Adenosine Metabolism in Phytohemagglutinin-Stimulated Human Lymphocytes

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ABSTRACT The association of a human genetic deficiency of adenosine deaminase activity with combined immunodeficiency prompted a study of the effects of adenosine and of inhibition of adenosine deaminase activity on human lymphocyte transformation and a detailed study of adenosine metabolism throughout phytohemagglutinin-induced blastogenesis. The adenosine deaminase inhibitor, coformycin, at a concentration that inhibited adenosine deaminase activity more than 95%, or 50 μM adenosine, did not prevent blastogenesis by criteria of morphology or thymidine incorporation into acid-precipitable material. The combination of coformycin and adenosine, however, substantially reduced both the viable cell count and the incorporation of thymidine into DNA in phytohemagglutinin-stimulated lymphocytes.

Incubation of lymphocytes with phytohemagglutinin for 72 h produced a 12-fold increase in the rate of deamination and a 6-fold increase in phosphorylation of adenosine by intact lymphocytes. There was no change in the apparent affinity for adenosine with either deamination or phosphorylation. The increased rates of metabolism, apparent as early as 3 h after addition of mitogen, may be due to increased entry of the nucleoside into stimulated lymphocytes. Increased adenosine metabolism was not due to changes in total enzyme activity; after 72 h in culture, the ratios of specific activities in extracts of stimulated to unstimulated lymphocytes were essentially unchanged for adenosine kinase, 0.92, and decreased for adenosine deaminase, 0.44. As much as 38% of the initial lymphocyte adenosine deaminase activity accumulated extracellularly after a 72-h culture with phytohemagglutinin. In phytohemagglutinin-stimulated lymphocytes, the principal route of adenosine metabolism was phosphorylation at less than 5 μM adenosine, and deamination at concentrations greater than 5 μM. In unstimulated lymphocytes, deamination was the principal route of adenosine metabolism over the range of adenosine concentrations studied (0.5–250 μM). These studies demonstrate the dependence of both the unstimulated and stimulated lymphocyte on adenosine deaminase activity for the metabolism of adenosine and may account for the observed sensitivity of mitogen-stimulated lymphocytes to the toxic effects of exogenously supplied adenosine in the presence of the adenosine deaminase inhibitor coformycin.

A single case of immunodeficiency disease has been reported in association with purine nucleoside phosphorylase deficiency. The catabolism of guanosine was also found to be enhanced in stimulated normal lymphocytes; phosphorolysis of guanosine to guanine by intact lymphocytes increased six fold after 72-h culture with phytohemagglutinin. The specific activity of purine nucleoside phosphorylase in extracts, with guanosine as substrate, was essentially the same in stimulated and unstimulated lymphocytes after 72 h of culture.

INTRODUCTION

Two inherited disorders of purine metabolism, adenosine deaminase (1–4) and purine nucleoside phosphorylase (5) deficiency, have recently been associated with immunodeficiency diseases. The concurrence of adenosine deaminase deficiency and severe combined immunodeficiency in at least 14 patients has suggested the possibility of a causal relationship between the enzyme deficiency and the immunological disorder. The report of a single case of purine nucleoside phosphorylase deficiency with defective T-cell immunity (5) adds further impetus to the investigation of purine nucleoside

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metabolism in relation to lymphocyte function. Adenosine is known to inhibit growth of and be ultimately toxic to mammalian cells in culture, and the inhibition of murine lymphocyte-mediated cytolysis by adenosine was potentiated by an inhibitor of adenosine deaminase (6). Work in this laboratory has shown the inhibition of lymphocyte transformation by adenosine to be potentiated by inhibitors of adenosine deaminase activity in concanavalin A-stimulated (7), and in the present study, phytohemagglutinin-stimulated human lymphocytes.

The present work describes changes of adenosine metabolism in both lysates and intact cells during phytohemagglutinin-induced transformation of human lymphocytes. The presently available description of adenosine metabolism during lymphocyte transformation is limited to observations of a 10–20-fold increase in the rate at which adenosine and guanosine are incorporated into RNA after 48 h culture with phytohemagglutinin (8). The metabolic pathways of adenosine and other purine nucleosides and bases are outlined in Fig. 1. Adenosine may either be phosphorylated to AMP by adenosine kinase or converted to inosine by the aminohydrolase adenosine deaminase. Adenosine is also a potential precursor of adenine (9). The metabolism of a second nucleoside, guanosine, has also been examined in phytohemagglutinin-stimulated human lymphocytes because the hereditary deficiency of purine nucleoside phosphorylase activity, for which guanosine and inosine are substrates, has been associated with immunodeficiency disease (5).

