiency in Cultured Human T and Null Leukemic Lymphocytes

A BIOCHEMICAL BASIS FOR THYMIDINE SENSITIVITY

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ABSTRACT Cultured leukemic T and null lymphocytes are highly sensitive to growth inhibition by thymidine, as well as the other deoxynucleosides, deoxyguanosine and deoxyadenosine. By contrast, Epstein-Barr virus-transformed B lymphocytes are relatively resistant to deoxynucleosides. Growth inhibition is associated with the development of high deoxyribotriphosphate pools after exposure to the respective deoxynucleosides. We show that malignant T and null lymphocytes are deficient in ecto-ATPase activity. We show this cell surface enzyme to be of broad specificity, capable of degrading both ribotriphosphates and deoxyribotriphosphates. High levels of this ecto-enzyme are found in deoxynucleoside-resistant, Epstein-Barr virus-transformed B lymphocytes. Ecto-ATPase deficiency may represent a mechanism for increased sensitivity to deoxynucleoside growth inhibition.

INTRODUCTION

The arrest of cell growth by millimolar concentrations of thymidine (TdR) is well established. The mechanism of this effect is believed to reflect inhibition of DNA replication by an increase in the cellular dTTP pool, which allosterically inhibits ribonucleotide reductase. This results in depression of the dCTP pool below a level critical for continued DNA replication (1).

More recently there has been interest in the clinical use of TdR as an antitumor agent following demonstration that TdR could inhibit human tumor xenografts in nude mice (2–4). We and others have reported that cultured leukemic lymphocytes of T and null cell type are extremely sensitive to growth inhibi-

tion by TdR at concentrations two orders of magnitude below millimolar levels. The mechanisms of this increased sensitivity appear to reflect the ability of these leukemic cells to accumulate excess dTTP at low concentrations of exogenous TdR (5–7).

These malignant cells are also similarly sensitive to low concentrations of deoxyguanosine (GdR) and deoxyadenosine (AdR). Again, the mechanisms of this inhibition appear to relate to accumulation of the triphosphates of these deoxynucleosides. An analogy has been drawn between the phenomena of deoxynucleoside-sensitive T and null leukemic cells and the immune-deficiency syndromes associated with adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiency. These defects in nucleoside catabolism result in accumulation of high levels of deoxyribonucleoside triphosphates (dNTP)—dATP in ADA deficiency and dGTP in PNP deficiency. It has been postulated that these excess dNTP act as feedback inhibitors of ribonucleotide reductase, causing inhibition of DNA replication in lymphocyte precursors, resulting in immune defects (5, 7, 8). Thus, these clinical immunodeficiency syndromes associated with inborn errors of purine catabolism and the deoxynucleoside sensitivity of leukemic lymphocytes indicate a specific vulnerability of lymphocytes (particularly T cells) at some stage in their differentiation to dNTP intoxication.

We now show that malignant T and null lymphocytes are deficient in ecto- (Ca^{++}, Mg^{++})adenosine triphosphate γ-hydrolase (Ecto-ATPase). We have found this ecto-triphosphatase to be a cell-surface enzyme of broad specificity capable of degrading both ribo- and deoxyribonucleoside triphosphates. High levels of this ecto-enzyme are found in deoxynucleoside-resistant Epstein-Barr virus (EBV)-transformed peripheral blood B lymphocytes. Ecto-ATPase de-

ficiency may represent a mechanism unifying the dNTP accumulation in malignant T and null cells, and ADA and PNP deficiency.

GLOSSARY

ADA	Adenosine deaminase
AdR	Deoxyadenosine
ADP	Adenosine diphosphate
CdR	Deoxycytidine
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNTP	Deoxyribonucleoside triphosphates
dTDP	Thymidine diphosphate
dTMP	Thymidine monophosphate
dTTP	Thymidine triphosphate
EBV	Epstein-Barr virus
Ecto-ATPase	Ecto-(Ca^{++}, Mg^{++})adenosine triphosphate-hydrolase
EHNA	Erythro-9-(3-(2-hydroxy-nonyl))adenosine
GdR	Deoxyguanosine
ID_{50}	Inhibitory dose, 50%
PEI	Polyethyleneimine
PNP	Purine nucleoside phosphorylase
TdR	Thymidine
TLC	Thin-layer chromatography

METHODS

Cell lines and culture

Cultured human leukemic lymphocytes derived from patients with various types of acute lymphoblastic leukemia, and lymphocytes transformed by EBV were kindly provided by Dr. H. Lazarus (Sidney Farber Cancer Institute, Boston, Mass.), J. Minowada (Roswell Park Memorial Institute, Buffalo, N. Y.), I. Jack (Royal Children's Hospital, Melbourne, Victoria), and H. Zola (Flinders Medical Centre, Adelaide, South America).

