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EFFECT OF ERYTHRO-9-(2-HYDROXY-3-NONYL) ADENINE ON PURINE AND PYRIMIDINE METABOLISM IN THE HUMAN PERIPHERAL LYMPHOCYTE DURING THE EARLY PHASES OF PHYTOHEMAGGLUTININ-MEDIATED BLASTOGENESIS

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Abstract—The effect of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) on purine, pyrimidine and ribonucleic acid metabolism in the human peripheral lymphocyte during the early phases of blastogenesis has been studied. Experiments that determine the incorporation of radioactive free base or nucleoside into their respective nucleotides indicated EHNA-inhibited phosphoribosylpyrophosphate-dependent reactions, nucleoside kinase reactions and RNA synthesis.

Studies utilizing the separating and quantitating ability of high pressure liquid chromatography demonstrated that 5–10 μM EHNA increased the nucleotide pool size of ADP without significantly changing the pool sizes of ATP, GTP or UTP. It also greatly increased the incorporation of deoxyadenosine into dADP and dATP. Higher concentrations of EHNA (15–100 μM) reduced all the measured nucleotide pool sizes when compared to the values observed at the lower EHNA concentration. In addition, 5–10 μM EHNA significantly reduced the energy level of the cell, which was not further altered by higher concentrations of EHNA. Our studies also determined that EHNA inhibited human lymphocyte guanase and indirectly increased glycolysis and respiration.

The transformation of small lymphocytes to lymphoblasts in culture media, induced by the mitogenic agent phytohemagglutinin (PHA), simulates cellular changes occuring *in vivo* after stimulation by antigen and thus provides an *in vitro* system for the study of problems basic to immunology. Adenosine deaminase and nucleoside phosphorylase are two enzymes that seem to be essential to the immune response. Individuals who show a deficiency in these enzymes also exhibit severe immune dysfunction [1, 2].

Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), a highly potent competitive inhibitor of adenosine deaminase (ADA), is coming into increasing use in studies of the metabolism of intact cells [3–5]. Human lymphocytes have been used with the aim of elucidating the biochemical events responsible for severe combined immunodeficiency disease associated with adenosine deaminase deficiency. In human lymphocytes undergoing blastogenesis and in cultured human lymphoblasts, it has been demonstrated that EHNA inhibits the incorporation of tritiated leucine into protein and tritiated thymidine into DNA. These inhibitory effects of EHNA were potentiated with adenosine and cyclic AMP [6–8].

The specificity of EHNA has been brought into question. Agarwal and Parks [9] stated that EHNA at 100 μM has no inhibitory effect on rabbit muscle adenylate deaminase. Henderson *et al.* [10], however, demonstrated that, in addition to adenosine deaminase, adenylate deaminase is also inhibited. Experiments with Ehrlich ascites tumor cells *in vitro*, as well as with cultured mouse lymphoma L5178Y cells, demonstrated that EHNA inhibited the conversion of adenosine, adenine, hypoxanthine and guanine to nucleotides, in addition to the *de novo* synthesis of purine nucleotides [10].

It is clear, therefore, that for a better understanding of experimental results in which this inhibitor is used, a study of all its possible effects in a particular cell system is needed. We have undertaken such a study, testing the effects of this inhibitor at various concentrations during the early phase of human lymphocyte blastogenesis, and have determined its effects on purine, pyrimidine and ribonucleic acid metabolism.

METHODS

Lymphocyte isolation

Aseptic cultures of human lymphocytes were prepared from 200 to 500 ml of blood collected, with heparin (The Upjohn Co., Kalamazoo, MI) as an anticoagulant, from laboratory-worker volunteers. The erythrocytes were sedimented by transferring the blood in 10 ml aliquots into 3 ml of 5% dextran (medium fraction, J. T. Baker Chemical Co., Phillipsburg, NJ) in saline using 15 × 150 mm screw cap tubes (Falcon 3026, Falcon Plastics, Division of Bio Quest, Oxnard, CA) and allowing the erythrocytes to sediment for 0.5 hr in an air incubator. The

leukocyte-rich plasma was withdrawn from the individual tubes in 2.5 ml portions and layered over 2.5 ml Ficoll-Hypaque, prepared by mixing 24 parts of 9% Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) and 10 parts of 34% (w/v) sodium diatrizoate (Winthrop Laboratories, Division of Sterling Drug, Inc., New York, NY), adjusted to a specific gravity of 1.077 with water. The tubes (12 × 75 mm, Falcon 2054) were centrifuged at 1000 *g* for 45 min. The resulting lymphocyte- and monocyte-containing interphase was recovered and washed with Hank's balanced salt solution buffered with Hepes* solution (Grand Island Biological Co., Grand Island, NY), using twice the cell suspension volume. The cells were recovered by centrifugation at 400 *g* for 10 min, washed two more times with the balanced salt solution, suspended in 10 ml of this solution, and counted in a hemocytometer.

Culture conditions

Lymphocytes were cultured in 100 × 20 mm tissue culture dishes (Falcon 3003) containing 10×10^6 cells in a final volume of 20 ml. Each individual determination was performed on at least 40×10^6 cells. EHNA obtained from the Burroughs Wellcome Co. (Research Triangle Park, NC) was added to the test cells 1 hr prior to PHA stimulation to obtain the desired micromolar solution.