METHODS

Chemicals. Radioactive bases and nucleosides were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.): [8-^{14}C]adenine, 58 mCi/mmol; [8-^{14}C]adenosine, 59 mCi/mmol; [8-^{14}C]hypoxanthine, 59 mCi/mmol; [8-^{14}C]-guanosine, 38.5 mCi/mmol; sodium [^{14}C]formate, 59 mCi/mmol; and from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. J.), [8-^{14}C]guanosine, 49 mCi/mmol, and [*methyl*-^{3}H]thymidine, 6 Ci/mmol. Coformycin was kindly provided by Dr. H. Umezawa, Institute for Microbial Chemistry, Tokyo, Japan; 6-((4-nitrobenzyl)-thio)-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine)[1] was provided by Dr. A. R. P. Paterson of the University of Alberta Cancer Research Unit, Edmonton, Canada. ATP, adenosine, adenine, inosine, hypoxanthine, guanosine, guanine and D-ribose-5-phosphate disodium salt were obtained from Sigma Chemical Co. (St. Louis, Mo.) and 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P), magnesium salt, from P—L Biochemicals, Inc. (Milwaukee, Wis.). Difco Laboratories (Detroit, Mich.) phytohemagglutinin P or Burroughs Wellcome purified phytohemagglutinin (Burroughs Wellcome Co., Research Triangle Park, N. C.), were used in these studies.

[1] *Abbreviations used in this paper:* EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; nitrobenzylthioinosine, 6-((4-nitrobenzyl)-thio)-9-β-D-ribofuranosylpurine; PP-ribose-P, ribosylpyrophosphate 5-phosphate.

Purification and culture of lymphocytes. Human lymphocytes were purified from 400 ml blood of healthy donors as previously described by Mendelsohn et al. (10, 11). The preparative steps involve: (*a*) defibrination with glass beads, which removes platelets and many polymorphonuclear leukocytes; (*b*) sedimentation in 1% dextran, followed by centrifugational banding on an aqueous solution of Ficoll and Hypaque sodium to remove erythrocytes; (*c*) passage through a nylon fiber column at 20°C to remove granulocytes and monocytes. The final cell population contained 95–99% small lymphocytes, with the remaining nucleated cells consisting of monocytes or granulocytes; the ratio of erythrocytes to lymphocytes was approximately 4:100 and platelets were not present.

Purified lymphocytes were resuspended at a density of $0.7–2.0 \times 10^6$ cells/ml in Eagle's minimum essential medium containing 10% horse serum (Flow Laboratories, Inc., Rockville, Md.), 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell counts were measured by hemocytometer and Coulter counter model Z_{B1} (Coulter Electronics, Inc., Hialeah, Fla.). Tests of cell viability, mitogen-induced incorporation of [^{3}H]thymidine into acid-insoluble material, and morphologic transformation identified two lots of horse serum suitable for utilization in these studies. Before use, the horse serum was heated at 56°C for 2 h. The cultures were stimulated by addition of phytohemagglutinin at concentrations producing optimal stimulation: 5 μg/ml (Difco) or 1 μg/ml (Burroughs Wellcome) (10, 11).

All assays were performed in duplicate. For studies of purine base and nucleoside metabolism, 0.2 or 0.4 ml of lymphocyte suspension was added to 10×75-mm plastic tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.); for thymidine labeling, 0.5 or 1.0 ml of lymphocyte suspension in 16×125-mm plastic tubes; and 2.0–5.0 ml of lymphocyte suspension/50-ml growth flask (Falcon Plastics) was used for enzyme assays on subsequent cell extracts or PP-ribose-P determinations. Cell suspensions were maintained in a 37°C incubator without agitation in an atmosphere of 5% CO_2 and 95% air. During assays with intact cells, the short-term incubations with radioactive precursors were performed for convenience in a 37°C water bath with gentle shaking, with tubes previously gassed with 10% CO_2 and stoppered.

Preparation of cell extracts and enzyme assays. At appropriate times, cells were harvested by transferring 5 or 10 ml cell suspensions from culture flasks to prechilled 50-ml conical centrifuge tubes, and rinsing the culture flask three times with 2 ml phosphate-buffered saline (Dulbecco's), containing 5 mg/ml bovine serum albumin. Cells were collected from the combined fractions by centrifugation, resuspended in phosphate-buffered saline lacking albumin (1.5–2.5 ml), transferred to 10×75-mm tubes, washed, and resuspended in 0.3–0.6 ml of phosphate-buffered saline at a final cell density of approximately $5–10 \times 10^7$ lymphocytes/ml. Lysates were prepared at 0°C by three 5-s pulses with a Biosonik sonifier (Will Scientific, Inc., Rochester, N. Y.) at 50–75% of maximum intensity; 5-μl samples were examined under the microscope for complete disruption. The extracts were centrifuged at 20,000 *g* for 40 min at 4°C in the Sorvall RC2B centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.) and supernates were used for enzyme assays.