The normal peripheral blood lymphocyte lines transformed by EBV were JE-Tg, WIL, JP, and LAZ 007. The lines derived from patients with acute lymphoblastic leukemia were (T cell) CCRF-CEM, 8402, CCRF-HSB, HPB-MLT, JM, and MOLT-4; and (null cell) Reh and KM-3. The origin and characteristics of the malignant cells have been previously described and summarized by Minowada (9). All cell lines were grown in suspension culture in RPMI 1640 media supplemented with 10% fetal calf serum. The lines had approximately similar doubling times (24–30 h). Cells were studied in the log-phase of growth.

Isotopes and chemicals

[Methyl-^{3}H]dTTP (45 Ci/mmol), D-[5-^{3}H]CTP (18.4 Ci/mmol), D-[8-^{3}H]ATP (13.8 Ci/mmol), D-[8-^{3}H]GTP (7.4 Ci/mmol), [8-^{14}C]AMP (59 mCi/mmol), [8-^{3}H]ATP (22 Ci/mmol), [methyl-^{3}H]ATP (45 Ci/mmol) and [6-^{3}H]TdR (5.0 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. Unlabeled dTTP, dCTP, dATP, dGTP, dTDP, dTMP, ADP, and AMP were obtained from Sigma Chemical Co., St. Louis, Mo., and P. L. Biochemicals, Milwaukee, Wis. Micrococcus luteus DNA polymerase and templates for the DNA polymerase assay for deoxytriphosphates poly (dA-dT) and poly (dI-dC) were purchased from Miles Laboratories, Elkhart, Ind. TdR, GdR, AdR, and CdR were purchased from Calbiochem Behring Corp., La Jolla, Calif. PEI cellulose thin-layer chromatography (TLC) plates were obtained from Merck, AG, Darmstadt, West Germany).

Erythro-9-(3-[2-hydroxy-nonyl]) adenosine (EHNA) was a gift from Dr. C. Nichol, Burroughs Wellcome Ltd., Research Triangle, N. C.

Enzyme assays

TdR kinase. This was assayed as described by Taylor et al. (10). Cells were harvested from log-growth cultures by centrifugation and resuspended in buffer (0.05 M Tris HCl, pH 7.5, 1 mM EDTA, and 5 mM β-mercaptoethanol) at a concentration of 1×10^7/ml. The cells were disrupted by sonication and then centrifuged (27,000 g for 10 min at 4°C). Aliquots of the supernate (without prior dialysis), containing 10–100 μg of protein, were added to a reaction mixture (final concentration 0.06 M Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, and 12 μM [6-^{3}H]TdR, 1 Ci/mmol. The final volume of the assay was 100 μl. The reaction mixtures were incubated for 30 min at 37°C and terminated by boiling for 4 min. 50 μl of reaction mixture was spotted onto Whatman DEAE paper squares, washed four times in ammonium formate (0.001 M), then four times in methanol, dried, and counted in a liquid scintillation counter. The assay was linear to 40 min incubation time and with protein.

TdR monophosphate kinase. This assay was performed as above with the exception that [methyl-^{3}H]dTMP (0.3 Ci/mmol, 30 μM) was used as substrate replacing [^{3}H]TdR. The reaction mixture was incubated for 20 min at 37°C and terminated as above. Aliquots of reaction mixture were spotted onto PEI TLC plates and chromatographed with 0.4 M LiCl. Markers consisted of cold dTMP, dTDP, and dTTP. After chromatography, the marker areas were identified under ultraviolet light, cut out, and counted in a liquid scintillation counter. This assay was linear with time and protein, and dTMP was saturating.

Ecto-ATPase. This was measured using a modification of the method of De Pierre and Karnovsky (12). Cells, in log growth, were harvested by centrifugation, and 0.8×10^4 cells were resuspended unwashed in a reaction mixture of 50-μl volume. Incubations were performed in duplicate at 37°C, and the reaction mixture consisted of RPMI 1640 culture medium containing 1 mM [8-^{3}H]ATP (0.5 μCi) or 1 mM [methyl-^{3}H]TTP (0.5 μCi). The mixture was incubated for 30 min, and the reaction terminated by heating at 80°C for 4 min, and the cells pelleted by centrifugation. 5 μl of supernate was spotted with the appropriate cold triphosphate, diphosphate, monophosphate, and deoxynucleoside onto PEI TLC plates. The compounds were separated by elution with $LiCl_2$. The ATP separation required 1 M $LiCl_2$, and for dTTP, 0.6 M $LiCl_2$ was used. This assay was linear with cell number and up to 40 min.