Lymphocyte blastogenesis was initiated by the addition of 0.5 ml phytohemagglutinin (PHA-M, Difco Laboratories, Detroit, MI) to each culture dish. The dishes were placed in a 5% CO_2 humidifier air incubator at 37°. All cultures contained 20% fetal calf serum in Eagle's minimal essential medium (MEM), Spinner-modified, supplemented with penicillin (100 μg/ml), glutamine (2 mM), streptomycin (100 units/ml) and sodium bicarbonate (0.23%), obtained from the Grand Island Biological Co. The culture media used for the adenosine, deoxyadenosine and guanosine incorporation studies were modified with fetal calf serum that had been heated for 1 hr at 56° prior to use.

Experimental protocol

Eighteen hr after initiation of blastogenesis with PHA, the cells were pulsed with various radioactive-labelled compounds for 2 hr. The pulse labeling was accomplished by adding 1.0 μCi/10×10^6 cells of [8-^{14}C]adenosine (48 mCi/mmole), [8-^{14}C]deoxyadenosine (37 mCi/mmole), [8-^{14}C]adenine (56 mCi/mmole), [8-^{14}C]guanosine (49 mCi/mmole), [8-^{14}C]inosine (35 mCi/mmole), [2-^{14}C]uracil (60 mCi/mmole), and [2-^{14}]cytosine (46.5 mCi/mmole), and 5.0 μCi/10×10^6 cells of [8-^{3}H]hypoxanthine (8 Ci/mmole), [8-^{3}H]guanine (6 Ci/mmole), [5-^{3}H]uridine (28 Ci/mmole), and [5-^{3}H]cytidine (27 Ci/mmole)—all purchased from Schwarz-Mann Orangeburg, NY. Five μCi/10×10^6 cells of [^{14}C-U]glycine (99.6 mCi/mmole) and [^{14}C-U]aspartic acid (192 mCi/mmole), purchased from New England Nuclear (Boston, MA), were used for measuring *de novo* purine and pyrimidine synthesis. At the end of all labeling periods the cells were harvested from the petri dishes: first the cells were scraped from the dish with a rubber spatula, and then the cell suspension was transferred to a centrifuge tube (Falcon 2070) and sedimented by centrifugation at 200 *g*. The aliquot of cells that would constitute a given determination was combined after washing two times with saline. A Schneider extraction [11], modified relative to the extraction of RNA [12], was carried out on a 1 ml H_2O suspension of 40×10^6 cells to obtain three fractions: an acid-soluble fraction (ASF) containing the nucleotides, and the 37° and 90° hydrolyzed fractions that contained the RNA and DNA respectively. The DNA was assayed by Burton's modification of the diphenylamine reaction using calf thymus DNA as a standard [13].

Radioactivity was determined with a Nuclear-Chicago Mark 1 scintillation counter by counting 0.5 ml samples in vials containing 10 ml Aquasol-2 (New England Nuclear). The cpm for all samples were counted to give 95 per cent, or more, confidence limits.

Radioactive distribution and nucleotide pool size in the acid-soluble fraction were determined by removing the trichloroacetic acid (TCA) with an alanine-freon solution [14] and evaporating to dryness on a rotovap under vacuum using a 10 ml pear-shaped flask. The flasks were stored in vacuum over Drierite (Fisher Scientific Co., Springfield, NJ) in the cold until reconstituted into 0.6 ml H_2O. Although samples stored in this way are stable for at least 1 week, all determinations were performed within 2 days. Aliquots of the reconstituted extract were loaded onto an ion-exchange column (Whatman Partisal 10SAX) (Whatman Co., Clifton, NJ) and chromatographed using the high pressure liquid chromotography (h.p.l.c.) system of Hartwick and Brown [15] with a Waters Associates Instrument (Waters Associates, Inc., Milford, MA). The instrument was equipped with a 254 nm filter, and the detector sensitivity was set at 0.05 AUF. The method was modified to a 10-min isocratic elution using 0.007 M KH_2PO_4 (pH 4.0). A linear gradient was created for a 35-min time period by adding a solution of 0.25 M KH_2PO_4 + 0.5 M KCl (pH 5.0). This was followed by an additional isocratic elution using the higher concentration buffer until GTP was eluted. The flow rate was kept at 2 ml/min throughout the chromatogram. The purity of the nucleotides was determined by their special data as recorded by a Cary spectrophotometer and compared to the values reported in the literature [16]. All nucleotides used as reference standards were purchased from Cal-Biochem (San Diego, CA). Quantitation was accomplished by measuring peak areas calculated from the width at half height multiplied by the height and relating to a standard curve.

To determine the radioactivity of the nucleotide pools, 2 ml aliquots were collected from the column from which 0.5 ml aliquots were used for counting. The radioactivity reported is the sum of the counts obtained for each nucleotide peak. The recovery of radioactivity from the chromatographic analysis varied from 95 to 105 per cent, within our experimental variation.

* Hepes *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

Glucose determinations were performed with the Glytel Reagent as outlined in the Pfizer Diagnostics Division bulletin (Pfizer Co., New York, NY).

Guanase determinations were performed utilizing [8-^{14}C]guanine (45 mCi/mmole) obtained from New England Nuclear and adjusted to a specific activity of 1 μCi/0.1 μmole, as well as a lymphocyte homogenate supernatant fraction obtained by homogenizing 1.5×10^9 cells in 5 ml Tris buffer, 0.16 M, pH 8.0, followed by a 5 min, 500 *g* centrifugation. The method entailed a 2-hr 37° enzyme reaction on 0.1 μmole of guanine in a final volume of 1 ml of 0.16 M Tris buffer, pH 8.0. The enzyme reaction was stopped by lowering the pH to 1.0 with 4 N HCl, followed by centrifugation for 5 min at 12,000 *g*. After addition of a carrier mixture of xanthine and guanine, the two purines were separated on a Dowex 50, H^+ form ion exchange resin (Bio-Rad, Richmond, CA) of 1 cm width and 10 cm height, using a flow rate of 2 ml/min. A concave gradient was generated using 25 ml H_2O in a mixing chamber to which was added 2.5 N HCl until xanthine was eluted. Guanine was eluted by extending the gradient to 4 N HCl. The eluates were u.v.-monitored using an Isco model UA-5 absorbance monitor (Instrumentation Specialties Co., Lincoln, NE). The xanthine and guanine peaks were collected and 1.0 ml aliquots were used for determining their radioactivity. The amount of xanthine formed was calculated from the known specific activity of guanine.

