Biochemical Pharmacology, Vol. 34, No. 17, pp. 3061–3070, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

NUCLEOTIDASE ACTIVITIES OF HUMAN PERIPHERAL LYMPHOCYTES*

FREDERICK W. BURGESS†, MAHMOUD H. EL KOUNI and ROBERT E. PARKS, JR.
Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

(*Received* 3 *October* 1984; *accepted* 3 *January* 1985)

Abstract—Several B lymphoblastic cell lines are known to be relatively resistant to the combination of 2′-deoxyadenosine with an adenosine deaminase inhibitor. These cell lines are believed to have a greater capacity to dephosphorylate 2′-deoxyadenosine nucleotides, thus preventing excessive accumulation of potentially toxic metabolites. In this study, the 2′-deoxynucleoside 5′-monophosphate dephosphorylating activities of human peripheral lymphocytes were examined. Peripheral lymphocytes have at least three nucleotide 5′-monophosphate nucleotidases distinguished by different pH optimums, substrate preference, Mg^{2+} requirement, inhibitors, and molecular weights. Two of the enzymes appeared to be cytosolic, only one of which had significant substrate activity with dAMP. This enzyme had an acidic pH optimum (5.0), no Mg^{2+} requirement, was inhibited by tartrate, and demonstrated broad substrate specificity. The other cytosolic nucleotidase required Mg^{2+}, had a pH optimum of 5.5 to 6.0, was activated by 2′-deoxyinosine, and demonstrated a substrate preference for 3′- and 5′-monophosphate 2′-deoxynucleosides of hypoxanthine, guanine, uracil, and thymine. The third enzyme, ecto 5′-nucleotidase, is associated with the cell membrane. Although the ecto 5′-nucleotidase activity was higher in the B lymphocytes, the cytosolic nucleotidases were similar in activity in the T and B lymphocytes.

Lymphoid cells that lack adenosine deaminase (ADA‡; adenosine aminohydrolase, EC 3.5.4.4) are extremely sensitive to the toxic effects of 2′-deoxyadenosine (dAdo) [1–6]. Intracellular dATP accumulation, and inhibition of ribonucleoside diphosphate reductase and DNA synthesis have been proposed as explanations of the cytotoxicity [7]. However, not all lymphoid cells are equally sensitive to dAdo. Many leukemic cell lines, especially those of B lymphocyte origin, are relatively resistant to dAdo cytotoxicity *in vitro* [1, 5, 8, 9], even in the presence of an ADA inhibitor, and accumulate far less dATP than do T lymphoblasts [5]. These, dAdo-resistant B cell lines do not differ from the more sensitive T lymphocyte cell lines in their capacity to phosphorylate dAdo [9]. This suggests that differences in the catabolism of dAdo nucleotides may contribute to the differential sensitivity among leukemic cell types. Several lymphoblastic cell lines of B-cell origin have considerably greater 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity associated with their plasma membranes than sensitive T cell lines [1, 9, 10]. It was postulated that the high ecto 5′-nucleotidase activity might prevent dATP accumulation in the B lymphoblasts [9, 11]. However, since this nucleotidase activity is believed to be found mostly on the external surfaces of these cells [12], it is not clear how it can serve to regulate intracellular dAdo metabolism [13].

Carson *et al.* [14] have described an inverse relationship between the activity of a cytosolic 5′-nucleotidase and dAdo sensitivity in various leukemic cell lines and have partially purified an enzyme that dephosphorylates 2′-deoxynucleoside 5′-monophosphates in preference to ribonucleoside 5′-monophosphates and is distinct from the lymphoblastic ecto 5′-nucleotidase. The existence of a similar enzyme and its distribution among peripheral T and B lymphocyte subpopulations have not been established. Tjernshaugen [15] has reported that human peripheral lymphocytes have a 5′-(3′)-nucleotidase that is activated by 2′-deoxyinosine. However, this enzyme has low activity with dAMP.

In this report we describe several distinct nucleotidase activities in human peripheral lymphocytes. At least two of these nucleotidases may contribute to the regulation of deoxynucleotide metabolism and possibly to the toxicity of deoxynucleosides in lymphocytes [16, 17].

MATERIALS AND METHODS

Chemicals

All purine and pyrimidine compounds, Histopaque, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesul-

* Supported by PHS Grants CA 07340, CA 31706, and CA 13943 awarded by the National Cancer Institute and by the American Cancer Society Grant IN 45W. This work has been submitted to the Graduate School of Brown University in partial fulfillment of the requirements for the Ph.D. degree (F. W. B.).

† Author to whom all correspondence should be addressed.

‡ Abbreviations: α,β-Me-ADP, α-β-methylene-ADP; Acid Nase, acid nucleotidase; ADA, adenosine deaminase; araA, arabinosyladenine; araAMP, arabinosyl AMP; araCMP, arabinosylcytosine 5′-monophosphate; Con A, concanavalin A; dCF, 2′-deoxycoformycin; DIAN, deoxyinosine activated nucleotidase; DTT, dithiothreitol; 2-F-araA, 2-fluoro-arabinosyladenine; 2-F-araAMP, 2-fluoro-arabinosyladenine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; 6MPR-P, 6-mercaptopurine ribonucleoside 5′-monophosphate; and PEI, polyethyleneimine.

fonic acid (HEPES), α, β-methylene ADP, concanavalin A (Con A), tetramisole, neuraminidase, levamisole, β-glycerophosphate, ribose-5-phosphate, dithiothreitol (DTT), cytochrome *c*, bovine serum albumin, bovine gamma globulin, catalase, and myoglobin were obtained from Sigma; Sephadex G-100 was from Pharmacia; polyethylenimine (PEI) anion exchange cellulose plates (Cel 300 PEI/UV_{254}) were purchased from Brinkmann; and sodium potassium tartrate, $MgCl_2$, EDTA, and trichloroacetic acid were purchased from Fisher. Roswell Park Memorial Institute Medium 1640 (RPMI-1640) containing 25 mM HEPES, penicillin (5000 units/ml)–streptomycin (5000 μg/ml) mixture, and fetal bovine serum were purchased from GIBCO. Betafluor liquid counting scintillant was purchased from National Diagnostics. Radiolabeled [U-^{14}C]dGMP (sp. act. 517 mCi/mmole) was obtained from Amersham.