Ecto-5'nucleotidase. This was measured by a modification of the method described by Wortmann et al. (7). Incubations were performed in duplicate at 37°C in a reaction volume of 50 μl containing 0.1–0.2 $\times 10^6$ intact lymphocytes. The reaction mixture consisted of RPMI 1640 culture medium containing 0.5 mM [8-^{14}C]AMP (0.05 μCi) or [8-^{3}H]dTMP (0.05 μCi). The mixture was incubated for 15 min (B cells) or 30 min (T cells). The reaction was terminated by heating at 80°C for 5 min. The cells were then pelleted by centrifugation. 5 μl of supernate was spotted with cold adenosine and AMP or thymidine and TMP onto PEI TLC plates. Nucleosides and nucleotides were separated by elution with methanol/water (1:1). The two ultraviolet absorbing spots were cut out and radioactivity counted in a liquid scintillation counter.

Deoxynucleoside inhibition of cell growth. This was performed as previously described by us (6).

DNA polymerase assay for deoxynucleoside triphosphates. This was performed as previously described by us (13) except

that cells were extracted with 60% ethanol rather than 60% methanol. This assay is a modification of the methods described by Solter and Handschumacher (14) and Lindberg and Skoog (15). Results are the means of duplicate assays.

Cytochemical stain for ATP triphosphatase. This was done by a modification of the method of Wachstein and Meisel (16). Cells were harvested by spinning onto glass slides using a cytocentrifuge (Shandon Southern Instruments Ltd., Frimley, Surrey, Eng.).

The slides were air dried and incubated in the reaction mixtures at 37°C for 1 h. After incubation the slides were rinsed in water, immersed in 0.2% aqueous ammonium sulphide for 1 min, water washed, and then rinsed in methanol for 30 s. The cell nuclei were counter-stained in a methylene blue solution (0.005%) in 0.005% borax and then rinsed in water and air dried.

TdR uptake studies. These were performed as described by Cohen et al. (17). 2 ml of cells in the log-growth phase (0.5 × 10⁶ cells/ml) were incubated in quadruplicate with 10 μM [*methyl*-³H]TdR (50 mCi/mol) at 37°C in culture medium for 30 min. The incubation was terminated by addition of 10 ml of cold phosphate-buffered saline, and the cells were washed three times with this cold phosphate-buffered saline. The cells were pelleted by centrifugation, and then lysed by addition of 0.5 ml of 2 N $HClO_4$. 200-μl aliquots were then counted in Aquasol.

RESULTS

Role of TdR kinase in TdR sensitivity. We have previously shown that sensitivity to TdR varied some 200-fold among different types of cultured human lymphocytes (6). The inhibitory dose (50%) (ID_{50}) of nonmalignant EBV-transformed lines was ~2 mM TdR (0.5–5), whereas acute lymphoblastic leukemic lines of T and null cell type were much more sensitive (ID_{50}-10–200 μM).

We studied an additional leukemic line of T cell type (JM), which was found to be resistant to growth inhibition by TdR. Its ID_{50} for TdR was 1.7 mM, a level similar to that of EBV-transformed peripheral blood B lymphocytes, but was 100–200-fold higher than that of TdR-sensitive cultured T and null cell leukemic lines (Table I).

TdR kinase activity was assayed in extracts of the various lymphocyte cell lines. The activity in the TdR-resistant T cell line (JM) was almost undetectable (0.2 nmol/mg per h) compared with the levels in the TdR-sensitive T and null cell lines (mean 11.47±7.16). However, the resistant EBV-transformed B cell lines had TdR kinase activity levels similar to those of the TdR-sensitive T and null cell lines (Table I).

Uptake of TdR by cultured lymphocytes. The TdR kinase-deficient T cell line (JM) had deficient uptake of [³H]TdR. There was no difference in uptake among the TdR-sensitive leukemic lines (T and null cells) and the TdR-resistant, EBV-transformed B cell lines. However, one of the T leukemic lines (MOLT 4), which had an intermediate level of TdR sensitivity (0.2 mM) had reduced uptake of radiolabeled TdR, despite a TdR

TABLE I
TdR Sensitivity, TdR Kinase Activity, and TdR Uptake of Cultured Lymphocytes

Cell lines	ID_{50} thymidine	TdR kinase	[³H]TdR uptake
	μ	nmol/mg protein/h	pmol/10⁶ cells/h
T leukemic lymphocytes			
CCRF-CEM	37 μm	6.17	67.7
8402	50 μm	10.8	72.2
CCRF-HSB	27	16.2	60.0
HPB-MLT	54	24.1	93.1
Molt 4	0.18 mM	16.9	11.0
JM	1.7 mM	0.023	3.0
Null leukemic lymphocytes			
Reh.	26 μm	4.7	19.3
KM-3	14 μm	17.8	55.0
EBV-transformed B lymphocytes			
Je-Tg	3.8 mM	5.47	99.0
WIL	2.2 mM	1.46	85.6
JP	3.6 mM	16.1	126.3
LAZ 007	1.0 mM	3.44	74.9

kinase level similar to the other TdR-sensitive T cell lines (Table I).

