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DIFFERENTIAL INCORPORATION OF 2′-DEOXYADENOSINE INTO HUMAN PERIPHERAL LYMPHOCYTES*

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Abstract—Human peripheral lymphocytes incubated with 2′-deoxycoformycin and 2′-deoxyadenosine (dAdo) reached a plateau in dATP accumulation after 4 hr that lasted for up to 24 hr. Total dATP accumulation did not exceed 15% of the control ATP concentration in the lymphocytes. In contrast, the human CCRF-CEM T lymphoblastic cell line and human erythrocytes showed a nearly linear pattern of dATP formation throughout the incubation period. By 6 hr the dATP concentration in the CCRF-CEM cells exceeded the control ATP concentration. A comparison of dATP accumulation in purified peripheral T and B lymphocytes indicated differences between these cells that favor greater dATP formation in the B lymphocytes. Incorporation studies with several adenosine analogs demonstrated that arabinosyladenine, 2-F-arabinosyladenine, tubercidin, formycin A, and 9-(2′-deoxy-2′-fluoro-β-D-ribofuranosyl)adenine form corresponding amounts of analog triphosphate in the T and B cell-enriched lymphocytes. 9-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine (2′-F-araA) was the only compound to show an incorporation pattern similar to that observed with dAdo by forming analog triphosphate only in the B cell-enriched lymphocyte population. Nucleoside kinase measurements showed no significant differences in dAdo, adenosine, or 2′-deoxycytidine kinase activities between the T and B lymphocytes. The inability of the T cells to incorporate dAdo or the analog 2′-F-araA into their nucleotide pools may indicate the existence of a highly specific catabolic enzyme(s).

Inherited deficiency of the catabolic enzyme adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) is associated with severe dysfunction of both cell-mediated and humoral immunity in humans [1]. ADA-deficient individuals are unable to degrade 2′-deoxyadenosine (dAdo) which results in elevated levels of dAdo in their plasma and the accumulation of dATP in their erythrocytes [2–4]. Similar elevations of dATP may also occur in the lymphocytes of these patients [2]. However, efforts to demonstrate this have produced varied results [2, 4]. Investigation of this clinical disorder is complicated by the fact that most ADA-deficient patients are profoundly lymphopenic, with tissue samples difficult to obtain. The availability of tight-binding ADA inhibitors, e.g. 2′-deoxycoformycin (dCF), has made it possible to reproduce ADA deficiency experimentally [5, 6]. *In vitro* studies have shown that many human leukemic cell lines, pretreated with a tight-binding ADA inhibitor, demonstrate increased susceptibility to the toxic effects of dAdo, which correlates directly with the amount of intracellular dATP accumulation [7, 8]. Leukemic cells resistant to the combination of dCF plus dAdo do not accumulate dATP, as is seen with many cell lines of B lymphocytic origin [7, 8].

Expansion of the dATP pools, especially in leukemic T lymphoblasts incubated with dCF and dAdo, alters the intracellular concentrations of other deoxynucleotides [9]. dATP is a known allosteric inhibitor of the enzyme ribonucleotide reductase [10–12], and it may inhibit DNA synthesis by selectively blocking the formation of dCTP [9]. dATP may also interfere with DNA synthesis by acting as a competitive inhibitor of other deoxynucleotides for binding sites on DNA polymerase [13]. Another possible mechanism of dATP-mediated toxicity involves the depletion of cellular ATP. Siaw *et al.* [14] noted that ATP levels declined in proportion to the amount of dATP accumulated by the erythrocytes of a patient undergoing a therapeutic trial with dCF. Bagnara and Hershfield [15] have reported that dATP may stimulate the catabolism of ATP by activating AMP deaminase and 5′-nucleotidase in the CCRF-CEM T lymphoblastic cell line. Recently, Brox *et al.* [16] have reported that dATP accumulation is in some way linked to the formation of DNA strand breaks in resting peripheral lymphocytes. The mechanism responsible for the formation of the DNA strand breaks has not been identified. Hershfield [17, 18] has proposed that dAdo inhibits essential DNA methylation reactions by binding irreversibly to the enzyme *S*-adenosylhomocysteine hydrolase, which catalyzes the formation of adenosine and homocysteine from *S*-adenosylhomocysteine. Inactivation of this enzyme by dAdo leads to accumulation of *S*-adenosylhomocysteine which can act as a product inhibitor of *S*-adenosylmethionine-mediated methylation reactions [19].