The following radiochemical assays were devised to minimize the amount of cellular extract required, and have these features in common: the final assay mix contained the equivalent of $5–20 \times 10^6$ cells/ml; the total assay volume or aliquot removed after appropriate periods of incubation

at 37°C from 10 × 75-mm plastic tubes was 0.050 ml; assays were terminated by the addition of 10 μl cold 8 M formic acid; and 25-μl aliquots were spotted for chromatographic analysis of products. Separated compounds were cut out and counted in Liquifluor toluene (Pilot Chemicals, Watertown, Mass.). Activities were calculated from a minimum of three time points during the linear portion (0–15 min) of the assay. Protein was determined according to the method of Lowry et al. (12).

Adenine phosphoribosyltransferase and hypoxanthine-guanine phosphoribosyltransferase activities were determined essentially as described by Fields and Brox (13) with assay mixtures containing 50 mM Tris-chloride (pH 7.4), 5 mM magnesium chloride, 0.5 mM PP-ribose-P, 66 μM [8-^{14}C]hypoxanthine or 50 μM [8-^{14}C]adenine and extract. Reactions were terminated by addition of formic acid, and 25-ml aliquots were spotted on polyethyleneimine cellulose thin layers (Polygram Cel 300, Brinkman Instruments, Inc., Westbury, N. Y.), and developed overnight in methanol: water (1:1) to remove unreacted bases from the origin where nucleotides remain. Nucleotides remaining at the origin were subsequently cut out and counted (13, 14).

Adenosine deaminase was assayed in 100 mM potassium phosphate (pH 7.0) with 300 μM [8-^{14}C]adenosine (15 mCi/mmol) and extract. After the reaction was stopped with formic acid, aliquots were spotted on cellulose thin layers (Eastman Kodak Co., Rochester, N. Y.) for separation of adenine, adenosine, hypoxanthine, inosine, and nucleotides, as previously described (9, 15). Due to the presence of purine nucleoside phosphorylase activity, the sum of radioactivity in inosine and hypoxanthine was used in determining adenosine deaminase activity.

Adenosine kinase was assayed in a reaction mixture containing 100 mM sodium phosphate (pH 5.8), 5 mM ATP, 1.0 mM $MgCl_2$, 40 μM [8-^{14}C]adenosine (59 mCi/mmol), 1 μg/ml coformycin, and extract. The assay conditions were optimized with respect to ATP, $MgCl_2$, adenosine concentrations, and pH with human lymphocyte lysates. The use of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (16) and coformycin (9, 15) in studying adenosine phosphorylation has previously been reported; these compounds prevent substrate depletion by inhibition of adenosine deaminase activity. Aliquots of formic acid-terminated assay mixtures were spotted on cellulose thin layers and developed as before (9) to measure nucleotide formation and monitor substrate deamination.

Adenosine cleavage was assayed in 100 mM potassium phosphate (pH 7.0) with 300 μM [8-^{14}C]adenosine, 1 μg/ml coformycin, and extract. Adenosine was separated from adenine as described (9), and radioactivity in adenine was used in determining adenosine cleavage.

Purine nucleoside phosphorylase activity was assayed by the conversion of guanosine to guanine. The assay mixture contained 100 mM potassium phosphate (pH 7.0), 200 μM [8-^{14}C]guanosine (10 mCi/mmol), and extract. Formic acid-terminated aliquots, 25 μl, were spotted on cellulose thin layers and chromatographed in acetonitrile: 0.1 M ammonium acetate: ammonia (60:30:10) to separate guanosine, guanine, and nucleotides (14). Purine nucleoside phosphorylase activity was calculated from the radioactivity in the free base guanine.