Isolation of lymphocytes

Lymphocytes from the whole blood of healthy human volunteers were separated from erythrocytes and granulocytes by centrifugation on Histopaque (ficoll-hypaque) for 30 min at 600 *g* [18]. The lymphocyte-rich mononuclear cells were removed from the Histopaque interface and washed three times with RPMI-1640 medium containing 25 mM HEPES (pH 7.4) by centrifuging at 400 *g* for 5 min and resuspending the cell pellet in fresh medium. RPMI-1640 medium containing 25 mM HEPES (pH 7.4) was used throughout this study and will henceforth be referred to as Medium.

T lymphocytes were isolated by their ability to form rosettes with neuraminidase-treated [19] sheep erythrocytes (Scott Labs) using a modification of the method developed by Gmelig-Meyling and Ballieux [20]. The lymphocyte suspensions (5×10^6 ml) were combined with equal volumes of 10% sheep erythrocytes in Medium supplemented with 10% fetal bovine serum. A portion (6 ml) of this cell suspension was layered over a Histopaque cushion (4 ml) and centrifuged at 8° for 30 min at 800 *g*. The T cell rosettes sediment through the Histopaque to form a pellet. After removing the upper layers, the sheep erythrocytes were lysed with HEPES-buffered ammonium chloride (0.8%), and the T lymphocytes were collected after repeated washing to remove the hemolyzed erythrocytes. The mononuclear cells at the Histopaque/Medium interface were collected and washed three times with RPMI-1640 containing 25 mM HEPES to remove traces of Histopaque and plasma. A second rosetting cycle was rarely required since 95% of the rosette-forming T lymphocytes sedimented in the first treatment. The non-rosetting (T lymphocyte-depleted) interface cells were further enriched for B lymphocytes by removal of plastic adherent monocytes by the following procedure: the interface cells were suspended in RPMI-1640 containing 25 mM HEPES at 1×10^7 cells/ml and added to 5 ml fetal bovine serum in 100×10 mm polystyrene petri dishes to give a final concentration of 5×10^6 cells/ml in 50% fetal bovine serum. The petri dishes were then incubated for 1–2 hr at 37° under a 5% CO_2 atmosphere after which B lymphocytes were recovered by gently swirling the dishes and collecting the non-adherent (B lymphocyte-enriched) lymphocytes with a plastic pipette. These T lymphocyte/monocyte depleted cells will be referred to as the B lymphocyte-enriched fraction.

Incorporation of nucleosides and nucleotides into intact T and B lymphocytes

All nucleoside and nucleotide incorporation studies were carried out with the T or B lymphocytes suspended at 1×10^7 cells/ml in RPMI-1640 containing 25 mM HEPES. Duplicate cultures were preincubated with 10 μM deoxycoformycin (dCF) for 30 min at 37° exposed to air in an oscillating water bath before the appropriate compound (dissolved in RPMI-1640 containing 25 mM HEPES) was added to yield a final concentration of 500 μM in a 1-ml volume. Lymphocyte viability during these incubations, as assessed by trypan blue exclusion, exceeded 90%. Analog incorporation into the cellular nucleotide pools was determined by anion exchange high-performance liquid chromatography (HPLC) after 90 min of incubation. The samples were centrifuged at 400 *g* for 5 min. The supernatant fluids were discarded, and the cell pellets were resuspended in 0.9% saline, followed by a second centrifugation. The cell pellets were then treated with 0.5 ml of cold perchloric acid (4%), and the acid-insoluble material was removed by centrifugation. The supernatant fluids were neutralized with 50 μl of potassium phosphate buffer (0.5 M; pH 7) and 50 μl KOH (5 N). The $KClO_3$ precipitate was removed by centrifugation. The neutralized extracts were stored frozen at −20° for analysis by HPLC. For HPLC analysis, a Varian model-5000 liquid chromatograph equipped with a Reeve-Angel Partisil-10 SAX (25 cm × 4.6 mm) column (Whatman, Inc.) was utilized. The nucleotides were separated by a linear gradient starting with a low concentrate eluate of 0.002 M potassium phosphate (pH 4.5) and increasing to a concentration of 0.5 M potassium phosphate (pH 4.5). The column eluate was monitored with a standard fixed wavelength detector at 254 nm.

Purification of cytosolic phosphohydrolase activities

Step 1. Lymphocytes were isolated from whole blood as described above. The cells were washed twice with 0.9% saline and resuspended in Tris-buffered ammonium chloride to lyse contaminating erythrocytes. After additional washing with saline, the lymphocytes were suspended in 50 mM Tris–Cl (pH 7.4) containing 2 mM DTT and 5% glycerol. This suspension was then frozen and thawed three times to disrupt the cell membranes. The freeze-thawed extracts were centrifuged for 1 hr at 100,000 *g* after which the supernatant fluid and membrane pellet were separated.

Step 2. The 100,000 *g* supernatant fluid from Step 1 was adjusted to pH 5.1 at 4° by slow addition of 1 M HCl. The protein precipitate was removed by centrifugation at 30,000 *g* for 20 min and discarded. The pH of the supernatant fluid was then readjusted to pH 6.5 with 1 M NaOH at 4°.

Step 3. The supernatant fraction of Step 2 was brought to 40% saturation by gradual addition of solid ammonium sulfate. The precipitate was removed by centrifugation at 20,000 *g* for 20 min;

additional ammonium sulfate was added to bring the concentration to 70% saturation, and the precipitate was recovered by centrifugation at 20,000 *g* for 20 min.