Phase I clinical trials with dCF revealed rapid increases in plasma levels of dAdo and accumulation

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of dATP in erythrocytes and leukemic cells [14, 20, 21]. Many dCF-treated patients, with various forms of advanced cancer, developed severe lymphopenia, suggesting that ADA inhibition is cytotoxic to non-dividing lymphocytes in man. *In vitro* studies indicate that peripheral blood lymphocytes show a dose-dependent loss of viability when incubated under non-stimulating conditions for 72–96 hr with an ADA inhibitor and dAdo [22]. Under these conditions it seems unlikely that dATP inhibition of ribonucleotide reductase alone can account for the toxicity. Carson *et al.* [23] have proposed that ATP depletion may be the primary mechanism of toxicity in ADA-inhibited resting lymphocytes [22].

In the present study we have examined the incorporation of dAdo into the nucleotide pools of human peripheral T and B lymphocytes and have compared it to the incorporation patterns of several Ado and dAdo analogs. A preliminary report of these findings has been presented [24, 25].

METHODS

Chemicals. The following items were purchased from the Sigma Chemical Co., St. Louis, MO: Histopaque 1077, dithiothreitol (DTT), Tris buffer, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer, B-lymphocyte test kit No. 95, bovine gamma globulin, ATP, 2′-deoxyadenosine, 7-deazaadenosine (tubercidin), arabinosyladenine (araA), formycin A, 2′-deoxycytidine, adenosine, 2′-deoxyguanosine, inosine, neuraminidase type IV, and xanthine oxidase. Fluorescein (FITC)-conjugated goat antihuman immune globulin (IgA, IgM, and IgG) was purchased from Cappel Laboratories, Cochranville, PA. Roswell Park Memoral Institute medium-1640 (RPMI-1640) containing 25 mM Hepes, penicillin (5000 units/ml) and streptomycin (5000 μg/ml), fetal bovine serum, and trypan blue (0.4%) were purchased from GIBCO, Grand Island, NY. Sterile 15-ml centrifuge tubes (Falcon 2095) and polystyrene petri dishes (10 × 100 mm) were purchased from American Scientific Products, Bedford, MA. [8-^{14}C]Adenosine (37.3 mCi/mmole), [U-^{14}C]-2′-deoxycytidine (29 mCi/mmole) and [U-^{14}C]-2′-deoxyadenosine (57 mCi/mmole) were purchased from the New England Nuclear Corp., Boston, MA. [8-^{3}H]-2′-Deoxyguanosine (14.5 Ci/mmole) was purchased from ICN, Radiochemical Division, Irvine CA. Betafluor counting scintillant was purchased from National Diagnostics, Somerville, NJ, and ACS aqueous counting scintillant from Amersham, Arlington Height, IL.

dCF was provided by Dr. John Douros of the Drug Development Branch of the National Cancer Institute. 9-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine (2′-F-araA) was synthesized and provided by Dr. Jack J. Fox of the Memorial Sloan-Kettering Cancer Center, NY. 9-(2′-Deoxy-2′-fluoro-β-D-ribofuranosyl)adenine (2′-F-riboA) was synthesized and provided by Dr. Morio Ikehara of Osaka University, Osaka, Japan. All other chemicals were of reagent grade and obtained through the Fisher Scientific Co., Medford, MA.

Cell isolation. Human erythrocytes were obtained by venipuncture from healthy volunteers using sodium citrate as the anticoagulant. The blood was washed repeatedly with 0.9% sodium chloride to remove platelets, leukocytes, and traces of plasma.

The CCRF-CEM leukemic cell line was grown in suspension culture in RPMI-1640 bicarbonate buffered medium supplemented with 10% fetal bovine serum under a 95% air and 5% CO_2 humidified atmosphere in the laboratory of Dr. Michael Wiemann of the Roger Williams General Hospital, Providence, RI. Cells used for the incorporation studies were in log growth phase. Before use, the cells were collected by centrifugation and suspended in fresh RPMI-1640 supplemented with 25 mM Hepes and 5% fetal bovine serum.