With modification of a previously reported assay (17), PP-ribose-P synthetase activity was measured by the ribose-5-phosphate-dependent conversion of [^{14}C]adenine to AMP. The assay mixture contained 50 mM potassium phosphate (pH 7.0), 5 mM magnesium chloride, 1 mM ribose-5-phosphate, 5 mM ATP, and 65 μM [8-^{14}C]adenine. The order of substrate additions was important in this assay (R. C. Willis, personal communication); assays were started by the simultaneous addition of ATP and [8-^{14}C]adenine after preincubation of extract and ribose-5-phosphate at 37°C for 10 min. Formic acid-terminated aliquots were spotted on polyethyleneimine cellulose thin layers and developed as in the phosphoribosyltransferase assays. This assay exhibited a lag period of 0–10 min and rates were therefore calculated from the linear 10–60 min interval.

Extracellular adenosine deaminase activity in culture medium was determined with 50-μl aliquots of medium from

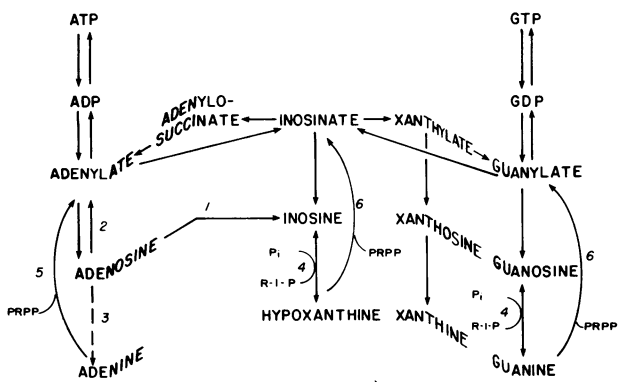

FIGURE 1 Pathways of purine metabolism.

Number in figure	Trivial name	Systematic name	Enzyme Commission number
1	Adenosine deaminase	Adenosine aminohydrolase	3.5.4.4
2	Adenosine kinase	ATP: adenosine 5′-phosphotransferase	2.7.1.20
3	Adenosine cleavage	—	—
4	Purine nucleoside phosphorylase	Purine-nucleoside: ortho phosphate ribosyltransferase	2.4.2.1
5	Adenine phosphoribosyltransferase	AMP: pyrophosphate phosphoribosyltransferase	2.4.2.7
6	Hypoxanthine-guanine phosphoribosyltransferase	IMP: pyrophosphate phosphoribosyltransferase	2.4.2.8

which cells had been removed (see below), to which 5 μl 1.0 M potassium phosphate (pH 7.0) and [8-^{14}C]adenosine were added. Parallel assays with complete incubation medium unexposed to cells were used as background. Incubations were carried out for 30 min at 37°C, reactions were stopped with 10 μl 8 M formic acid addition and 25 μl aliquots were spotted on cellulose thin layers as described for the adenosine deaminase assay. These procedures were also followed in studies of [^{14}C]adenosine metabolism by human and horse serum.

Determination of intracellular PP-ribose-P concentration. PP-Ribose-P was measured by a modification of the method of Henderson and Khoo (18). At various times cell suspensions containing 4–8 × 10^6 cells were transferred from culture flasks to 10 × 75-mm glass tubes on ice. Culture flasks were rinsed three times with 0.5 ml of phosphate-buffered saline containing 5 mg/ml bovine serum albumin and combined fractions were centrifuged for 3 min at 600 *g*. The supernate was poured off, tubes were rapidly dried with a swab, and the cell pellets were immediately resuspended in 0.200 ml of cold 100 mM Tris-chloride, pH 7.4, containing 1.0 mM EDTA, and extracted by placing the tubes in a boiling water bath for 30 s and then returning them to ice. After a further centrifugation, additions were made so that the final assay mixture contained 5 mM magnesium chloride, 100 μM [8-^{14}C]adenine, and partially purified adenine phosphoribosyltransferase (prepared essentially free from 5′-nucleotidase activity from WI-L2 cultured lymphoblasts by Dr. R. C. Willis). Incubations were carried out to completion at 37°C for 30–45 min, and 50-μl aliquots were spotted on polyethyleneimine cellulose thin layers for separation of nucleotides from unreacted adenine as described. Results are expressed in terms of cell count per culture determined on the initial day.

Metabolism of radioactive precursors by intact lymphocytes. Because of the appearance of extracellular activity capable of metabolizing adenosine, the medium was changed immediately before assays involving radioactve nucleoside precursors. Lymphocyte suspensions were centrifuged for 3 min at 650 *g* at 25°C, 0.100 ml of the medium was removed for assay of extracellular adenosine deaminase activity, and the remainder poured off: the tubes were quickly dried with swabs and the cells were resuspended in fresh prewarmed complete incubation medium containing 10% horse serum, gassed with 10% CO_2, stoppered, and incubated at 37°C. After these manipulations, a 30-min recovery period was allowed before initiation of assays by the addition of radioactive precursors.