Effect of TdR on the dTTP pools of cultured lymphocytes. The various cell lines were incubated with TdR (50 μM) for 24 h, and then the dTTP pools were assayed. The T and null leukemic lines showed marked elevation of their dTTP pools; however, the EBV B cell lines failed to elevate their dTTP pool (Table II). In contrast, little change was seen in the dTTP pools of the TdR-resistant TdR kinase-deficient T cell line (JM) (Table III).

TdR monophosphate kinase. TdR monophosphate kinase activity, measured by the conversion of dTMP to dTDP and dTTP, was similar in the TdR-sensitive T cell line (CEM) with a value of 57 nmol/h per mg protein, and 36 nmol/h per mg protein in the TdR-resistant B cell line (LAZ).

Relationship of TdR sensitivity to deoxyguanosine and deoxyadenosine sensitivity. As well as being extremely sensitive to growth inhibition by TdR, the cultured leukemic lymphocytes of T and null type were sensitive to GdR and AdR, though resistant to CdR. The growth of EBV-transformed peripheral blood B lymphocytes was not inhibited by these deoxy-nucleosides (Table IV). The leukemic T cell lines showed increased sensitivity to deoxyadenosine if treated with the ADA inhibitor EHNA.

Following exposure to growth-inhibitory concentrations of GdR or AdR (in the presence of EHNA), there was a rapid rise in the intracellular pool of dGTP or

TABLE II
dTTP Accumulation by Cultured Cell Lines following Incubation with TdR

Cell lines	Control dTTP pool	dTTP increase
	pmol/10⁶ cells	% control level
T leukemic lymphocytes		
CCRF-CEM	20	585
8402	13	479
CCRF-HSB	27	359
Null leukemic lymphocytes		
Reh	19	1,116
KM-3	19	200
EBV-transformed B lymphocytes		
Je Tg	27	104
WIL	32	134
LAZ 007	25	120
JP	23	113

The cell lines were cultured as described and then incubated with 50 μM TdR for 24 h, and the cells harvested by centrifugation. The dTTP pools were then assayed by the DNA polymerase method. Control dTTP pool variation during the incubation period was ±8%.

dATP, respectively, in a T cell line compared with little change in a B cell line. This was similar to the rise in dTTP level after incubation with TdR (Table V). These elevated levels of dNTP fell, after the cells were resuspended in fresh media, to control levels by 48–72 h.

Degradation of dTTP by cultured lymphocytes. The breakdown of dTTP by a leukemic T cell line (CCRF-CEM) and an EBV B cell line (LAZ 007) was studied using cells disrupted by sonication. There was rapid degradation of dTTP, via dTDP, to dTMP by sonicated LAZ 007 line (1.5 × 10⁵ cells/ml). There was <1%

TABLE III
Influence of TdR on the dTTP Pools in Cultured Lymphocytes

Time of incubation	T cell, TdR sensitive (CCRF-CEM)	T cell, TdR resistant (JM)	EBV-transformed B cell (LAZ 007)
h	dTTP pool % control		
1	370	115	260
2	460	120	180
24	1150	105	130
48	580	110	95
72	300	85	70

Cells were incubated with 60 μM TdR harvested at the time intervals indicated, ethanol extracted, and their dTTP pools assayed using the DNA polymerase assay. Results are expressed as percent change from untreated controls. Control dTTP pool variation during the incubation period was ±8%.

TABLE IV
Deoxynucleoside Sensitivity of Cultured Lymphocytes

Cell line	I.D.₅₀		
	Deoxy-guanosine	Deoxy-adenosine	Deoxy-cytidine
T leukemic lymphocytes			
CCRF-CEM	23 μM	0.35 mM	>3 mM
8402	30 μM	0.20 mM	>3 mM
CCRF-HSB	75 μM	0.32 mM	>3 mM
HPB-MLT	16 μM	0.18 mM	>3 mM
Null leukemic lymphocytes			
Reh	40 μM	0.5 mM	>3 mM
KM-3	50 μM	0.2 mM	>3 mM
EBV-transformed B lymphocytes			
Je-Tg	1.5 mM	>3 mM	>3 mM
WIL	0.10 mM	>3 mM	>3 mM
GK	0.85 mM	>3 mM	>3 mM
LAZ-007	0.24 mM	>3 mM	>3 mM

conversion of dTMP to TdR under the conditions of this assay (Fig. 1A). The CCRF-CEM line sonicate failed to degrade dTTP (<1%) at 1.5 × 10⁵ cells/ml; however, by increasing the concentration of these cells to 0.5 × 10⁸/ml, 30% of the dTTP was degraded to dTMP after 30 min incubation. The concentration of dTTP in these experiments was 0.5 mM. Thus, the relative degradation rates were 5.3 μmol dTTP/10⁶ cells/h in the B cell line compared with 0.006 μmol/10⁶ cells/h in the T cell line.