Peripheral lymphocytes were isolated from the buffy coat layer of blood drawn from healthy human volunteers. The buffy coat blood was provided by Dr. Manfred Steiner of The Memorial Hospital, Pawtucket, RI. RPMI-1640 medium containing 25 mM Hepes (pH 7.4) was used throughout these studies and is henceforth referred to as Medium. The buffer layer (10–15 ml) was diluted 1:4 with Medium and processed according to the method of Boyum [26] to isolate the peripheral blood mononuclear cells. The lymphocyte-enriched cells were recovered from the Histopaque (Ficoll-Hypaque) medium interface and washed at least three times by repeated suspension in fresh Medium and centrifugation at 400 *g* for 5 min. The cell suspensions were then adjusted to an appropriate cell number for use in drug incorporation studies or for further purification of the T and B lymphocytes.

T lymphocytes were isolated from rosettes formed with neuraminidase-treated [27] sheep erythrocytes (Scott Laboratories, Fiskville, RI) using a modification of the method described by Gmelig-Meyling and Ballieux [28]. The lymphocyte suspensions (5×10^6 cells/ml) were combined with equal volumes of 10% sheep erythrocytes in Medium supplemented with 10% fetal bovine serum. A 6-ml portion of this cell suspension was layered over a 4-ml Histopaque cushion and centrifuged at 8° for 30 min at 800 *g* to sediment the T cell rosettes. 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 T lymphocytes isolated by this method were completely free of B lymphocytes and contained less than 1% monocytes as determined by published methods [29, 30]. The mononuclear cells at the Histopaque interface were collected and washed three times with Medium to remove traces of Histopaque and plasma. A second rosetting cycle was rarely required since 95% of the rosette-forming T lymphocytes were recovered in the first treatment.

The T lymphocyte-depleted mononuclear cells were further enriched for B lymphocytes by depleting them of plastic adherent monocytes. The T lymphocyte-depleted mononuclear cells (5×10^6 cells/ml) were suspended in Medium supplemented with 30% fetal bovine serum and incubated for 2 hr in polystyrene petri dishes at 37° under a 95% air and 5% CO_2 humidified atmosphere. The B lymphocyte-enriched cells were recovered by gently swirling the dishes and removing the nonadherent (T

lymphocyte/monocyte depleted) cells with a plastic pipette. Most B lymphocyte-enriched preparations contained approximately 70% B lymphocytes (surface immunoglobulin positive cells) and 15–30% contaminating monocytes.

To verify the results obtained from the nucleoside incorporation studies on the B lymphocyte-enriched cells, further purification of the B lymphocyte population was performed using the Affi-Gel Cell Sorting System (Bio-Rad Laboratories, Richmond, CA) as described in the brochure of the manufacturers. Because of the large numbers of cells needed, it was not feasible to use affinity purified B lymphocytes for all the incorporation studies.

Nucleoside incorporation studies. To compare the abilities of the various cell types to incorporate dAdo and various adenosine analogs into their nucleotide pools, all cells were preincubated for 30 min at 37° in loosely-capped polystyrene centrifuge tubes with 1.0 ml of Medium containing 10 μM dCF. The incubations (duplicates or triplicates) were started with the addition of 1.0 ml of Medium containing the appropriate concentration of the nucleoside and were placed in a humidified incubator at 37° exposed to a 95% air/5% CO_2 atmosphere. Lymphocytes were incubated at a concentration of 5×10^6 cells/ml, CCRF-CEM leukemic cells at 1.5×10^6 cells/ml, and the erythrocytes as 1% cell suspensions. Incubations were terminated by centrifuging the cells at 600 *g* for 5 min. The cell pellets were resuspended in 1.0 ml of 0.9% sodium chloride and again centrifuged. After aspiration of the saline supernatant fluid, the cell pellets were treated with 0.5 ml of 4% perchloric acid and placed on ice for 10 min. The acid-insoluble material was removed by centrifuging at 1000 *g* for 10 min at 4°. The supernatant fluids were neutralized with 50 μl of 5 N KOH and 50 μl of potassium phosphate buffer (0.5 M; pH 7), and the $KClO_3$ precipitate was removed by centrifugation. The neutralized extracts were stored at −20° and analyzed by anion exchange high performance liquid chromatography (HPLC) [31, 32].