Incubations were terminated by addition of 10 μl cold 4.4 M perchloric acid/0.200 ml culture; the mixture was vortexed and placed on ice. After centrifugation, 0.100 ml of the supernate was neutralized by transfer to a tube containing 5 μl of 4.2 M KOH on ice, and after a further 5 min the potassium perchlorate was removed by centrifugation and 25-μl aliquots were spotted on cellulose for chromatography. The metabolism of [^{14}C]adenosine was followed by chromatography of extracts to separate adenine, adenosine, hypoxanthine, inosine, and nucleotides as previously described (9, 15). The phosphorolysis and possible phosphorylation of [^{14}C]guanosine were followed by chromatographic separation of guanosine, guanine, and nucleotides, as described for the purine nucleoside phosphorylase assay. These assays exhibit linear rates over 90 min in control and phytohemagglutinin-stimulated cultures, and 30 min was used as the standard assay period.

The metabolism of purine bases was followed by incubation of lymphocytes with [^{14}C]adenine or hypoxanthine as

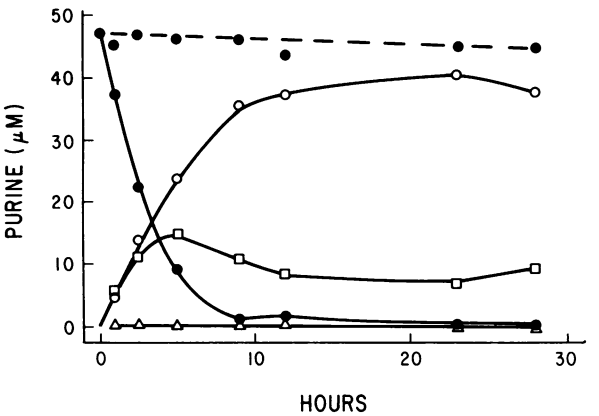

FIGURE 2 The metabolism of [^{14}C]adenosine in Eagle's minimum essential medium containing 10% human (——) or 10% horse (- - - -) serum at 37°C was followed by removing samples at various times, deproteinizing and chromatographing on cellulose thin layers to separate adenosine (●), inosine (□), hypoxanthine (○) and adenine (△).

described for nucleoside metabolism. Neutralized aliquots of the perchloric acid extracts, 25 μl, were spotted on polyethyeneimine cellulose thin layers for separation of GTP, ATP, GDP, ADP, GMP, XMP, IMP, AMP, and NAD as previously described (14). Radioactivity in each compound was determined and results are expressed as nanomoles per 10^9 cells.

As a measure of *de novo* purine and nucleic acid synthesis, the incorporation of sodium [^{14}C]formate into nucleic acid adenine and guanine was measured by a modification of a previous method (19). To 0.400-ml lymphocyte suspensions, 20 μCi sodium [^{14}C]formate was added and after 120 min incubation at 37°C, 40 μl 4.4 M perchloric acid was added. The acidified material was quantitatively transferred to 1.0-ml glass ampules, acid-insoluble material was washed three times with 1.0 ml 0.4 M perchloric acid and dried *in vacuo*, 0.100 ml of 1 N hydrochloric acid was added, and ampules were sealed and placed in a boiling water bath for 60 min. Portions of the nucleic acid hydrolysates, 50 μl, were chromatographed on cellulose thin layers, as for the adenosine deaminase assay, to separate adenine and guanine.

Assays of lymphocyte transformation. After 64–72 h of incubation, the mitogenic effects of phytohemagglutinin were assayed by morphologic criteria and by [^{3}H]thymidine incorporation into acid-insoluble material. Aliquots of phytohemagglutinin-stimulated and control cells were tested for viability by the trypan blue method; cells were incubated with 0.1% trypan blue for 5 min and scored for their ability to exclude dye. Microscopic slide preparations for morphologic studies were made with a Shandon cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.), and after staining with Wright's stain, the percent blast transformation was determined by counting 400 cells. Cells scored as transformed were enlarged to over 15 μm in diameter, with nucleus: cytoplasm ratios less than 1:1; diffuse rather than condensed nuclear chromatin staining; and increased cytoplasmic basophilia. Phytohemagglutinin-stimulated and control cultures were incubated in triplicate for 4 h with [^{3}H]thymidine (6 Ci/mM, 2 μCi/ml), then washed with saline by centrifugation, precipitated in 5% trichloroacetic acid, disrupted by sonication for 15 s at

TABLE I

Effect of Adenosine and Inhibition of Adenosine Deaminase upon Parameters of Human Lymphocyte Blastic Transformation