Step 4. The 70% ammonium sulfate precipitate from Step 3 was dissolved in 10 ml of elution buffer (100 mM KCl, 2 mM DTT, and 50 mM imidazole chloride buffer, pH 6.5). Portions (1 or 2 ml) were then applied to a 1.5 × 89 cm Sephadex G-100 column equilibrated with the elution buffer. The column was eluted with the same buffer, and 1- or 2-ml fractions were collected at a flow rate of 10 ml/hr at 4°.

Relative molecular weight estimation

The Sephadex G-100 column was calibrated with catalase (M_r 240,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), myoglobin (M_r 16,900), and cytochrome *c* (M_r 12,400). Then 0.5 ml of the 70% ammonium sulfate fraction of Step 3 was applied to the column, and 1-ml fractions were collected.

Enzyme assays

Phosphohydrolase activities with various substrates were assayed by determining liberated inorganic phosphate. Membrane-bound ecto 5′-nucleotidase activity was measured in the 100,000 *g* pellet of the freeze-thawed lymphocytes. The assay mixture contained 1 mM 5′-AMP, 20 mM $MgCl_2$, and 50 mM Tris–Cl, pH 7.5, in a total volume of 0.5 ml. Cytosolic nucleotidase activity was measured in the 100,000 *g* extract supernatant fraction. The assay mixture contained 1 mM dAMP, 20 mM $MgCl_2$, and 50 mM imidazole choride, pH 6.0, in a final volume of 0.5 ml. The reactions were run for 20 min at 37° and were stopped by the addition of 0.5 ml of 10% trichloroacetic acid. The samples were centrifuged to remove precipitated proteins, and the liberated phosphate was determined by the method of Chen *et al.* [21]. To each tube, 1 ml of the freshly prepared color reaction mixture (1 part 10% ascorbate; 1 part 6 N sulfuric acid; 1 part 2.5% ammonium molybdate; 2 parts distilled water) was added and allowed to develop for 30 min at 45°. The absorbance of each tube was measured at 820 nm with a red filter. The amount of phosphate was determined from a standard curve prepared with each assay.

The standard assay mixture for the partially purified enzymes included: 50 mM buffer (either Tris acetate, pH 5.0, imidazole chloride, pH 6.0, or Tris–Cl, pH 8.0), 20 mM $MgCl_2$, 1 mM substrate, and 50–100 μl of enzyme (approximately 0.15 mg of protein for cell extracts or 20 μg of purified enzyme) in a final volume of 0.5 ml. The reaction was started with the addition of substrate and allowed to run for 20–40 min (the reaction rate was linear for at least 50 min) at 37°. The incubations were terminated by the addition of 0.5 ml of a 10% TCA, and the liberated phosphate was determined as described above. To examine the effects of phosphate and ATP on the partially purified enzymes, a radioisotopic assay was used with [U-^{14}C]dGMP (1 mCi/mmole). Assay conditions were similar to those of the colorimetric assay except that the final volume was 75 μl. The reactions were stopped with 25 μl of 4 M formic acid. Protein was removed by centrifugation, and 10 μl of supernatant fluid was spotted on PEI cellulose. The plates were developed in 1:1 (v/v) methanol and water. The nucleoside and nucleotide spots were cut out and added to Betafluor for counting in a Packard TriCarb model 3320 liquid scintillation counter.

Protein estimation

Protein concentrations were determined by the method of Bradford [22] as described by Bio-Rad Laboratories using bovine gamma globulin as a standard. When accuracy was not critical (column fractions), protein concentrations were estimated by absorbance at 280 nm.

Statistical analysis

Due to large variations in enzyme activities between different individuals, a paired *t*-test was used to determine the significance of the enzyme activity differences between T and B lymphocytes [23].

RESULTS

Nucleotidase activities in T and B cell-enriched lymphocytes

Table 1 shows that the cytosolic extracts of T and B lymphocytes from the same individual dephosphorylated dAMP at similar rates. In contrast, the membrane-associated dephosphorylation of AMP was significantly higher ($P < 0.001$) in the B lymphocytes confirming the results of Rowe *et al.* [24]. To examine possible metabolic effects of the difference in membrane-associated phosphohydrolase activities, the uptake and metabolism of various 5′-monophosphate nucleotides were studied in both cell types. Since nucleotides are highly anionic, they do not readily cross membranes and must first be dephosphorylated. As shown in Table 2, the conversion of dAMP and tubercidin 5′-monophosphate to their respective intracellular nucleoside 5′-triphosphates was more pronounced in B than in T lymphocytes. On the other hand, neither araAMP nor 2-F-araAMP was incorporated by either cell type. In contrast to the 5′-monophosphate nucleotides, the respective nucleosides of these two arabinosyl analogs were readily converted to intracellular nucleotides in both cell types. This indicates that both T and B lymphocytes have the capacity to transport and phosphorylate these analog nucleosides and suggests that the failure of either lymphocytic subpopulation to convert the arabinosyl nucleotides to intracellular nucleotides is due to the inability of the membrane-associated nucleotidase to dephosphorylate these particular nucleotides.

Membrane associated and cytosol phosphohydrolase activities from pooled peripheral lymphocytes with various substrates

Table 3 compares the rates of dephosphorylation for various substrates by lymphocyte membrane and cytosolic activities. The most notable difference was the preference for ribonucleoside 5′-monophosphates by the membrane enzyme and for

Table 1. Comparison between nucleotidase activities in human T and B cell-enriched lymphocytes isolated from individual donors*

Nucleotidase	Activity† (nmoles/min/10^6 cells)		
	B lymphocytes	T lymphocytes	Difference (B-T)
Membrane (AMP)	0.150	0.040	0.110
	0.160	0.030	0.130
	0.195	0.010	0.185
	0.250	0.030	0.220
	0.260	0.000	0.260
	0.340	0.160	0.180
	0.360	0.290	0.070
Mean	0.246	0.080	0.165‡
Cytosol (dAMP)	0.125	0.163	−0.038
	0.162	0.195	−0.033
	0.125	0.153	−0.028
	0.101	0.133	−0.032
	0.200	0.130	0.070
Mean	0.143	0.155	−0.012

* T cells were >95% pure with <1% B cell and monocyte contamination. The B cell-enriched lymphocytes contained 70 ± 20% surface immunoglobulin (IgA, IgM, and IgD) positive lymphocytes.