Enzyme assays. All enzyme assays were carried out on extracts of T lymphocytes and B cell-enriched lymphocytes isolated as described above. Cell extracts were prepared by suspending a known quantity of cells in a 1:5 ratio with Enzyme Buffer (5% glycerol, 2 mM DTT, and 50 mM Tris–HCl, pH 7.4). The cells were disrupted by three cycles of freezing and thawing, and then centrifuged for 60 min at 105,000 *g*. The supernatant fluids were assayed for enzyme activity. Protein estimations were made with the Bio-Rad Protein Assay kit, a colorimetric method based on dye-binding to proteins [33]. Bovine gamma globulin was used as the protein standard.

The enzymatic assays employed were minor modifications of published procedures [34–37]. Adenosine deaminase activity was measured spectrophotometrically with formycin A as the substrate according to the method described by Chassin *et al.* [34]. Purine nucleoside phosphorylase was measured by following the rate of hypoxanthine formation from inosine as monitored by its conversion to uric acid by xanthine oxidase. The reactions were carried out at 30° with 0.05 M potassium phosphate buffer, pH 6.5, and 0.02 to 0.04 units of xanthine oxidase per ml [35]. The nucleoside kinase enzymes were assayed by modifications of the methods of Ives *et al.* [36] and Lukey and Snyder [37]. This assay utilized radiolabeled nucleosides which were phosphorylated by the kinases in the presence of ATP and Mg^{2+}. The phosphorylated nucleosides were then trapped on DE-81 (Whatman, Inc.) filter discs, washed free of unreacted nucleoside with 1 mM ammonium formate, and counted. 2′-Deoxyguanosine kinase was assayed with [8-^{3}H]-2′-deoxyguanosine (60 mCi/mmole) at a concentration of 0.3 mM with 3.5 mM ATP, 10 mM $MgCl_2$, 15 mM NaF, and 50 mM Tris–HCl at pH 8.0 in a total volume of 0.25 ml. To avoid the phenomenon of substrate inhibition by adenosine (Ado), Ado kinase was assayed with [8-^{14}C]Ado (5 mCi/mmole) at a concentration of 40 μM with 5 mM ATP, 1.0 mM $MgCl_2$, 15 mM NaF, 5 μM dCF, and 50 mM Tris–HCl (pH 7.4). dAdo kinase was assayed with [8-^{14}C]-2′-dAdo (5 Ci/mmole) at a concentration of 450 μM with 1 mM ATP, 5 mM $MgCl_2$, 15 mM NaF, 5 μM dCF, and 50 mM Tris–HCl (pH 7.4). 2′-Deoxycytidine kinase was assayed with 0.3 mM [U-^{14}C]-2′-deoxycytidine (6 mCi/mmole) in the presence of 3.5 mM ATP, 10 mM $MgCl_2$, and 50 mM Tris–HCl (pH 8.0). In all cases, the cell extracts (approx. 0.6 mg/ml protein) were preincubated at 37° for 5 min with the buffer, dCF, and NaF. The reactions were initiated by the addition of the Mg^{2+}, ATP, and substrate. Samples (25 μl) were taken at selected time intervals over 30 min and added directly to the DE-81 discs where they were allowed to dry. The discs were rinsed ten times with 1 ml of ammonium formate on a Millipore filter support. Tritium-labeled substrates were eluted with 0.8 ml of 0.1 M HCl and 0.2 M NaCl into scintillation vials to which 9 ml of ACS aqueous counting solution was added. Discs with ^{14}C nucleotides were added directly to 10 ml of Betafluor for counting in a Packard TriCarb model 3320 liquid scintillation spectrometer.

RESULTS

Comparison of dATP accumulation in peripheral lymphocytes, erythrocytes and the CCRF-CEM lymphoblast cell line. After 30 min of preincubation with the ADA inhibitor, dCF, all three cell types accumulated dATP following the addition of 100 μM dAdo. As indicated in Fig. 1, peripheral lymphocytes showed a non-linear pattern of dATP accumulation over the 24-hr time course. These cells reached a maximal level of dATP at 4 hr that did not exceed 10% of the control ATP concentration. In contrast to the lymphocytes, both the erythrocytes and the CCRF-CEM lymphoblasts displayed an approximately linear accumulation of dATP. The formation of dATP in the CCRF-CEM cells proceeded rapidly, exceeding slightly the ATP concentration of the control cells at 6 hr. No viable cells remained in the 16- and 24-hr time samples. The erythrocytes accumulated dATP about four to five times more slowly than the CCRF-CEM cells. However, by 24 hr the dATP concentration in the erythrocytes had reached 97% of the control cell ATP concentration, whereas the ATP levels had decreased about 60%. Despite the marked increase in dATP and decrease in ATP