Additions	PHA	Morphologic transformation	Relative thymidine incorporation	[^{14}C]Formate incorporation into nucleic acid purines		Acid-soluble nucleotide synthesis from purine bases		PRPP concentration
				Adenine	Guanine	[^{14}C] Adenine	[^{14}C] Hypoxanthine	
		% blasts	*(cpm sample/ cpm PHA) × 100±SEM*	*cpm/10^6 cells/120 min*		*nmol/10^6 cells/120 min*		*nmol/10^6 cells*
None	+	40–70	100	5,532	8,783	752	897	52
Coformycin	+	40–75	102±15	5,301	7,562	768	857	68
Adenosine	+	35–70	101±21	698	1,820	476	621	14
Coformycin +adenosine	+	30–35	5.1±1.9	754	1,700	<5	46	2
None	−	<4	2.6±1.2	440	794	130	156	6
Coformycin	−	<4	1.0±0.9					
Adenosine	−	<4	1.3±0.7					
Coformycin +adenosine	−	<4	0.4±0.1					

Adenosine, 50 μM; or coformycin, 1 μg/ml, were added to lymphocyte cultures concurrent with phytohemagglutinin at the initiation of incubation. Where adenosine was added in combination with coformycin, the cells were preincubated with inhibitor for 15 min. Cultures were harvested and assayed for incorporation of [^{3}H]thymidine after 66 h of incubation, as described in Methods. The relative incorporation is the percent of thymidine incorporated into acid-precipitable material of test cultures, relative to the phytohemagglutinin-stimulated cultures receiving no other additions. The absolute values of thymidine incorporation as acid-insoluble cpm per 1.0 ml culture were 66,400±16,900 for phytohemagglutinin-stimulated cultures and 1,780±1,162 for unstimulated cultures. The total number of viable cells per culture was calculated as per cent viable cells × total cell number. In control cultures the number of viable cells remained constant within±5%. Although the cell count is difficult to assay accurately in phytohemagglutinin-stimulated cultures due to clumping, estimates of reductions in the total number of viable cells in the series of phytohemagglutinin-stimulated cultures (relative to phytohemagglutinin without any other additions) are as follows: coformycin, adenosine, 15–20% reduced; coformycin + adenosine, 65% reduced. The data are from four experiments. Acid-soluble nucleotide synthesis from 100 μM [^{14}C]adenine or hypoxanthine, 0.8 mM sodium [^{14}C]formate incorporation into nucleic acid adenine and guanine and intracellular PP-ribose-P concentrations were measured after 66 h incubation as described in Methods and are the average of duplicate determinations.

maximal intensity, and collected on glass fiber disks for liquid scintillation counting (10, 11).

RESULTS

Freshly purified human lymphocytes were cultured in Eagle's minimum essential medium supplemented with 10% horse serum because of the negligible adenosine deaminase activity in serum from this source compared to human or fetal calf serum. Less than 5% of 50 μM [^{14}C]adenosine was metabolized after 30 h incubation at 37°C in medium containing 10% horse serum (Fig. 2), compared to over 95% deamination to inosine and subsequent phosphorolysis to hypoxanthine after 9 h incubation in complete medium containing 10% human serum (Fig. 2). After 72 h of incubation in medium containing 10% horse serum, 90–95% of control and phytohemagglutinin-stimulated cells were viable. By morphological examination, the unstimulated culture contained 2–3% blastic-transformed cells, whereas phytohemagglutinin-stimulated cultures had 40–70% blasts. In stimulated cultures, [^{3}H]thymidine incorporation into acid-precipitable material showed a 20- to 50-fold increase and the incorporation of [^{14}C]formate into nucleic acid adenine and guanine increased more than 10-fold (Table I).

Effect of inhibition of adenosine deaminase and effect of exogenous adenosine on phytohemagglutinin-induced transformation. Because of the immunological disorders associated with a gross deficiency of adenosine deaminase activity (1–4), it was of interest to determine whether inhibition of this activity could prevent mitogen-induced transformation of human lymphocytes. The structure and synthesis of coformycin (20) and EHNA (21), two potent inhibitors of adenosine deaminase activity, have recently been reported, as have examples of their utility in studying adenosine metabolism (6, 9, 15, 16, 22, 23). A concentration of 50 μM adenosine has been found to suppress growth of the WI-L2 cultured

TABLE II

Effect of Nucleoside Transport and Adenosine Deaminase Inhibitor on Adenosine Metabolism in Intact Lymphocytes