Oxygen uptake was measured polarographically with a Clerk oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) and a Biological Oxygen Monitor (model 52). The measurements were performed at 25° in MEM Spinner-modified reaction media containing 1.5×10^8 cells in a 1 ml volume.

Since quantitative values of lymphocytes vary among individual donors, each experiment was performed on cells obtained from a single donor, divided into test and control populations, and reported as percent of control values. One radioactive purine or pyrimidine base, or nucleoside, was added as labeling material per experiment, and the effect of EHNA on their conversion to nucleotides and RNA was determined. All results were expressed in terms of 100 μg DNA to correct for variations in cell recovery (100 μg DNA was found to be equivalent to 13×10^6 cells of initial culture cells).

RESULTS

The high pressure liquid chromatographic method used in these experiments allowed us to quantitate with excellent reproducibility the ATP, ADP, UTP and GTP pools of the human peripheral lymphocyte. The method demonstrated a variation of 15 per cent at most. Quantitation of the other nucleotides under these experimental conditions was less reliable since the peak areas were too small for accurate spectral analysis.

When ITP, dADP and dATP were included in a standard mixture of nucleotides (1 nmole/nucleotide), the modified method used produced a distinct peak for ITP which eluted before ATP, a distinct peak for dADP which eluted between ADP and GDP, and a peak for dATP whose descending limb overlapped the ascending limb of GTP. Neither u.v. nor radioactive monitoring demonstrated any significant amount of ITP. However, radioactive monitoring, counting 0.5 ml aliquots of the eluate and relating the counts to a u.v. scan, demonstrated a dADP and a dATP peak when the cells were pulsed with [8-^{14}C]deoxyadenosine. The uptake was negligible in the uninhibited (control) cells (dADP, 20 cpm; dATP, 55 cpm). However, when the cells were inhibited with 5 μM EHNA, the incorporation increased 20-fold (dADP, 400 cpm; dATP, 1150 cpm). Higher concentrations of EHNA did not alter the amount of incorporation of labeled deoxyadenosine.

When guanine, guanosine, adenine or adenosine was used as a pulse label, 95 per cent of the radioactivity was always found in the nucleotide whose base corresponded to the pulse labeled base used. When hypoxanthine or inosine was used, 70 per cent of the label was in the adenine base nucleotides, while 25 per cent of the label was in the guanine base nucleotides.

Human peripheral lymphocytes obtained from ten different individuals showed a wide variation in nucleotide pool size after 20 hr of PHA stimulation. Values obtained from quadruplicate determinations on each sample showed ATP varying from 4.9 ± 0.7 to 28.2 ± 3.1 nmoles/100 μg DNA, whereas ADP varied from 1.03 ± 0.2 to 6.0 ± 0.7; UTP, 1.3 ± 0.1 to 4.0 ± 0.6; and GTP, 1.0 ± 0.1 to 3.8 ± 0.5.

Many effects were produced by EHNA on purine and pyrimidine metabolism in this cell system. These effects were determined by the amount of radioactive base or nucleoside incorporated into their respective nucleotides per unit time. Only the results from the nucleoside triphosphates are presented because the majority of radioactivity in the ASF for each experiment was always in these compounds, and the radioactive data obtained from the nucleoside mono- and diphosphates paralleled those of the triphosphates.

It was observed that varying the concentrations of EHNA in the culture media produced a variety of effects that were both inhibitory and stimulatory and which increased in scope as the concentration increased. The effects were found to be reproducible in direction and magnitude when the experiments were performed on lymphocytes obtained from two to three different individuals.

Effect of EHNA on purine metabolism

Incorporation of free purine bases. EHNA markedly increased the incorporation of guanine into GTP. This effect was maximal at the 50 μM concentration. Minor changes suggesting first stimulation and then inhibition were noted in guanine incorporation into RNA, but the degree was slight and of questionable significance (Fig. 1). The control cells incorporated 24,700 cpm/100 μg DNA in 2 hr, of which 57 per cent was in the ASF while 43 per cent was in the RNA fraction.

The adenine and hypoxanthine incorporation into ATP and RNA was inhibited at all concentrations, with the initial effect observed at 5 μM, and with progressive continuation in RNA to concentrations of 100 μM. At low concentrations of EHNA the