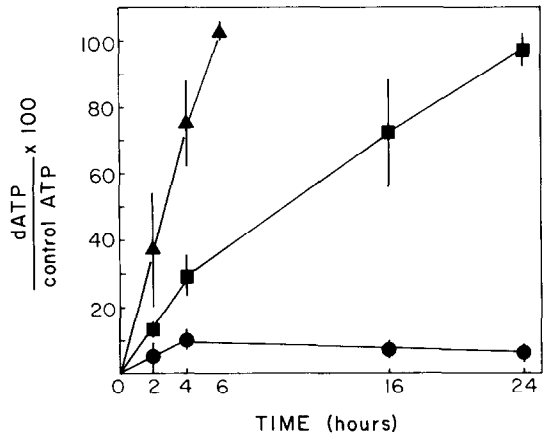

Fig. 1. dATP accumulation in human peripheral lymphocytes (●), erythrocytes (■), and CCRF-CEM lymphoblastic cells (▲) incubated with 100 μM dAdo, following a 30-min preincubation with 10 μM 2′-deoxycoformycin. The plotted dATP values are expressed as a percentage of the ATP concentrations of appropriate control cells incubated in the absence of dAdo. The standard deviations are represented by the bars (N = 3–5 experiments for each point).

concentrations, there was no evidence of hemolysis in the 24-hr samples.

dATP accumulation in isolated T and B lymphocytes. The relatively limited capacity of the peripheral lymphocytes to accumulate dATP suggested that they have a mechanism to prevent excessive dATP formation and/or that a particular lymphocytic subpopulation may account for most of the dATP. To address the latter possibility, peripheral blood lymphocytes were separated into T and B cell-enriched populations. The results in Table 1 indicate that the T lymphocytes did not synthesize amounts of dATP detectable by HPLC. By contrast, the B cell-enriched lymphocytes and the affinity column-purified B lymphocytes synthesized readily measurable amounts of dATP. Figure 2 compares the HPLC profiles of erythrocytes, CCRF-CEM lymphoblasts, T lymphocytes, and B cell-enriched lymphocytes after 4 hr of incubation with 100 μM dAdo and 10 μM dCF. There was no accumulation of dATP in the T lymphocytes even after 24 hr of co-incubation with dAdo and dCF. In Fig. 3, the formation of dATP by B cell-enriched lymphocytes is shown in relation to changes in the ATP concentrations. Although the dATP concentration achieved a maximum between 4 and 16 hr the ATP level decreased linearly to about

Table 1. dATP accumulation in human peripheral blood lymphocyte subpopulations incubated for 90 min with 2′-deoxycoformycin (10 μM) and 2′-deoxyadenosine (250 μM)

Lymphocyte population	dATP (pmoles/10^6 cells)
Peripheral lymphocytes (unfractionated)	10
Purified T lymphocytes	<10*
B Cell-enriched lymphocytes	34
Affinity-purified B lymphocytes	49
Non-B lymphocytes	<10

* No detectable dATP accumulation. dATP concentrations <10 pmoles/10^6 cells could not be detected.

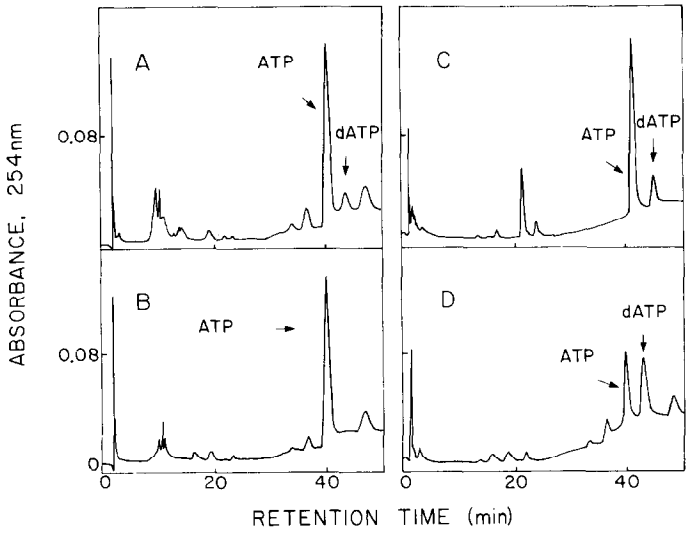

Fig. 2. High performance liquid chromatographic profiles of dATP formed during 4 hr of incubation with 2′-deoxycoformycin (10 μM) and dAdo (100 μM) in human B lymphocytes (A), T lymphocytes (B), erythrocytes (C), and CCRF-CEM lymphoblastic cells (D).