TABLE V
Change in Deoxynucleotide Pool after Incubation with Deoxynucleoside

Deoxynucleoside	Deoxy-nucleotide	T cell (CCRF-CEM line)	B cell (LAZ-007 line)
		% control level	
TdR (50 μM)	dTTP	572±30	138±22
GdR (60 μM)	dGTP	2887±322	127±18
AdR (60 μM) plus EHNA (5 μM)	dATP	1845±96	272±14

The cell lines were cultured as described and then incubated with TdR, GdR, or AdR at the concentrations indicated for 4 h and the cells harvested by centrifugation. The respective dNTP pools in the cells were then assayed by the DNA polymerase method. The base-line dNTP pool levels (pmol/10⁶ cells) in counted cells were (a) CCRF-CEMcells; dTTP 64±3, dGTP 33±1, dATP 44±1 and, (b) LAZ 007 cells; dTTP 36±8, dGTP 3±0.4 and dATP 12±2, p mol/10⁶ cells, respectively. All incubations were performed in quadruplicate.
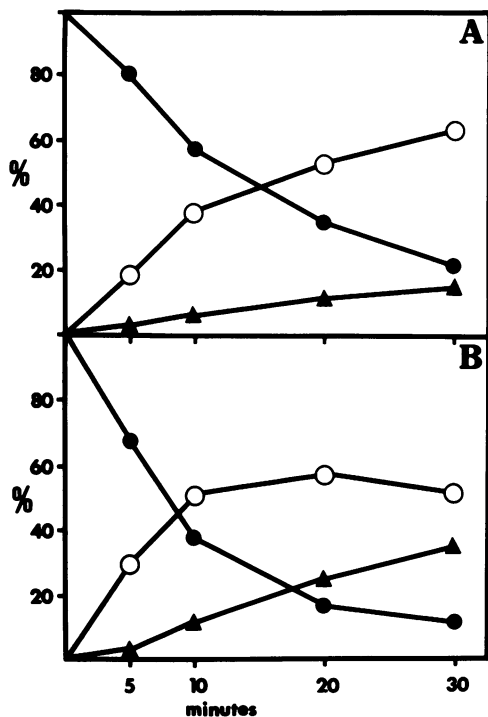
FIGURE 1 An EBV-transformed B lymphocyte line (LAZ 007) was harvested in log-growth phase and resuspended in fresh medium at 1.4 × 10⁵/ml. In experiment A the cells were disrupted by sonication, and in B they were allowed to remain intact. The degradation of [³H]dTTP was studied after incubation at 37°C for varying time intervals, as described in Methods. ●, dTTP; ○, dTDP; ▲, dTMP.

TABLE VI
Ecto-enzyme Activities of Cultured Cell Lines

Cell lines	Ecto-triphosphatase substrate		Ecto-5'-Nucleotidase	
	ATP	dTTP	AMP	dTMP
	$\mu mol/10^6\ cells/h$		$nmol/10^6\ cells/h$	
T leukemic lymphocytes				
CCRF-CEM	<0.1	<0.1	<1	<1
8402	<0.1	<0.1	2.2	<1
CCRF-HSB	<0.1	<0.1	2.4	<1
HPB-MLT	<0.1	<0.1	4.0	<1
Molt 4	<0.1	<0.1	<1	<1
JM	<0.1	<0.1	6.0	<1
Null leukemic lymphocytes				
Reh	0.128	0.1	12.4	10.9
KM-3	<0.1	<0.1	9.2	15.6
EBV-transformed B lymphocytes				
JeTg	0.684	0.76	16.4	21
WIL	3.30	2.05	7.6	19
LAZ-007	2.90	3.03	14.4	7.3
JP	0.979	1.23	6.6	12.9

We had previously noted, in a study of uptake of dNTP into EBV-transformed B lymphocytes made permeable by DEAE-dextran, that after incubation of intact cells with dTTP there was rapid extracellular degradation of dTTP via dTDP to dTMP (18). This breakdown of dTTP appeared to represent activity of a cell surface phosphatase, since its activity was not reduced by washing the cells nor was enzyme activity found in the growth medium (18). We therefore compared the breakdown of dTTP by sonicated LAZ 007 (EBV B lymphocytes) with intact cells (Fig. 1B). The rate of degradation of dTTP was similar although slightly more rapid in the intact cells.