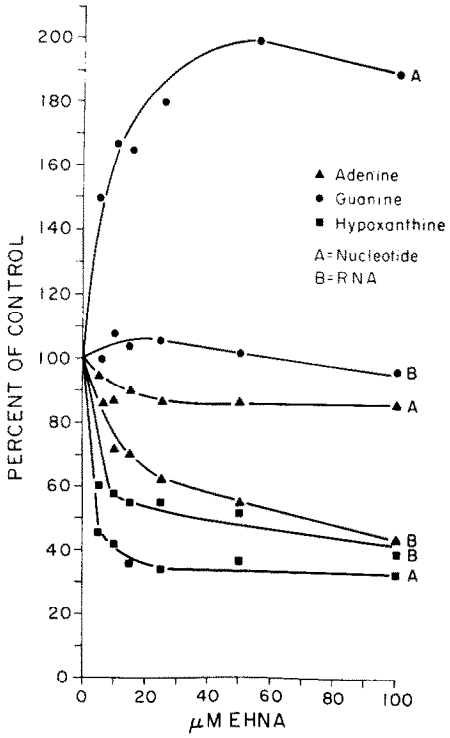

Fig. 1. Effects of EHNA on incorporation of radioactive-labeled purine bases into nucleoside triphosphates and RNA. Peripheral human lymphocytes (0.5×10^6 cells/ml) were incubated in Eagle's MEM media (Spinner-modified) and varying concentrations of EHNA for 1 hr. Blastogenesis was started with the mitogen PHA and the incubation was continued for an additional 18 hr. Radioactive-labeled purines were added for an additional 2-hr period, after which the cells were harvested and fractionated as described in Methods. Radioactivity in the nucleoside triphosphates (A) and in RNA (B) was determined. Each point for the incorporation of adenine (▲), guanine (●) and hypoxanthine (■) is presented as percent of control (lymphocytes without EHNA) and represents the mean of two determinations with a deviation of not more than 15 per cent.

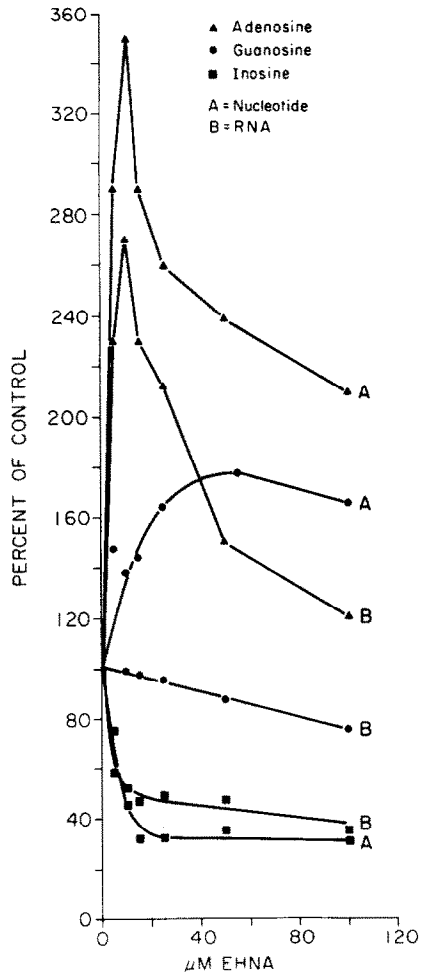

Fig. 2. Effects of EHNA on incorporation of radioactive-labeled purine nucleosides into nucleoside triphosphates and RNA. Conditions were as described in the legend of Fig. 1. Radioactivity in the nucleoside triphosphates (A) and in RNA (B) was determined. Each point for the incorporation of adenosine (▲), guanosine (●) and inosine (■) is presented as percent of control (lymphocytes without EHNA) and represents the mean of two determinations with a deviation of not more than 15 per cent.

incorporation of hypoxanthine into ATP was inhibited more than the incorporation of adenine—65 per cent as compared to 10 per cent. The incorporation of both adenine and hypoxanthine into RNA was inhibited to the same degree at intermediate to high concentrations of EHNA, although at low concentrations the inhibition of hypoxanthine was greater than that of adenine (Fig. 1). The control cells incorporated 298,800 cpm/100 μg DNA for adenine and 36,000 cpm/100 μg DNA for hypoxanthine, of which 59 per cent was in the ASF for adenine and 72 per cent in the ASF for hypoxanthine.

Incorporation of purine nucleosides. EHNA stimulated the incorporation of adenosine and guanosine into ATP and GTP respectively. The effect on adenosine incorporation was maximal at a concentration of 10 μM and for guanosine at 50 μM. The EHNA effect found relative to the incorporation of adenosine and guanosine into RNA was one of stimulation for adenosine and inhibition for guanosine. Adenosine incorporation into RNA paralleled its incorporation into ATP, whereas the guanosine incorporation into RNA was progressively inhibited by intermediate to high concentrations of EHNA (Fig. 2). For these nucleosides the control cells incorporated 74,000 cpm/100 μg DNA for adenosine and 12,300 cpm/100 μg DNA for guanosine, of which 87 per cent was in the ASF for adenosine, while 54 per cent was in the ASF for guanosine.

The effect of EHNA on the incorporation of inosine was the same as for hypoxanthine, inhibiting its incorporation into ATP and RNA (Fig. 2). The control cells incorporated 6300 cpm/100 μg DNA, of which 67 per cent was in the ASF and 33 per cent in the RNA fraction.

In all these experiments in which the free purine bases or purine nucleosides were used, the effects of EHNA on the incorporation into GTP paralleled the effects observed for their incorporation into ATP.

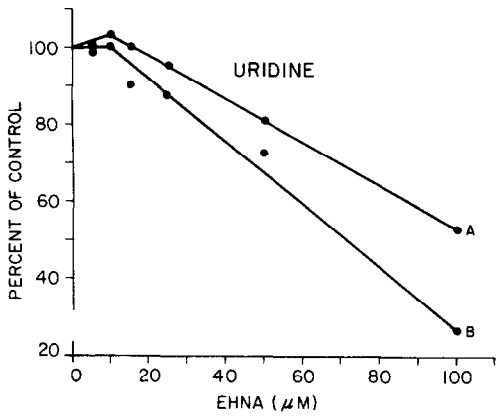

Fig. 3. Effect of EHNA on the incorporation of radioactive-labeled uridine. Conditions were as described in the legend of Fig. 1. Radioactivity in UTP (A) and RNA (B) was determined. Each point for the incorporation of uridine (●) is presented as percent of control (lymphocytes without EHNA) and represents the mean of two determinations with a deviation of not more than 15 per cent.