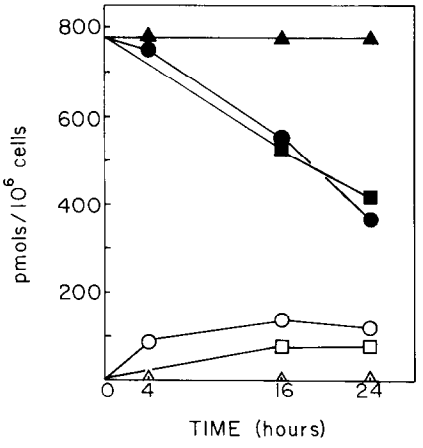

Fig. 3. Alterations in dATP (open symbols) and ATP (closed symbols) concentrations in human B cell-enriched lymphocytes incubated with 10 μM dAdo (□, ■) or 100 μM dAdo (○, ●) and in control cells (△, ▲). dATP concentrations <10 pmoles/10^6 cells could not be accurately quantitated with our HPLC system.

50% of the control values in 24 hr. Cell viability remained unchanged throughout the 24-hr incubation period (>90%). In agreement with the findings of others, viability declined after 24 hr [22].

Formation of nucleoside 5'-triphosphates in T and B cell-enriched lymphocytes incubated with dAdo and various adenosine analogs. To determine whether T and B lymphocytes differ in their abilities to transport and phosphorylate nucleosides, several adenosine analogs were compared with dAdo. The results are shown in Table 2. AraA and 2-F-araA were incorporated to a similar extent in both the T and B lymphocytes. 2'-F-riboA, a presumed dAdo analog, behaved like tubercidin and formed large amounts of nucleoside triphosphate in both cell types. Formycin A, an adenosine analog, was phosphorylated equally by T and B lymphocytes. 2'-F-araA was the only analog that resembled the unique incorporation pattern of dAdo by forming small amounts of triphosphate nucleotide only in the B cell-enriched lymphocytes.

Comparison of nucleoside-metabolizing enzymes in the cytosolic extracts (100,000 g) *of T and B cell-enriched lymphocytes.* Table 3 compares the activities of adenosine deaminase, purine nucleoside phosphorylase, and several deoxynucleoside kinases in T and B cell populations. No significant differences were seen between the T and B cell-enriched lymphocytes in the activities of these enzymes of nucleoside metabolism. Lymphocyte nucleotidase activities were also studied and are the subject of a separate publication [38].

Table 2. Incorporation of dAdo and several adenosine analogs into 2'-deoxycoformycin-pretreated T and B cell-enriched lymphocyte nucleoside triphosphate pools*

Nucleoside (100 μM)	Nucleoside 5'-triphosphate†	
	T lymphocytes	B lymphocytes
dAdo	0	11
AraA	2	3
2-F-araA	4	6
2'-F-riboA	38	53
2'-F-araA‡	0	0.5
Tubercidin	48	52
Formycin A	17	17

* T and B cell-enriched lymphocytes were isolated as described in Materials and Methods. The cells were preincubated with 5 μM dCF for 30 min before addition of the appropriate nucleoside. Duplicate cultures were incubated for 4 hr at 37° without agitation in a humidified 5% CO_2 atmosphere. The analog nucleoside 5'-triphosphate accumulation was determined by HPLC analysis as described in Materials and Methods.

† Nucleoside 5'-triphosphate accumulation is expressed as a percentage of the ATP peak area from nucleoside-free control cultures. Control T and B lymphocytes had 780 pmoles of ATP/10^6 cells. Values are averages of duplicate measurements and are representative of results from two or three experiments.

‡ These results are from a single experiment in which the T and B lymphocytes were incubated for 2 hr with 500 μM 2'-F-araA; all other conditions were the same as those described above. The limited supply of 2'-F-araA prevented further study of this compound.