† AMP (1 mM) was the substrate with the membrane enzyme and dAMP (1 mM) with the cytosol.

‡ Significantly different at $P < 0.001$ as determined by a paired *t*-test [23].

2′-deoxynucleoside 5′-monophosphates and nucleoside 3′-monophosphates by the cytosol enzyme(s). The membrane nucleotidase was most active with pyrimidine ribonucleotides. 3′-AMP and 3′-UMP were poor substrates. The arabinosyl nucleotides (araAMP, 2-F-araAMP and araCMP) were unreactive with the membrane nucleotidase. This is consistent with the failure of arabinosyl nucleotides to form analog nucleotides in either the T or B lymphocytes (Table 2). Table 3 also shows that the cytosol readily dephosphorylated dGMP, dUMP, and 3′-UMP. Relatively low substrate activity was seen with AMP, 3′-AMP, and the arabinosyl nucleotides. Dephosphorylation of β-glycerophosphate, an indicator of nonspecific phosphatase activity, was negligible with the membrane-associated enzyme, which indicates that this enzyme has high specificity for 5′-monophosphate nucleotides.

Partial purification of cytosolic 2′-deoxynucleotide phosphohydrolase(s)

Partial purification of the cytosolic nucleotidase activities was undertaken as shown in Table 4, which indicates that 60% of the total deoxynucleotidase activity resides in the cytosolic fraction of the lymphocyte. Figure 1 presents the profiles of dGMP, dAMP, and dIMP nucleotidase activities eluted from a Sephadex G-100 column. Two peaks of activity for dIMP and dGMP were observed. The optimal pH of activity for each peak was determined with several

Table 2. Incorporation of analog nucleoside and nucleoside 5′-monophosphates into the nucleoside triphosphate pools of intact T and B cell-enriched lymphocytes

Compound (500 μM)	% of Control ATP*	
	T cell	B cell
Tubercidin	86	86
Tubercidin 5′-monophosphate	24	75
2-F-araA	20	20
2-F-araAMP	0	0
araA	20	20
araAMP	0	0
dAdo	0	24
dAMP	0	10

* Cell extracts were prepared following a 90-min incubation and analyzed by HPLC, as described in Materials and Methods. The area of the analog triphosphate peak was determined and expressed as a percentage of the control ATP area. Control cultures contained 7.8 nmoles ATP/10^7 cells. Values are the average of at least two different experiments.

Table 3. Phosphohydrolase activities of the human lymphocyte membrane bound (100,000 *g* pellet) and cytosol (100,000 *g* supernatant) with various substrates*

Substrate	Activity	
	Membrane enzyme	Cytosol enzyme(s)
AMP	100 (8.4 nmoles/min/mg)	100 (18.2 nmoles/min/mg)
GMP	74	170
IMP	82	235
6-MPR-P	130	159
UMP	175	115
CMP	208	125
dAMP	98	200
dGMP	46	465
dUMP	110	500
dTMP	35	210
dCMP	88	210
2-F-araAMP	0	64
araAMP	1	75
araCMP	0	69
3′-AMP	8	93
3′-UMP	10	465
β-Glycerophosphate	4	74

* Activities are expressed as the percentage of the activity with AMP (1 mM). Values represent the averages of two or three separate experiments.

Table 4. Partial purification of the deoxynucleotide (dGMP) phosphohydrolase activity from human lymphocytes

Purification step	Total protein (mg)	Total activity (nmoles/min)	Yield (%)	Specific activity (nmoles/min/mg)
(1) Freeze/Thaw	471	5833	100	12.4
(2) 100,000 *g* Supernatant	193	3513	60	18.2
(3) pH 5.1 Treatment	155	3518	61	22.7
(4) 70% $(NH_4)_2SO_4$	121	2783	48	23.0
(5) Sephadex G-100				
Peak 1 (Acid Nase)	2.1	160	3	76.4
Peak 2 (DIAN)	10	2080	36	208.0

buffers at 37° (data not shown). Optimal activity for the first peak (Acid Nase) occurred between pH 5.0 and 5.5. At pH 7.0 this activity was only 30% of that at pH 5.5. The activity of the second peak, as shown below, was enhanced by the addition of dIno, and is referred to as DIAN (for deoxyinosine-activated nucleotidase) in accord with other investigators who have studied similiar enzymes [15, 25]. DIAN had a sharp activity optimum between pH 5.5 and 6.0. At pH 7.0 DIAN retained 60% of its optimal activity. Because of differences in their pH optima, Acid Nase was assayed at pH 5.0 and DIAN at pH 6.0 in all subsequent experiments. Acid Nase corresponded to M_r 73,000 and DIAN to M_r 47,000 as estimated by gel filtration (Fig. 2).

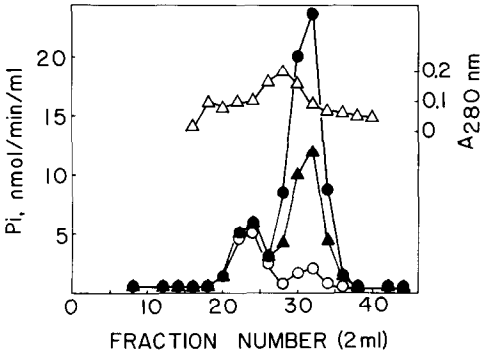

Fig. 1. Elution patterns of dIMP (●), dAMP (○), and dGMP (▲) nucleotidase activities from human lymphocyte extract on a Sephadex G-100 column. Fractions (2 ml) were collected and assayed with 1 mM substrate. Protein content was determined by absorbance at 280 nm (△).