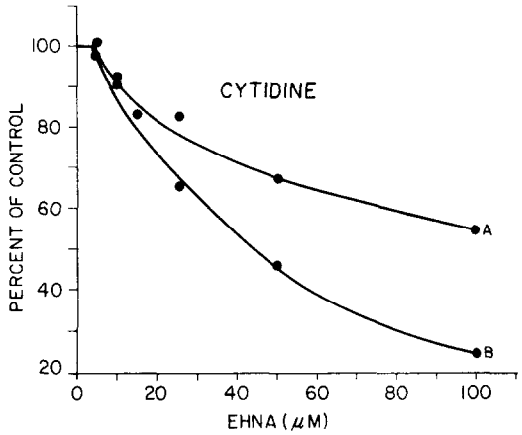

Fig. 4. Effect of EHNA on the incorporation of radioactive-labeled cytidine. Conditions were as described in the legend of Fig. 1. Radioactivity in CTP (A) and RNA (B) was determined. Each point for the incorporation of cytidine (●) is presented as percent of control (lymphocytes without EHNA) and represents the mean of two determinations with a deviation of not more than 15 per cent.

Effect of EHNA on pyrimidine metabolism

Incorporation of free pyrimidine bases. Uracil and cytosine were not incorporated in the free base form by the lymphocyte regardless of the presence or absence of EHNA. Trace radioactivity in the cells was determined to be either the free base or degradation products. No radioactivity was observed in UTP, CTP or RNA in these studies.

Incorporation of pyrimidine nucleosides. The predominant effect of EHNA on the incorporation of uridine and cytidine into UTP, CTP and RNA was one of inhibition. This was first observed at concentrations of 10 μM and increased progressively up to 100 μM. However, the inhibitory effect on the incorporation rate into RNA was greater than that into their respective triphosphate nucleotides. At concentrations of 100 μM half the amount of radioactive pyrimidine nucleosides was incorporated into RNA when compared to incorporation into their respective triphosphates (Figs. 3 and 4). The control cells incorporated 31,800 cpm/100 μg DNA for uridine and 27,800 cpm/100 μg for cytidine, of which 57 and 32 per cent, respectively, were in the ASF, while 43 and 68 per cent were in the RNA fraction.

Effect of EHNA on nucleotide pools

At low concentrations of EHNA (5–10 μM), no significant changes were observed in the pool sizes of the triphosphate nucleotides. However, the elevation observed in the ADP pool was significant. In the intermediate to high concentrations (15–100 μM), there was a significant decrease in all but the ADP

Table 1. Nucleotide pools of human lymphocytes

	ATP (nmoles/100 μg DNA)			nucleotide ADP (nmoles/100 μg DNA)			GTP (nmoles/100 μg DNA)			UTP (nmoles/100 μg DNA)		
	EHNA (μM)			EHNA (μM)			EHNA (μM)			EHNA (μM)		
Expt.	0	5	50	0	5	50	0	5	50	0	5	50
1	15.8	16.0	10.8	4.7	5.8	4.0	1.8	2.2	1.5	2.2	2.4	1.5
2	8.4	10.3	7.3	1.3	2.6	1.7	1.0	1.0	0.7	1.7	1.8	0.9
3	12.7	14.9	7.0	3.1	5.1	2.5	2.1	2.1	1.7	1.8	1.9	1.0
4	7.3	6.9	6.3	1.4	2.2	1.8	2.7	1.5	2.3	2.0	1.3	1.4
5	16.5	12.1	9.4	4.4	3.7	2.8	3.8	2.8	3.4	2.2	2.0	1.3
6	4.9	5.5	4.2	1.8	2.4	1.8	1.0	0.8	0.7	1.3	1.3	0.7
7	9.5	7.6	3.9	2.0	2.6	1.3	1.6	1.4	1.3	2.3	1.7	1.5
8	11.7	14.1	7.6	2.8	3.8	2.0	1.6	2.2	1.3	2.0	3.1	1.3
9	28.2	31.3	20.8	6.0	6.8	4.6	2.7	3.4	2.3	4.0	4.0	2.4
10	7.6	8.3	4.6	1.4	1.7	0.9	1.0	0.9	0.7	2.0	2.2	1.2
Mean	12.3	12.7	8.2	2.8	3.7	2.3	1.9	1.8	1.6	2.2	2.2	1.3
±S.D.	±6.7	±7.5	±5.0	±1.8	±1.7	±1.2	±0.9	±0.9	±0.9	±0.7	±0.8	±0.5

nucleotide pool (Table 1). This decrease occurred at 15–25 μM EHNA and was not magnified with increasing concentrations of EHNA. For simplicity of presentation, the values shown in Table 1 are restricted to those obtained for the control studies and those with 5 and 50 μM EHNA.

Statistical analysis of the observed changes in the nucleotide pools was done using the Wilcoxan Signed Ranks Test for Matched Pairs [17]. When compared to control cells, the increases observed for ADP at 5 μM EHNA and the decreases observed in ATP, GTP and UTP at 50 μM EHNA were all significant, with a P value of <0.01; also, the decrease in ADP observed at 50 μM EHNA and the changes observed for ATP, GTP and UTP at 5 μM EHNA were not significant. However, the ADP value observed at 50 μM EHNA was significantly lower than the value observed at 5 μM EHNA.