DISCUSSION

In view of earlier studies that examined lymphoblastic cell lines of T and B cell origin, the findings reported here on normal non-dividing peripheral blood T and B lymphocytes were both unexpected and provocative. As reported elsewhere and confirmed here, lymphoblastic cell lines, e.g. CCRF-CEM, accumulate large amounts of dATP when exposed to dAdo in the presence of an ADA inhibitor [7, 8]. Attempts have been made to explain the

Table 3. Activities of nucleoside-metabolizing enzymes in T and B lymphocytic extracts

Enzyme	Activities*	
	T lymphocytes	B lymphocytes
Adenosine deaminase	860 ± 280	940 ± 160
Purine nucleoside phosphorylase	1470 ± 480	1350 ± 540
Adenosine kinase	31 ± 14	31 ± 14
Deoxyguanosine kinase	5 ± 3	7 ± 1
Deoxycytidine kinase	4 ± 2	4 ± 3
Deoxyadenosine kinase	17 ± 3	19 ± 3

* Activities are expressed in terms of pmoles/min/10^6 cells. Each value represents the mean activity of four to six different individuals ± the standard deviation.

immunosuppressive actions of the ADA inhibitors on the basis of the behavior of the lymphoblastic cell lines. The present findings, that B cells rather than T cells accumulate large amounts of dATP under such conditions, indicate that these interpretations must be reconsidered. Recently, a preliminary report has been presented of similar findings with peripheral T and B lymphocytes [39]. We have not yet been able to explain the difference between T and B cells in their abilities to accumulate dATP on the basis of enzyme patterns, as shown in Table 3 and in other studies to be reported elsewhere on the nature of the membrane-associated and cytosolic nucleotidase activities of T and B lymphocytes [24, 38]. In agreement with other laboratories [40], we do not find any difference in purine nucleoside phosphorylase (PNP) activity between T and B lymphocytes, which contradicts the early report of Borgers *et al.* [41]. The finding that peripheral B lymphocytes accumulate substantial levels of dATP when exposed to dAdo and dCF may have special significance with regard to recent reports that leukemias of B cell origin, including nodular lymphomas, chronic lymphocytic leukemia and "hairy cell" leukemia, are responsive to treatment with low doses of dCF [42–44].

The CCRF-CEM lymphoblastic T cell line has been used as a model system for studying the lymphocyte toxicity encountered in ADA-deficient patients. With respect to deoxynucleotide metabolism, the CCRF-CEM cells resemble developing human thymocytes [22]. However, similarities between human peripheral lymphocytes and the lymphoblastic cell lines are less striking. The CCRF-CEM cells are extremely susceptible to the toxic effects of dAdo and are lethally affected after a short period of exposure [45]. Peripheral lymphocytes are clearly susceptible to dAdo toxicity in the presence of an ADA inhibitor [14, 20, 21]; however, the lethal effects are demonstrable only after 24 hr of exposure [22, 23]. As shown in Fig. 1, the CCRF-CEM cells also differ from peripheral lymphocytes in the ability to incorporate dAdo. The CCRF-CEM cells accumulated dATP in a rapid and linear fashion, unlike the peripheral lymphocytes which restricted dATP accumulation. Furthermore, the data in Fig. 2 and Table 1 suggest that peripheral T lymphocytes accumulate very little (<10 pmoles/10^6 cells) dATP, underscoring the lack of correlation between the CCRF-CEM cells and T lymphocytes. This disparity in dATP accumulation between the T lymphocytes and the CCRF-CEM cell line may be partly attributed to the greater synthetic capacity of the actively dividing cells. However, the progressive accumulation of dATP by human erythrocytes (Fig. 1), the prototype of a terminally differentiated cell and one that does not contain a specific deoxynucleoside kinase [46, 47], suggests that dATP accumulation may depend more on the rate of dAdo nucleotide catabolism.