To determine whether the cytosolic deoxynucleotidases of T and B lymphocytes differ, extracts of T and B lymphocyte-enriched cells were subjected to molecular sieving. The cells were isolated as described in Materials and Methods, processed as in Step 1 of the enzyme purification scheme, and 1-to-2-ml aliquots (representing about 5×10^7 cells) of the 100,000 *g* supernatant fraction were applied to the Sephadex G-100 column. Assay of the eluted fractions with dAMP, dIMP, and dGMP resulted in nucleotidase activity profiles that were essentially identical to that shown in Fig. 1. No additional nucleotidase peaks were observed in either the T or

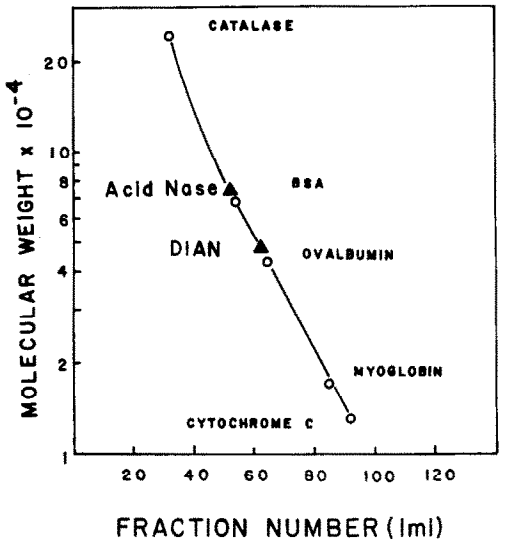

Fig. 2. Separation and molecular weight estimation of DIAN and Acid Nase from the $(NH_4)_2SO_4$ pellet of human lymphocytes by molecular sieving on Sephadex G-100. A Sephadex G-100 gel column (1.5 × 89 cm) was prewashed with elution buffer containing 100 mM KCl, 50 mM imidazole–HCl, and 2 mM DTT and calibrated with the standard proteins: catalase, M_r 240,000; bovine serum albumin, M_r 67,000; ovalbumin, M_r 43,000; myoglobin, M_r 16,900; and cytochrome *c*, M_r 12,400. The column was eluted with the same buffer, and 1-ml fractions were collected at 4° at a flow rate of 9 ml/hr. The sample (6 mg of the 70% $(NH_4)_2SO_4$) was applied to the column in a 0.5-ml volume. Fractions were collected as before and assayed for nucleotidase activity with dIMP as substrate. The peak for DIAN activity corresponded to M_r 47,000 and Acid Nase to M_r 73,000.

B lymphocyte-enriched extracts (data not shown). These studies showed no differences in the activities of the cytosol nucleotidases between T and B lymphocytes. Subsequent examinations of the individual enzymes were performed with fractions from the unresolved mixtures of T and B cells.

Characterization of the phosphohydrolase activities

Table 5 shows the effects of Mg^{2+} on Acid Nase and DIAN. The dIMP-dephosphorylating activity of Acid Nase was enhanced slightly (10%) by the omission of Mg^{2+} and was not altered by the addition of EDTA. Therefore, Acid Nase was assayed in the absence of Mg^{2+} in all subsequent experiments. On the other hand, the omission of Mg^{2+} caused an 80% reduction in DIAN activity towards dIMP. Addition of EDTA (5 mM) further reduced the activity to less than 10%. DIAN was subsequently assayed with 20 mM $MgCl_2$.

The effects of several known phosphatase inhibitors on lymphocytic ecto 5′-nucleotidase, Acid Nase and DIAN are shown in Table 5. Sodium fluoride was an effective inhibitor of all nucleotidase activities. Con A, α,β-methylene ADP, and tetramisole were all ineffective as inhibitors of the cytosol nucleotidases, DIAN and Acid Nase. In contrast, Con A and α,β-methylene ADP inhibited the ecto 5′-nucleotidase. Sodium potassium tartrate selectively inhibited Acid Nase at 1 mM, but concentrations as high as 100 mM had no effect on the activity of DIAN. It has been proposed that the nucleotidase activities of many tissues are regulated by the cellular energy charge, particularly by fluctuations in ATP concentrations [26, 27]. The influence of ATP and inorganic phosphate on dGMP dephosphorylation by Acid Nase and DIAN was determined by a radioisotopic assay (Table 5). ATP at 0.5 mM was slightly more inhibitory to Acid Nase than to DIAN. Doubling the ATP concentration, however, did not increase the inhibition (data not shown). Potassium phosphate (5 mM) was an effective inhibitor of both Acid Nase and DIAN. Increasing the phosphate concentration to 10 mM reduced both activities to less than 10% of the control values (data not shown).

Both Acid Nase and DIAN lost activity in the

Table 5. Effects of various compounds on the partially purified nucleotidase activities from lymphocyte cytosol*

Compound	(ecto) Nucleotidase	Cytosol nucleotidases	
		Acid Nase	DIAN
None	100 (8.4 nmoles/min/mg)	100 (76 nmoles/min/mg)	100 (456 nmoles/min/mg)
No Mg^{2+}	ND†	111	21
No Mg^{2+} plus EDTA (5 mM)	ND	111	7
Fluoride (15 mM)	0	2	4
Concanavalin A (1 mg/ml)	40	98	98
Tetramisole (1 mM)	99	100	100
Tartrate (1 mM)	ND	17	98
α,β-Me-ADP (2 mM)	0	93	100
ATP (1 mM)	ND	93	83
PO_4^{2-} (15 mM)	ND	26	31

* The (ecto) nucleotidase activity was measured with 1 mM AMP; Acid Nase, and DIAN were measured with 1 mM dIMP. Studies with ATP and PO_4^{2-} were performed with [U-^{14}C]dGMP (1 mM) as the substrate. The values presented are the average of at least two different experiments. The standard deviations were less than 10%.

† Not determined.