Additions	Deamination			Phosphorylation		
	0 h	−PHA 72 h	+PHA 72 h	0 h	−PHA 72 h	+PHA 72 h
	nmol/10⁹ cells/30 min					
None	566	2,612	8,473	108	188	525
Coformycin, 1 μg/ml	*	51	<10	*	183	190
Nitrobenzylthioinosine						
3 nM	488	2,709	7,099	90	130	387
30 nM	420	2,768	6,887	55	125	454
300 nM	262	2,136	5,708	40	98	328
3,000 nM	252	2,199	2,446	22	82	104

At various times lymphocytes were resuspended in fresh medium with or without inhibitor, after 30 min incubation, 250 μM {^{14}C}adenosine was added and after a further 30 min cells were extracted and radioactivity in the products of deamination, inosine plus hypoxanthine, and phosphorylation, acid-soluble nucleotides, were measured.

* Activities were not measured for these samples.

human lymphoblast line, when the nucleoside is added together with either of the adenosine deaminase inhibitors coformycin or EHNA, which have little effect on growth when added singly.[2] The effects of these growth-inhibitory concentrations of coformycin and adenosine on phytohemagglutinin-induced lymphocyte transformation were therefore examined (Table I).

In the presence of coformycin (1 μg/ml), essentially no changes were observed in transformation to a blastic morphology, [^{3}H]thymidine incorporation into DNA, [^{14}C]formate incorporation into nucleic acid purines, or nucleotide synthesis from purine bases. When stimulated

[2] Snyder, F. F., and J. E. Seegmiller. 1976. The adenosine-like effect of exogenous cyclic AMP upon nucleotide and PP-ribose-P concentrations of cultured human lymphoblasts. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* In press.

TABLE III

Effect of Phytohemagglutinin-Induced Transformation on Lymphocyte Enzyme Activities

Enzyme	Activities			Ratio, 72-h, +PHA/−PHA
	0 h (*n*)	72 h − PHA (*n*)	72 h + PHA (*n*)	
	nmol/mg protein/min			
A.				
Adenosine deaminase	45.0±10.0 (3)	54.2±7.6 (6)	24.1±4.6 (6)	0.44
Adenosine kinase	5.50 (1)	5.78±1.78 (3)	5.32±0.88 (3)	0.92
"Adenosine cleavage"	0.07 (1)			
Purine nucleoside phosphorylase	55.8±10.5 (3)	58.3±7.0 (5)	52.8±5.9 (5)	0.91
B.				
Adenine phosphoribosyltransferase	6.8 (1)	9.2±1.2 (3)	7.2±1.3 (3)	0.78
Hypoxanthine-guanine phosphoribosyltransferase	1.82±0.13 (2)	2.71±0.44 (3)	4.64±0.32 (3)	1.71
PP-ribose-P synthetase	0.84±0.34 (3)	1.34±0.58 (3)	1.42±0.74 (3)	1.06

Extracts were made of freshly prepared lymphocytes or lymphocytes which had been incubated for 72 h in the presence and absence of 1 μg/ml phytohemagglutinin and enzyme activities were assayed on the day of harvesting. Enzyme activities are reported as the mean±1 SD where *n* is the number of lymphocyte donors. The term "adenosine cleavage" refers to the conversion of adenosine to adenine; purine nucleoside phosphorylase activity was measured using guanosine as substrate and hypoxanthine-guanine phosphoribosyltransferase activity using hypoxanthine. The 20,000 × g supernatant of 10^9 freshly prepared lysed lymphocytes was equivalent to approximately 27 mg protein; after 72 h culture supernatant protein was 2.9-fold greater in stimulated than unstimulated lymphocyte lysates whereas cell number remained approximately constant in control and stimulated cultures.

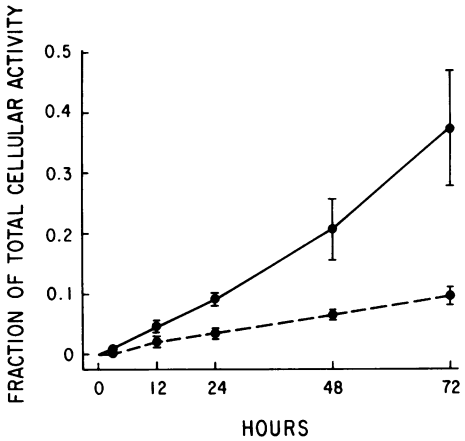

FIGURE 3 Effect of phytohemagglutinin on accumulation ot extracellular adenosine deaminase activity. Lymphocytes were incubated with (——) and without (- - - -) 1 μg/ml phytohemagglutinin in Eagle's minimum essential medium with 10% horse serum. At various times cells were removed from the medium by centrifugation and adenosine deaminase activity in the medium was assayed as described in Methods. Results are the mean±1 SD of duplicate determinations on lymphocyte cultures from three donors.