We subsequently studied the activity of this ecto-dTTPase in T, null, and B type cultured lymphocytes, as well as assayed the activity of ecto-ATPase (19). The EBV-transformed B lymphocyte lines had high levels of ecto-triphosphatase activity using either ATP or dTTP as substrate (Table VI). Activity ranged from 1–3 μmol substrate consumed/10^6 cells per h. The malignant T and null cell lines, which were sensitive to deoxynucleosides, had very low levels of activity to both ATP and dTTP (<0.1 μmol/10^6 cells per h). These differences were confirmed cytochemically by an ATPase stain (Fig. 2). Levels of ecto-5'-nucleotidase, using AMP and dTMP as substrate, were also assayed in these cells. Higher activity was found in the null leukemic cell lines and the EBV-transformed B cell lines (Table VI).

Characteristics of the EBV-transformed B lymphocyte ecto-triphosphatase. Some characteristics of this ecto-triphosphatase present in the EBV-transformed B lines were studied in the LAZ 007 cells. The Michaelis constants (K_M) for ATP and dTTP were similar (~0.5 mM), and ATP could inhibit the degradation of dTTP and vice versa. It is not possible to demonstrate unequivocally whether these inhibitions were competitive or noncompetitive. This reflected difficulty in obtaining straight line initial rates, a feature of ecto-enzymes kinetics previously noted (19).

The LAZ 007 line could degrade dATP, dCTP, and dGTP with apparently similar facility to dTTP (Table VII). The activity against ATP or dTTP was inhibited only 10% by the nonspecific phosphatase substrate p-nitrophenylphosphate (5 mM) and was not inhibited by Ouabain (2 mM). The specificity of this ecto-triphosphatase for triphosphates was demonstrated by the ability of the ATP analogue, γ,β methylene ATP (5 mM) to inhibit both ATPase and dTTPase activity by 80%.

Ecto-triphosphatase activity was distinguishable from ecto-5'-nucleotidase activity. Ecto-triphosphatase activity was sensitive to inhibition by detergents. When Triton X-100 (0.1–0.5%) was added to the assay
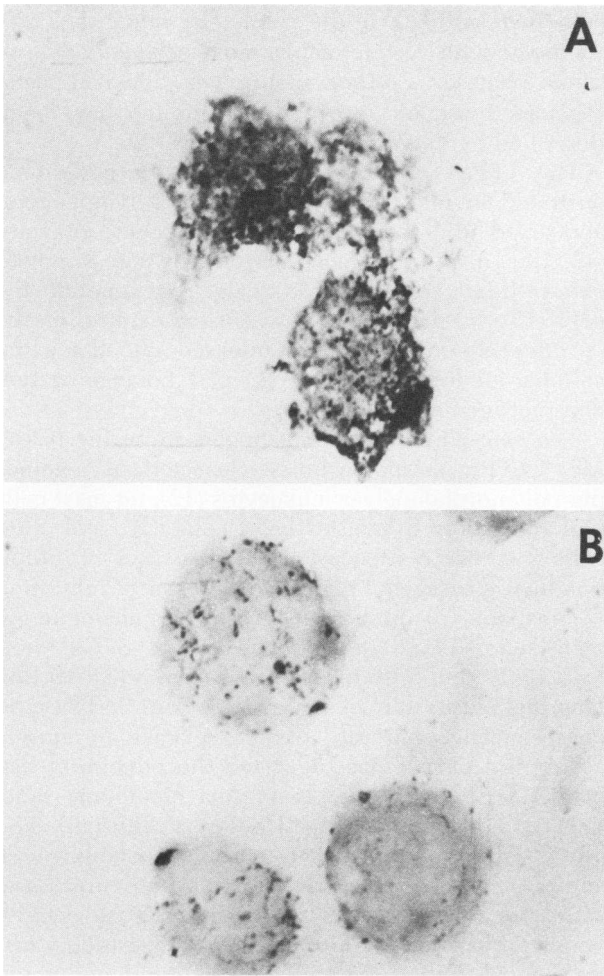
FIGURE 2 Cytochemical demonstration of ATPase activity. Cells were harvested onto glass slides using a cytocentrifuge and stained for ATPase activity as described in Methods. (A) EBV-transformed B lymphocyte line (LAZ 007). (B) T acute lymphoblastic leukemic line (CCRF-CEM).