Table 6. Substrate specificity of Acid Nase and DIAN partially purified from lymphocyte cytosol*

Base	Ribose						2′-Deoxyribose			
	5′-Monophosphate		3′-Monophosphate		2′-Monophosphate		5′-Monophosphate		3′-Monophosphate	
	Acid Nase	DIAN	Acid Nase	DIAN	Acid Nase	DIAN	Acid Nase	DIAN	Acid Nase	DIAN
Adenine	57	4	74	4	69	1	92	9	97	2
Guanine	61	5	74	40	63	3	98	49	104	59
Hypoxanthine	64	12	—†	—	—	—	100	100	—	—
Cytosine	52	1	—	—	—	—	78	4	98	4
Thymine	—	—	—	—	—	—	76	30	70	96
Uracil	41	4	61	47	—	—	45	43	—	—
4-Thiouracil	71	4	—	—	—	—	—	—	—	—
5-F-Uracil	—	—	—	—	—	—	65	72	—	—
Other substrates										
Pseudo UMP	63	32	—	—	—	—	—	—	—	—
Ribose-5-PO_4	1	1	—	—	—	—	—	—	—	—
β-Glycerophosphate	5	5	—	—	—	—	—	—	—	—

* All values are expressed as a percentage of dIMP (1 mM) activity with each enzyme assayed under optimal conditions. The specific activity of DIAN was 456 nmoles/min/mg and 76 nmoles/min/mg for Acid Nase. All substrates were assayed at 1 mM.
† Lines indicate nucleotides that were not studied.

absence of the thiol agent, DTT. Purified DIAN was unstable and lost total activity within a week even in the presence of DTT and 20–50% glycerol at 4°. Neither purified Acid Nase nor DIAN was stable to freezing even in the presence of 50% glycerol or 0.5 mg/ml bovine serum albumin. Acid Nase lost activity somewhat more slowly than DIAN, retaining 40% of the original activity after 2 weeks at 4°.

Substrate specificity of Acid Nase and DIAN

The activities of Acid Nase and DIAN with a wide range of substrates were examined under optimal assay conditions for each enzyme. A comparison of the activities of Acid Nase and DIAN with various nucleoside monophosphates is shown in Table 6. Acid Nase was relatively nonspecific for the various nucleotides, but had little activity with either ribose-5-phosphate or β-glycerophosphate. This indicates that it is a true nucleotidase rather than a non-specific phosphatase. Acid Nase dephosphorylated ribonucleoside and deoxyribonucleoside 2′- and 3′-monophosphates more readily than the corresponding 5′-substituted compounds. In contrast, DIAN appeared more specific for 2′-deoxynucleoside monophosphates. Of all the nucleoside 3′-monophosphates tested, only 3′-GMP and 3′-UMP reacted with DIAN. DIAN was inactive with all adenine and cytosine nucleotides (see Tables 6 and 7), and it displayed the greatest activity with 3′- or 5′-nucleotides of guanine, uracil, and thymine. These compounds are all characterized by the presence of an oxygen on *C(4)* of the pyrimidine ring or in the corresponding *C(6)* of the purine ring. Nucleotides of the adenine and cytosine series have amino groups on *C(6)* and *C(4)* respectively, and demonstrate minimal activity with DIAN. All three of the arabinosyl nucleotides tested (araAMP, 2-F-araAMP, and araCMP) were relatively inactive with Acid Nase and were completely inactive with DIAN (Table 7). This confirms the results observed with the crude extracts (Table 3).

Table 8 shows K_m and V_{max} values for several substrates with partially purified Acid Nase and DIAN assayed under optimal conditions. The kinetic parameters were estimated by the method of least squares [28]. The apparent discrepancy between the specific activity of the enzymes assayed directly from the column and the estimated V_{max} values for dIMP reflects the loss of activity of both enzymes during storage.

dIno activation of DIAN 3′-nucleotide hydrolysis

The effect of 4 mM dIno on the activity of DIAN with 3′-nucleotides is shown in Table 9. dIno approximately doubled the rate of phosphate release from each nucleotide tested, with 3′-dTMP showing the greatest increase (230%). Reactions with nucleoside 5′-monophosphates were neither inhibited nor activated by dIno. Acid Nase activity with nucleoside 5′- and 3′-monophosphates was not influenced by dIno (data not shown).

DISCUSSION

Based on work with cultured leukemic cells, it was proposed that the higher specific activity of the ecto

Table 7. Substrate activity of several arabinosyl nucleotides, dAMP, and dIMP with the partially purified lymphocyte cytosolic nucleotidases

	Activity (% of dIMP control)	
Nucleotide (1 mM)	Acid Nase	DIAN
dIMP	100*	100
dAMP	92	13
araAMP	28	0
2-F-araAMP	28	0
araCMP	30	0

* The specific activity of Acid Nase was 76 nmoles/min/mg and 456 nmoles/min/mg for DIAN.

Table 8. Kinetic parameters of the partially purified cytosolic nucleotidases (Fraction 5)

Enzyme	K_m (μM)	V_{max} (% of dIMP)
Acid Nase		
dIMP	372	100 (55.9 nmoles/min/mg)
dAMP	395	86
dGMP	1150	160
DIAN		
dIMP	350	100 (63.8 nmoles/min/mg)
dGMP	2300	131
3'-dGMP	126	69
3'-UMP	75	55

* Results are representative of at least two separate determinations.