The relative changes in ATP and ADP created an altered ATP/ADP ratio. The control cell ratios varied from 2.7 to 6.5, the cells inhibited with 5 μM EHNA gave ATP/ADP ratios of 2.3 to 4.9, and the cells inhibited with 50 μM EHNA gave ATP/ADP ratios of 2.3 to 5.1 (Table 2). This decrease in the ATP–ADP ratio was significant, with a $P < 0.01$ when controls were compared to the 5 or 50 μM EHNA-inhibited cells; however, the differences observed between the 5 and 50 μM EHNA-inhibited cells were not significant.

Miscellaneous studies

De novo *synthesis of purine and pyrimidine nucleotides.* The observation that the incorporation of radioactive purine nucleosides into nucleotides was increased by the addition of EHNA, coupled with a decrease in the nucleotide pool sizes, prompted a study of *de novo* synthesis. Two cultures of 3×10^8 lymphocytes obtained from two different donors were stimulated to blastogenesis and incubated with both [^{14}C]glycine and [^{14}C]aspartic acid for 48 hr. Half of each of the cultures was incubated with either 5 or 50 μM EHNA, while the other half served as a control. Radioactive incorporation into ATP and UTP was compared.

Five μM EHNA decreased ^{14}C incorporation into ATP by 41 per cent (1100 cpm to 650 cpm), while radioactive incorporation into UTP decreased by 32 per cent (983 cpm to 665 cpm). Fifty μM EHNA decreased ^{14}C incorporation into ATP by 31 per cent (650 cpm to 450 cpm), while radioactive incorporation into UTP decreased by 99 per cent (1600 cpm to 20 cpm).

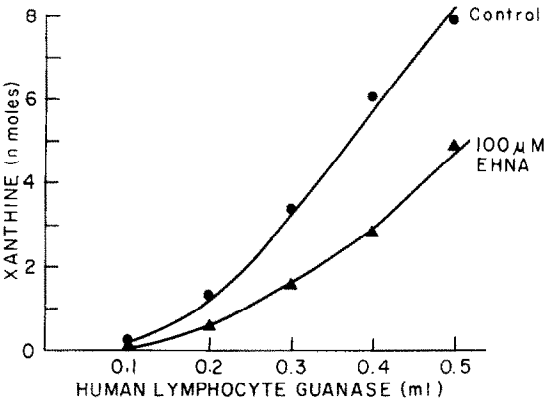

Fig. 5. Guanase activity curve. Five assays done at different enzyme concentrations in the presence of 100 μM EHNA gave values (▲) which demonstrated inhibition of enzyme activity (●).

Guanase activity. In view of the marked increase in guanine incorporation in the presence of EHNA, the effect of the latter compound on the activity of human guanase was studied using [8-^{14}C]guanine as a substrate. An enzyme activity curve was obtained, and five concentrations of the enzyme were tested with 100 μM EHNA in the enzyme mixture. A 50 per cent inhibition of activity was observed (Fig. 5).

Glucose utilization. Glucose concentrations were determined in the growth media of the control and EHNA-containing lymphocyte cultures. Its utilization was determined after 20 hr of incubation by comparison of the concentration to that found in the media alone. The media contained a glucose concentration of 110 mg/100 ml. After 20 hr of incubation the cells in the control group utilized 2.1 mg of glucose/10×10^6 cells. EHNA-treated cells increased their utilization, and at a concentration of 50 μM EHNA, glucose utilization rose to 4.6 mg/10×10^6 cells (Fig. 6). This slight but definite increase was noted consistently in six separate studies with minor variation.

Table 2. Change in ATP/ADP ratios

	ATP/ADP ratios		
	EHNA (μM)		
Expt.	0	5	50
1	3.4	2.8	2.7
2	6.5	4.0	4.3
3	4.1	2.9	2.8
4	5.2	3.1	3.5
5	3.8	3.3	3.4
6	2.7	2.3	2.3
7	4.8	2.9	3.0
8	4.2	3.7	3.8
9	4.7	4.6	4.5
10	5.4	4.9	5.1

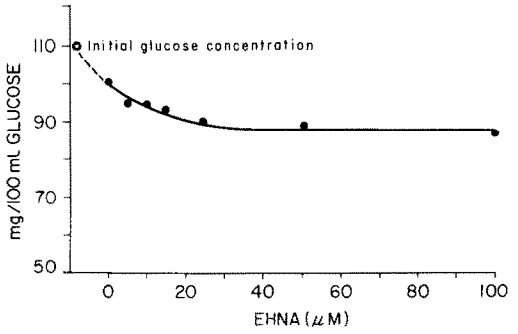

Fig. 6. Glucose utilization curve. Presence of EHNA in varying concentrations in the culture media increased glucose utilization by the lymphocytes.

Oxygen uptake. Oxygen consumption by 1.5×10^8 cells suspended in 1 ml of MEM growth media was compared to the oxygen consumption by 1.5×10^8 cells suspended in the same media containing 10 μM EHNA. The rate of oxygen uptake was determined by monitoring the decline in the oxygen tension of the media over a given time period. The decline was linear for the first 10 min of measurement, and the determinations, which were done in duplicate, gave values of 4.1 and 4.3 nmoles O_2 utilized/min for the control cells, and 4.9 and 5.1 nmoles O_2 utilized/min for the 10 μM EHNA-treated cells.