TABLE VII

Ecto-triphosphate Activity in an EBV-transformed B Cell Line (LAZ 007)

Deoxyribotriphosphate substrate	Specific activity
	μmol substrates degraded/10^6 cells/h
dTTP	4.6
dCTP	2.9
dGTP	3.9
dATP	3.1

(Table VIII). We have previously shown this analogue to inhibit ecto-5'-nucleotidase activity for both AMP and dTMP in these cell lines (20).

The influence of divalent cations on ecto-triphosphatase activity in an EBV-transformed B cell line was investigated by washing and resuspending the LAZ 007 line in a cation-free buffer. In a series of separate experiments, Ca^{++} and Mg^{++} ions were added at a concentration of 0.1–10 mM, using either ATP or dTTP as substrate. Dependence of the reaction on cation was demonstrated, and both ions were equally capable of restoring activity. Maximal activity was achieved at 0.5 mM, and this activity was not altered by increasing the cation concentration to 10 mM. The activity of ATPase and dTTPase at optimal Mg^{++} and Ca^{++} concentration was the same when assayed with cells suspended in culture medium, which contain Mg^{++} and Ca^{++}.

DISCUSSION

The phenomenon of TdR growth inhibition at low concentration of exogenous TdR appears to reflect the ability of a cell to accumulate and sustain an increase

system containing an intact, EBV-transformed B cell line, there was 80% inhibition of ATPase activity and complete inhibition of dTTPase activity. Similarly the detergent Nonidet P40 (0.25–1.0%) inhibited ATPase activity by 80% and dTTPase activity by 88%. Neither of these detergents inhibited the activity of ecto-5'-nucleotidase.

Furthermore, ecto-triphosphatase activity for ATP was not inhibited by mononucleotide substrates (AMP or TMP) of the ecto-5'-nucleotidase reaction. The ecto-triphosphatase activity for dTTP was not inhibited by TMP, but was partially inhibited by AMP (Table VIII). The reason for this discrepancy is not apparent. The specific inhibitor of ecto-5'-nucleotidase, adenosine 5'-(α,β-methylene) diphosphate (1–5 mM) did not inhibit ecto-triphosphatase activity using ATP as substrate but ~20% when dTTP was substrate

TABLE VIII

EBV-transformed B Lymphocyte Ecto-triphosphatase Characteristics

Addition	ATPase	TTPase
	% control activity	
AMP		
1 mM	114	83
5 mM	91	56
10 mM	97	35
dTMP		
1 mM	98	105
5 mM	105	90
10 mM	90	100
$\alpha\beta$-Methylene ADP		
1 mM	114	85
5 mM	106	76

in the dTTP pool. This allosterically affects ribonucleotide reductase, resulting in increased levels of dGTP and a reduction in the level of dCTP. We have shown that reversal of TdR inhibition of cell growth in these sensitive cells by CdR correlates with return of the dCTP pool to normal (21).

The mechanism by which particular cells elevate their dTTP pool at low exogenous TdR concentrations in comparison to insensitive cells could reflect differences in (a) membrane transport of TdR, (b) anabolism of TdR to dTTP, or (c) catabolism of TdR and/or its phosphorylated metabolites.

We found a leukemic T cell line (JM) which appears to be a spontaneous mutant for TdR kinase. This cell line is resistant to TdR and does not elevate its dTTP pool after exposure to TdR. This cell line demonstrates the necessity for TdR kinase activity in the development of TdR toxicity, which is thus nucleotide dependent. However, significant differences in TdR kinase activity between the sensitive T and null cell lines and the insensitive B cell lines were not found. This implies that the kinase step, though necessary, is not pivotal in explaining TdR sensitivity. Similarly, there were no significant differences in cell uptake of TdR or in TdR monophosphate kinase activities that could explain TdR sensitivity.

Recently, Carson et al. (5) and Wortmann et al. (7) have postulated reduced breakdown of deoxyribotriphosphate as a mechanism for the development of a high deoxynucleoside triphosphate pool following exposure to either deoxyadenosine or deoxyguanosine (5, 7). They have shown cultured leukemic T cell lines to be deficient in ecto-5'-nucleotidase compared with high levels in EBV-transformed B lymphocyte lines, and have correlated these findings with deoxynucleoside sensitivity and resistance. We have confirmed these findings, and have reported that the cultured leukemic lymphocytes of null type which are deoxynucleoside sensitive have levels of ecto-5'-nucleotidase similar to those of resistant EBV-transformed B lymphocytes (20); however, the null type lymphocytes are deficient in ecto-triphosphatase activity. We have therefore inferred that ecto-5'-nucleotidase does not have a central role in determining deoxynucleoside sensitivity (20).