5'-nucleotidase of the B lymphoblasts conferred on these cells an increased resistance to deoxynucleoside toxicity [1, 9]. Subsequent studies of human leukemic cells indicate that ecto 5'-nucleotidase activity is unrelated to dAdo sensitivity and dATP accumulation [29, 30]. Although our results confirm the presence of higher ecto 5'-nucleotidase activity in peripheral B lymphocytes than in T lymphocytes (Table 1), it appears unlikely that this enzyme regulates nucleoside incorporation [9, 10, 24]. As shown in Table 2, the arabinosyl nucleosides and tubercidin readily accumulated in the intracellular nucleotide pools of both lymphocyte populations. Despite the greater ecto 5'-nucleotidase activity of the B lymphocyte-enriched cells, there was no significant difference in the relative amounts of analog nucleoside triphosphate formed by the T or B lymphocytes. Tubercidin 5'-monophosphate, however, was incorporated to a greater extent by the B lymphocyte-enriched cells. This observation probably results from the higher ecto 5'-nucleotidase activity of B lymphocytes and is consistent with the proposed role of this enzyme in the salvage of extracellular purine and pyrimidine nucleotides [13, 31]. The results in Table 2 also indicate that the B lymphocytes, unlike the T lymphocytes, are capable of forming dATP when incubated with dAMP. This pattern of incorporation is evidently not determined by the ecto 5'-nucleotidase activity since the formation of dATP from the dAdo nucleoside was also limited to the B cell-enriched lymphocytes. Additional studies on the incorporation of dAdo into human lymphocytes have been presented elsewhere [16, 17].

The inability of araAMP and 2-F-araAMP to be incorporated into either lymphocyte population (Table 2) agrees with the failure of ecto 5'-nucleotidase to accept arabinosyl nucleotides as substrates (Table 3). As nucleotides, these agents carry a strong anionic charge that prevents transport across the non-polar cellular membrane. Both araAMP and 2-F-araAMP, because of their increased solubility, are currently under study as prodrug forms of araA and 2-F-araA for the treatment of cancer. Studies of arabinosyl nucleotide metabolism in animals and man indicate that araAMP is rapidly dephosphorylated to araA by phosphatases believed to be located primarily in the kidney [32–34]. Therefore, the inability of the lymphocytic ecto 5'-nucleotidase to dephosphorylate arabinosyl nucleotides should not prevent these and similar cells from forming the respective analog nucleotides *in vivo*. However, *in vitro* studies of arabinosyl nucleotides must be con-

Table 9. Effect of dIno on partially purified DIAN activity with nucleoside 3'-monophosphates

	DIAN activity* (nmoles/min)		
Nucleotide (1 mM)	−dIno	+dIno (4 mM)	% Fold activation
3'-dTMP	0.96	2.22	230
3'-GMP	0.62	1.18	192
3'-dGMP	0.84	1.57	186
3'-UMP	0.85	1.63	192

* The specific activity of DIAN was 456 nmoles/min/mg.

cerned with bioavailability of these agents, even with cell lines known to have ecto 5′-nucleotidase activity.

The results in Tables 3 and 6 demonstrate that 2′-deoxynucleoside 5′-or 3′-monophosphates are the preferred substrates of the cytosolic nucleotidases. Acid Nase closely resembled the lysosomal enzyme purified from rat liver by Arsenis and Touster [35]. It displayed broad substrate specificity, did not require Mg^{2+}, had a low pH optimum (pH 5.0), and was inhibited by tartrate. Tjernshaugen [36] has purified a similar nucleotidase from rat spleen. However, his results indicate that the rat spleen acid nucleotidase, unlike the enzyme studied by Arsenis and Touster [35], is located in the cytosol. We have not attempted to identify the subcellular location of Acid Nase and cannot exclude the possibility that it may be a lysosomal enzyme.

Our findings with DIAN from peripheral human lymphocytes were similar to those made by Fritzson and Smith [25] with the rat liver enzyme and by Tjernshaugen [37] with the rat spleen enzyme. The pH optimum, Mg^{2+} requirement, molecular weight, dIno activation, substrate specificity, and K_m values of human lymphocyte DIAN closely resembled those described for the highly purified (2000-fold) rat liver enzyme [11, 25]. The lack of activity with adenine and cytosine nucleotides suggests that DIAN may function in sequence with AMP deaminase (EC 3.5.4.6) and dCMP deaminase (EC 3.5.4.12) for the metabolism of these compounds. Several nucleotidases are known to operate in conjunction with AMP deaminase to maintain the cellular adenylate charge [26, 27, 38]. Unlike these nucleotidases, DIAN did not appear to be regulated by ATP and had little activity with 5′-ribonucleotides. Tjernshaugen and Gautvik [39] have shown that DIAN activity varies during rat liver regeneration. The activity of this enzyme was inversely related to growth rate, possibly indicating that it is primarily a catabolic rather than a salvage enzyme [39]. Increases in DIAN activity have also been noted in late log and plateau phase cultured cell lines of non-hepatic origin [39]. A possible explanation centers on the need of the cell to eliminate excess nucleotides generated from RNA and DNA fragments, requiring coordination between the nucleases and the nucleotidase. As the cell begins to degrade RNA or DNA fragments, the accumulation of dIno, dIMP, and other nucleotides in the cell would then activate the dephosphorylation of the nucleoside 3′-monophosphates by DIAN. The ability of DIAN to cleave ribonucleoside and 2′-deoxyribonucleoside 3′-monophosphates would be advantageous in recycling these nucleotides since the 3′-nucleotides are not anabolized directly and must be dephosphorylated before the nucleoside may be salvaged. The low reactivity of DIAN with 5′-ribonucleotides is consistent with this hypothesis, since they are important metabolic substrates for energy metabolism and synthetic reactions (e.g. ATP, GTP, and CTP) and appear to be regulated by a different set of enzymes in lymphocytes [26, 27, 38].