DISCUSSION

Studies utilizing the separating and quantitating ability of high pressure liquid chromatography have shown that EHNA has a wide range of effects on the purine and pyrimidine metabolism of human lymphocytes in the early phases of blastogenesis. Our observations indicate that EHNA inhibits adenine, hypoxanthine and inosine incorporation into ATP, nucleoside kinase reactions, RNA synthesis, and *de novo* synthesis of the purine and pyrimidine nucleotides. It also produces acceleration of ATP turnover, guanase inhibition, change in nucleotide pool size, and a decrease in the energy level of the cell.

Although it has been demonstrated and confirmed in this laboratory that EHNA greater than 10^{-6} M has an increasingly inhibitory effect on thymidine incorporation into DNA [7], this does not necessarily indicate an inhibition of PHA stimulation. Our results show no correlation between EHNA concentrations greater than 10 μM and the rate of adenine, hypoxanthine or inosine incorporation into ATP. There is no significant difference in inhibition of purine *de novo* synthesis or energy level of the cell between 5 and 50 μM EHNA, and the stimulatory effects observed for adenosine incorporation into its nucleotides, as well as increased glucose utilization, do not support inhibition of PHA stimulation by EHNA.

Rogler-Brown *et al.* [18] stated that the ADA inhibitor, 2-deoxycoformycin, is transported into the human erythrocyte cell via the nucleoside transport system. Henderson *et al.* [10] have asserted that EHNA does not inhibit adenosine kinase but instead inhibits adenosine entry into their cell system. In the transforming human peripheral lymphocyte cell, 5–10 μM EHNA inhibited only adenine and hypoxanthine incorporation into nucleotides, which is not further affected by increasing the EHNA concentration. If a "transport" system is inhibited for these purine bases, its effect is maximal at 10 μM EHNA. At this same concentration of EHNA, while cytidine and uridine showed no inhibition of incorporation into their respective nucleotides, guanine, guanosine and adenosine demonstrated a stimulatory effect. The concept of transport inhibition, therefore, is not supported by the results observed at this concentration of EHNA for these compounds. Above 10 μM EHNA, however, adenosine, uridine and cytidine are inhibited when compared to the 10 μM EHNA concentration, and the inhibitory effects of EHNA on these nucleosides observed at the high concentration of EHNA could be attributed to inhibition of the nucleoside transport system.

Another explanation for the observed inhibition of these nucleosides could be the fall in pool size of ATP. While the lower concentration of EHNA (5–10 μM) had no effect on the pool sizes of the nucleoside triphosphates, the higher concentrations lowered these pool sizes. However, this fall in the ATP pool was observed at the 15–25 μm level of EHNA and was not further affected at the 50–100 μM level. Since the magnitude of inhibition of incorporation of adenosine, uridine and cytidine was proportional to the concentration of EHNA in the 50–100 μM region whereas the decrease of the ATP pool was not, it seems unlikely that the decrease in ATP, the other substrate in these reactions, is solely responsible for this inhibition; however, it may be a factor.

The nuleoside deoxyadenosine increases its incorporation into dATP twenty times in the presence of 5 μM EHNA and is not further affected with increasing concentrations of EHNA. The possible transport inhibition shown for adenosine, uridine and cytidine at the higher concentrations of EHNA, therefore, is not seen with deoxyadenosine.

It has been reported that mammalian cells do not exhibit inosine or guanosine kinase activity [19, 20]. EHNA, therefore had no apparent effect on the purine nucleoside phosphorylase reactions since the effects of EHNA on hypoxanthine and inosine, as well as on guanine and guanosine incorporation into nucleotides, were similar. The observed effects of EHNA on the incorporation into both GTP and ATP were also parallel when either adenine, guanine, adenosine or guanosine was used as a pulse label. This indicated that EHNA had no specific effect on the interconversion of AMP and GMP through IMP. In addition, the parallelism demonstrated among the nucleoside mono-, di- and triphosphates showed no effect of EHNA on the formation of the nucleoside triphosphate from its monophosphate.

It is reasonable to assume that one factor responsible for the cellular level of phosphoribosylpyrophosphate (PRPP) is the enzymatic activity of PRPP synthetase. It has been shown that the PRPP synthetase activity in both *Escherichia coli* and Ehrlich ascites tumor cells is dependent upon the energy level of the cell [21, 22]: therefore, the depressed energy level demonstrated in these cells can also result in a depressed PRPP level. However, it is apparent from the results that PRPP is not the common rate-limiting factor. Whereas 5 μM EHNA inhibited the hypoxanthine–guanine transferase reaction for hypoxanthine incorporation into ATP by 65 per cent, it accelerated guanine incorporation into GTP by 150 per cent. In addition, while hypoxanthine incorporation into ATP is inhibited by 65 per cent, adenine is inhibited by only 10 per cent, suggesting that adenylosuccinate synthetase is the rate-limiting factor for hypoxanthine incorporation into ATP, rather than the PRPP pool activity which is common to both reactions. Under these same experimental conditions, adenine incorporation into ATP was inhibited by 10 per cent, while adenosine incorporation was accelerated 290 per cent. Since

adenosine and adenine are not directly interconvertible [23, 24] and both from AMP, the adenine phosphoribosyl transferase reaction is indicated as the rate-limiting factor for adenine incorporation into ATP.