The finding that cultured leukemic null lymphocytes, as distinct from the T lymphocytes, possess ecto-5'-nucleotidase while both are deficient in ecto-triphosphatase activity is consistent with the view that ecto-5'-nucleotidase and ecto-triphosphatase are distinct enzymes. This view is supported by (a) the destruction of ecto-triphosphatase activity but not ecto-5'-nucleotidase activity by detergent, (b) the failure of AMP or dTMP, substrates for the ecto-5'-nucleotidase, to inhibit ecto-triphosphatase activity, (c) activity of

ecto-5'nucleotidase in the nanomolar range (5, 7, 20) compared with the micromolar range of activity of ecto-triphosphatase. Further distinction between ecto-5'-nucleotidase and ecto-ATPase activities have been described by DePierre and Karnovsky (22).

The ATPase and dNTPase activity we have demonstrated in intact EBV-transformed B lymphocytes appears to fulfill the accepted criteria for ecto-enzymes (12, 19): (a) the enzyme(s) act on substrate supplied extracellularly; (b) the substrate does not penetrate the cells (18); (c) the products are located extracellularly; (d) the ecto-enzyme is not released into the extracellular medium (18); and (e) cell homogenization does not increase its activity.

The ecto-ATPase activity appears to be the (Ca^{++}, Mg^{++}) ATP γ-phosphohydrolase characterized in guinea pig polymorphonuclear leukocytes (12), rat mast cells (23) and cultured hamster heart cells (24), and other cells (19). The evidence for this is (a) lack of inhibition by p-nitrophenyl phosphate, (b) lack of inhibition by ouabain, (c) inhibition by the ATP analogue γ,β methylene ATP, (d) dependence on Mg^{++} or Ca^{++} ions for activity, and (e) inhibition by Triton X (12, 19, 23). The biological role of ecto-(Ca^{++}, Mg^{++})ATPase is obscure. The similarity of ecto-ATPase to muscle actomysin ATPase has suggested the possibility that ecto-ATPase contributes to plasma membrane function in cell contact and cell deformability (19). The biological significance of its absence in leukemic T and null cells is apparently then more obscure. However, the possibility that low ecto-ATPase activity is related to some T cell function is suggested by histochemical studies of normal human lymph nodes and spleens demonstrating negative ATPase in T cell regions compared with positive B cell regions (25). Furthermore, the ATPase activity of human peripheral blood B lymphocytes is higher than T lymphocytes (26).

The apparent ability of ecto-ATPase to also degrade dNTP does not appear to have been previously reported. We have not unequivocally demonstrated a single enzyme to be responsible for this broad ribo- and dNTP specificity; however, the similarity in activity, effects of inhibitors, metal-ion requirement, and cell distributions suggests this may be so. The rapid and sustained accumulation of dNTP on exposure to deoxynucleosides correlates with growth inhibition and lack of ecto-triphosphatase activity. The way in which an ecto-triphosphatase could influence intracellular dNTP pool levels is not clear, however. It should be noted that Skoog and Bjursell (27) have shown during the G_1 phase of the cell cycle the largest amounts of dNTP are in the cytoplasm, whereas during S phase the relative amounts of dNTP in the nucleus increase. Could these phenomena and dNTP pools be controlled at the plasma membrane? There is little known about

the possibility of an ecto-enzyme on the plasma membrane influencing levels of intracellular nucleotides by degradation. Thus, the possibility remains that the absence of triphosphatase activity in T lymphoblasts and null cells may be a marker for cells susceptible to deoxynucleoside toxicity rather than play a role in actual intracellular nucleotide metabolism. It should also be noted that the studies described in this report relate to cultured cell lines. Clearly, future investigations should be directed at T and B lymphocytes isolated directly from human blood and tissues. In particular, note that these cultured cell models represent distinct forms of transformation, i.e., leukemic and viral.

The similarity of the selective toxicity of TdR, GdR, and AdR for cultured T and null cells and the predominant T cell defect in ADA and PNP deficiency has been previously commented upon (5, 7). It appears necessary to invoke a biochemical phenomenon unique to T cells (and null cells) in addition to defects in deoxynucleoside catabolism—e.g., ADA deficiency, ADA inhibition by drugs, PNP deficiency, and TdR phosphorylase deficiency—to explain deoxynucleoside sensitivity (6). If leukemic T and null lymphocytes mirror a particular stage in normal lymphocyte biochemical ontogeny, then ecto-triphosphatase deficiency may represent a biochemical mechanism common to the abnormality of lymphoid cell function in ADA and PNP deficiency. However the possibility that the manifestations of ADA deficiency are mediated by a nucleotide-independent process inactivation of *S*-adenosylhomocysteine hydrolase with accumulation of *S*-adenosylhomocysteine remains to be determined (28).

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