It does not appear that ecto 5′-nucleotidase plays a major role in the regulation of intracellular nucleotide metabolism in human lymphocytes. The most widely held view to explain the differential metabolism of dAdo between lymphoid cells is that cells which accumulate the most dATP have the lowest specific activity of cytosolic deoxynucleotidase [14]. Our evidence suggests that peripheral T and B lymphocytes do not differ in their abilities to dephosphorylate dAMP. Acid Nase, the enzyme accounting for most of the dephosphorylation of dAMP in the peripheral lymphocytic cytosol, does not appear to have adequate specificity or affinity for dAMP to play a major regulatory role in these cells. In several respects, the Acid Nase reported here resembles the deoxynucleotidase activity reported by Carson *et al.* [14] in human lymphoblasts. Both enzymes are more active with deoxynucleoside 5′-monophosphates, have acidic pH optima, and are inhibited by fluoride. However, in contrast to the lymphoblastic deoxynucleotidase, the Acid Nase of peripheral lymphocytes does not require Mg^{2+}, was inhibited by tartrate, and retained less than 30% of control activity in the presence of 5 mM phosphate. The deoxynucleotidase identified by Carson *et al.* [14] was not sufficiently characterized to permit a unifying explanation at this time. It seems unlikely that the regulation of dATP accumulation is controlled at the level of dAMP; however, this does not exclude other catabolic enzymes. Consideration should be given to the possibility that an enzyme specific for dATP, resembling the nucleotide pyrophosphohydrolase enzymes [deoxyuridine 5′-triphosphate pyrophosphohydrolase (EC 3.6.1.23) and inosine 5′-triphosphate pyrophosphohydrolase (EC 3.6.1.19)] may exist [40, 41]. This possibility is presently under investigation.

Acknowledgements—We wish to express our gratitude to Dr. Manfred Steiner and his associates for their invaluable assistance with these studies.

REFERENCES

1. D. A. Carson, J. Kaye, S. Matsumoto, J. E. Seegmiller and L. Thompson, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2430 (1979).
2. A. Cohen, R. Hirschorn, S. D. Horowitz, A. Rubenstein, S. Polmar, R. Hons and D. Martin, *Proc. natn. Acad. Sci. U.S.A.* **75**, 472 ((1978).
3. R. Hirschorn, V. Roegner, A. Rubenstein and P. Papargeorgiou, *J. clin. Invest.* **65**, 768 (1980).
4. D. W. Martin and E. W. Gelfand, *A. Rev. Biochem.* **50**, 845 (1981).
5. B. S. Mitchell, E. Mejias, P. E. Dadonna and W. N. Kelley, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5011 (1978).
6. B. Ullman, L. J. Gudas, A. Cohen and D. W. Martin, *Cell* **14**, 365 (1978).
7. L. Thelander and P. Reichard, *A. Rev. Biochem.* **48**, 133 (1979).
8. D. A. Carson, J. Kaye and J. E. Seegmiller, *J. Immun.* **121**, 1726 (1978).
9. R. L. Wortmann, B. S. Mitchell, N. L. Edwards and I. H. Fox, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2434 (1979).
10. J. Dornand, J. Bonnafous, J. Favero and J. Mani, *Biochem. Med.* **28**, 144 (1982).
11. P. Fritzson, *Adv. Enzyme Regulat.* **16**, 43 (1978).
12. N. L. Edwards, E. Gelfand, L. Burk, H. Dosch and I. H. Fox, *Proc. natn. Acad. Sci. U.S.A.* **76**, 3474 (1979).
13. A. C. Newby, *Biochem. J.* **186**, 907 (1980).
14. D. A. Carson, J. Kaye and D. B. Wasson, *J. Immun.* **126**, 348 (1981).

15. H. Tjernshaugen, *Int. J. Biochem.* **13**, 417 (1981).
16. F. W. Burgess and R. E. Parks, Jr., *Fedn Proc.* **41**, 4512 (1982).
17. F. W. Burgess, *Ph.D. Thesis*. Brown University, Providence, RI (1983).
18. A. Boyum, *Scand. J. Immun.* **5**, (Suppl. 5), 9 (1976).
19. M. S. Wiener, C. Bianco and V. Nussenzweig, *Blood* **42**, 939 (1973).
20. F. Gmelig-Meyling and R. E. Ballieux, *Vox Sang.* **33**, 5 (1977).
21. P. S. Chen, T. Y. Toribara and H. Warner, *Analyt. Chem.* **28**, 1756 (1956).
22. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
23. R. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics*, p. 78. McGraw-Hill, New York (1960).
24. M. Rowe, C. G. DeGast, T. A. E. Platts-Mills, G. I. Asherson, A. D. B. Webster and S. M. Johnson, *Clin. expl Immun.* **36**, 97 (1979).
25. P. Fritzson and I. Smith, *Biochim. biophys. Acta* **235**, 128 (1971).
26. D. A. Carson and D. G. Wasson, *Cancer Res.* **42**, 4321 (1982).
27. A. G. Chapman and D. E. Atkinson, *J. biol. Chem.* **248**, 8309 (1973).
28. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
29. S. S. Matsumoto, A. L. Yu, L. C. Bleeker, B. Bakay, F. H. Kung and W. L. Nyhan, *Blood* **60**, 1096 (1982).
30. R. L. Wortmann, J. Holcenberg and D. G. Poplack, *Cancer Treat. Rep.* **66**, 387 (1980).
31. H. Fleit, M. Conklyn, R. D. Stebbins and R. Silber, *J. biol. Chem.* **250**, 8889 (1975).
32. D. H. W. Ho and E. Frei, *Cancer Res.* **30**, 2852 (1970).
33. G. A. LePage, Y-T. Lin, R. E. Orth and J. A. Gottlieb, *Cancer Res.* **32**, 2441 (1972).
34. G. A. LePage, S. R. Naik, S. B. Katakkar and A. Khaliq, *Cancer Res.* **35**, 3036 (1975).
35. C. Arsenis and O. J. Touster, *J. biol. Chem.* **243**, 5702 (1968).
36. H. Tjernshaugen, *Biochem. J.* **169**, 597 (1978).
37. H. Tjernshaugen, *Acta chem. Scand.* **B33**, 384 (1979).
38. A. S. Bagnara and M. S. Hershfield, *Proc. natn. Acad. Sci. U.S.A.* **79**, 2673 (1982).
39. H. Tjernshaugen and K. M. Gautvik, *J. cell. Physiol.* **88**, 13 (1976).
40. C. J. Chern, A. B. MacDonald and A. J. Morris, *J. biol. Chem.* **244**, 5489 (1969).
41. W. V. Williams and Y-C. Cheng, *J. biol. Chem.* **254**, 2897 (1979).