PRPP is also involved in the *de novo* synthesis of purines and pyrimidines. Five μM EHNA inhibited ATP and UTP syntheses 41 and 32 per cent respectively, while 50 μM EHNA inhibited ATP synthesis 31 per cent and UTP synthesis 99 per cent. In view of the reproducibility of the method, there is no significant difference between 5 μM EHNA inhibition of ATP and UTP *de novo* synthesis and 50 μM EHNA inhibition of ATP *de novo* synthesis. However, 50 μM EHNA did inhibit *de novo* synthesis of UTP to a significantly greater extent.

Of the triphosphate pools measured, UTP showed the greatest percentage decrease—41 per cent compared to 33 per cent for ATP, 16 per cent for ADP and 15 per cent for GTP. The greater degree of inhibition of UTP *de novo* synthesis shown at the higher concentration of EHNA could explain the greater degree of fall observed for UTP. This decrease is also reminiscent of that seen in pyrimidine starvation experiments produced by excess adenosine [25].

In our studies, although uridine and cytidine were readily utilized for RNA and nucleotide synthesis, uracil and cytosine were not. Uracil is utilized for UMP synthesis in rat tissue *in vitro* and *in vivo* [26, 27]. UMP may be formed by a reversible pyrophosphorylase reaction between uracil and 5-phosphoribosylpyrophosphate. However, in mammalian tissues other than human, the most important pathway is the formation of uridine from uracil catalyzed by uridine phosphorylase, which is then phosphorylated to UMP by uridine kinase [27]. Our studies did not demonstrate this reaction pathway.

A decrease in RNA synthesis is also demonstrated above 10 μM EHNA. All the bases and ribosides tested showed a greater inhibition of incorporation into RNA than into their respective nucleoside triphosphates. The interrelationships of protein synthesis and RNA are well established. Carson and Seegmiller [6] have shown that 10 μM EHNA inhibits approximately 35 per cent of protein synthesis, with a marked increase in inhibition above this concentration. Whether inhibition of RNA synthesis is causally related to the inhibition of protein synthesis cannot be determined from our experiments, since we did not study transfer and messenger RNA specifically.

Adenosine incorporation into ATP and RNA increased 350 and 270 per cent, respectively, with 10 μM EHNA. Since this increase represented a total cellular increase and not a redistribution of radioactivity due to ADA inhibition, and since the pool sizes of ATP and RNA were not significantly altered, increased ATP turnover is demonstrated. This indicates either increased glycolysis and/or increased respiration. Measurement of glucose in the growth media showed an increase in its utilization with increasing concentrations of EHNA, and measurement of oxygen utilization increased 20 per cent when the cells were inhibited with 10 μM EHNA. EHNA also increased guanine and guanosine incorporation into GTP, with a maximum effect at 50 μM; however, unlike adenosine, this increase is not reflected in the RNA fraction. Since EHNA affects human guanase activity, this increase may be the result of guanase inhibition upon a limited supply of guanine during the incubation period. However, the increased incorporation of radioactivity into only the GTP fraction, whose pool size did not increase, may also indicate increased GTP turnover associated with succinyl CoA synthetase activity, which forms GTP from GDP and P_i in the tricarboxylic acid cycle, and fatty acid activation, which utilizes GTP to form GDP and P_i.

EHNA always lowered the energy level of the cell when measured 20 hr after PHA stimulation. This was first observed at 5 μM EHNA and was created by an increase in the ADP pool. Since respiration in EHNA-inhibited cells is increased and ATP turnover is accelerated, the possibility exists that in these inhibited cells oxidative phosphorylation has been uncoupled, altering the ATP/ADP ratio. Since the same reduction in ATP/ADP ratio is observed in the lymphocytes of children with adenosine deaminase deficiency [28], it would appear that the effect observed in our cell system may not have been due to the EHNA directly, but rather was a result of the inhibition of adenosine deaminase. The mechanism of this postulated relationship is not clear. An alternative explanation that could relate adenosine deaminase deficiency or inhibition to a depressed energy level is the relatively simple one of an uncontrolled reconstitution of adenosine to ADP. The rate of reconstitution of adenosine to ATP in the absence of adenosine deaminase depends on the enzymatic activities of adenosine kinase, adenylate kinase, nucleoside diphosphokinase and the rate of oxidative phosphorylation. Although our [^{14}C]adenosine incorporation experiments demonstrate an acceleration in the rate of ATP production, the oxidative phosphorylation process can be rate-limiting when compared to the rate at which ADP is formed in the absence of adenosine deaminase. Nucleoside diphosphokinase, which is a non-specific enzyme, does not seem to be a factor at this low concentration of EHNA. The formation of the other nucleoside triphosphates from the salvage pathway was not inhibited and there was no indication of any other increased nucleoside diphosphates. An ineffectual control mechanism for the rate of ADP formation from adenosine through AMP can make the oxidative phosphorylation process rate-limiting in the formation of ATP. In the presence of adenosine deaminase, reutilization of adenosine through IMP falls under the control of feedback inhibition by the cellular level of AMP [29,30].

Carson and Seegmiller [6] have shown that 10 μM EHNA completly inhibits human lymphocyte ADA activity. Therefore, the effects of EHNA that appear only at this level of concentration can be related to blockage of ADA and can be used a a guide or model for the relationship between immunodeficiency and ADA deletion.

The recent finding of an elevated dATP and dADP in erythrocytes [31], lymphocytes and bone marrow [32] from patients with severe combined immunodeficiency is probably related to the adenosine

deaminase deficiency of the cell. Our results demonstrate that inhibition of adenosine deaminase with 5 μM EHNA produced a marked increase in incorporation of deoxyadenosine into dADP and dATP. This illustrates an alteration in the normal equilibrium which in uninhibited cells favors the formation of deoxyinosine.

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