Previous studies on T and B lymphoblastic cell lines indicated a greater tendency for the cell lines to synthesize and accumulate dATP [48]. However, recent reports suggest that the observed differences in dAdo incorporation may be due to inherent differences between individual cell lines that are independent of T or B lymphocyte origin [49, 50]. As shown in the HPLC profiles in Fig. 2, dATP accumulated only in the B cell-enriched lymphocytes. In these cells, the incorporation of dAdo reached an apparent steady-state level after 4 hr (Fig. 3), sharing the same pattern seen with the pooled peripheral lymphocytes (see Fig. 1). The results of Table 1 confirm the B lymphocyte as the principal cell type responsible for the dATP accumulation. Comparison of the nucleoside kinase activities in T and B cell-enriched lymphocyte extracts does not explain the differential incorporation of dAdo into these cells. The lack of a significant difference in dAdo or Ado kinase activity is further illustrated by the comparable incorporation of several dAdo analogs into both lymphocyte subpopulations. The analogs, 2-F-araA and araA, formed small but equivalent amounts of analog nucleoside triphosphate in both B and T cell-enriched lymphocytes. This was surprising since both the arabinosyl nucleosides have been reported to share the same phosphorylation pathway as dAdo, e.g. adenosine kinase and/or 2′-deoxycytidine kinase [51–53]. Tubercidin, a substrate for adenosine kinase, also formed a substantial quantity of nucleotide in both cell types. The results of these experiments demonstrate that both the T and B lymphocytes have similar capacities for synthesizing adenine nucleotides. These findings, in agreement with the nucleoside kinase measurements, suggest that the differential incorporation of dAdo is not explained by differences in nucleoside phosphorylation.

Additional evidence for this proposal comes from the incorporation patterns of the dAdo analogs, 2′-F-araA and 2′-F-riboA. Stoeckler *et al.* [32] have shown previously that both compounds are good substrates for human erythrocyte ADA. When the ADA is inhibited by dCF, human erythrocytes and murine Sarcoma 180 cells readily convert both analogs to their respective nucleoside 5′-triphosphates. In erythrocytes the 2′-F-araATP replaced ATP which was reduced by 70%. However, 2′-F-riboinosine and 2′-F-arainosine showed low and no activity, respectively, with human erythrocyte purine nucleoside phosphorylase, despite the fact that, on the basis of the close steric resemblance of hydrogen and fluorine atoms, one might assume that both analogs closely resemble 2′-deoxyinosine, an excellent substrate for purine nucleoside phosphorylase [32]. The present findings illustrate further the complex behavior of this type of analog. One might expect on steric grounds that both 2′-F-riboA and 2′-F-araA would closely resemble dAdo. However, the incorporation patterns shown in Table 2 indicate that 2′-F-riboA resembles the Ado analog tubercidin, whereas only trace amounts of 2′-F-araA were converted to the 5′-triphosphate nucleoside by B lymphocytes with no detectable formation of analog nucleotides by T lymphocytes. This pattern correlates with that observed earlier with mixed peripheral lymphocytes [32]. It is likely that further detailed examination of the biochemical behavior of this type of dAdo analog will yield important insights into the routes of dAdo metabolism in lymphocytes.

Previously, Kefford and Fox [22, 54] had shown that peripheral T and B cell-enriched lymphocytes accumulate nearly equivalent amounts of dATP

when incubated with dAdo and an ADA inhibitor. It is difficult to account for the discrepancies with the results reported above, since their methods for isolating lymphocyte populations were nearly identical to those employed in the present study. A significant difference in methodology is their practice of incubating the lymphocytes overnight prior to the addition of dAdo, which could have contributed to the incorporation of dAdo by the T lymphocytes. The process of isolating T lymphocytes by the E-rosette method has been shown to stimulate DNA synthesis [55]. By allowing the T cells to incubate overnight, especially if cells from multiple donors are used, a significant number of T lymphocytes may have become activated [56]. The early phase of lymphocyte activation triggers increased protein synthesis, including the synthesis of adenosine kinase and other nucleoside kinase enzymes [57, 58]. Activated lymphocytes have also been shown to expand their deoxynucleotide pools in preparation for DNA synthesis, which may explain the difference between our results and those previously reported [59]. To avoid introducing artifacts related to lymphocyte stimulation, in our studies the lymphocytes were isolated from individual donors and nucleoside incubation experiments were initiated immediately following isolation. Incubation of lymphocytes with dAdo and dCF has been shown to block the early changes associated with lymphocytic activation, including protein synthesis [60]. Perhaps the absence of detectable dATP accumulation in the T lymphocytes, compared with the B cell-enriched lymphocytes and, more importantly, with actively dividing T lymphocytes (e.g. CCRF-CEM lymphoblastic cell line) is explained by the presence of an active dAdo nucleotide catabolizing pathway which may be low or absent in the latter cell types. Evidence for such a pathway is entirely circumstantial, since no such enzyme(s) has yet been identified. Future studies that examine dAdo incorporation into T lymphocytes in the early phases of mitogen-induced activation may provide additional insights into this question.

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