or unstimulated intact lymphocytes were incubated with coformycin (1 μg/ml) for either 0.5 (Table II) or 72 h, the deamination of [^{14}C]adenosine was inhibited by over 95%. Incubation with adenosine (50 μM) also caused no change in morphologic transformation or thymidine incorporation into DNA. Adenosine decreased nucleotide synthesis from purine bases and [^{14}C]formate incorporation into nucleic acid purines, presumably because adenosine provided a preformed purine and also competed for PP-ribose-P after sequential deamination and phosphorolysis to hypoxanthine. Addition of coformycin to lymphocyte cultures concurrently with adenosine, however, resulted in 65% cell death, as indicated by a decrease in cell count and a marked rise in cells unable to exclude trypan blue. There was also a reduction in the percent transformed blasts, accompanied by a 95% inhibition of [^{3}H]thymidine incorporation into acid-precipitable material. In unstimulated cultures, [^{3}H]thymidine incorporation was suppressed in the presence of coformycin plus adenosine, but cell death was not observed. These results demonstrate that low concentrations of adenosine are toxic to lymphocytes stimulated to divide in the presence of the adenosine deaminase inhibitor, coformycin.

Enzyme activities in lymphocyte extracts. As a first approach to characterize changes in adenosine metabolism during phytohemagglutinin-induced lymphocyte transformation, the specific activities of several enzymes were determined in lymphocyte lysates after 72 h incubation, when maximal changes in activities due to protein synthesis have been attained (24, 25). The specific activities of adenosine kinase and purine nucleoside phosphorylase showed less than 10% change and the specific activity of adenosine deaminase was reduced approximately 55% in lymphocytes incubated with mitogen, compared to unstimulated lymphocytes (Table IIIA). In these experiments, phytohemagglutinin caused approximately a 2.9-fold increase in protein per culture lysate after 72 h; thus, the actual amount of adenosine deaminase activity increased slightly per culture but decreased with respect to specific activity measurements. Of the three possible routes of adenosine metabolism (Fig. 1), the ratio of specific enzyme activity for adeno-

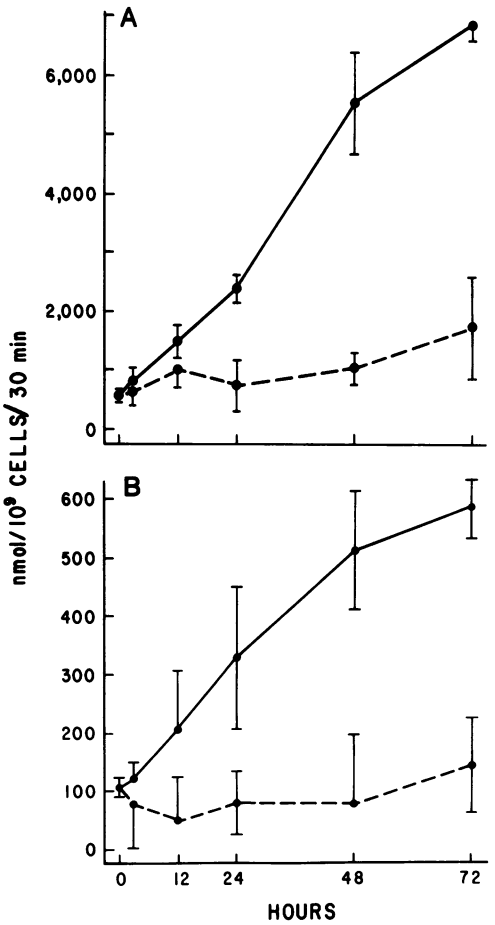

FIGURE 4 Phytohemagglutinin-induced changes in the rate of deamination (A) and phosphorylation (B) of adenosine in intact lymphocytes. At various times after culture in the presence (——) or absence (- - - -) of 1 μg/ml phytohemagglutinin, cells were resuspended in fresh medium and incubated for 30 min with 85 μM [^{14}C]adenosine. Radioactivity in the products of deamination (A), inosine plus hypoxanthine, and phosphorylation (B), acid-soluble nucleotides, were measured and results are the mean±1 SD of duplicate determinations on lymphocytes from three donors. There was less than 10% deviation from the mean in duplicate results from lymphocytes of a single donor.

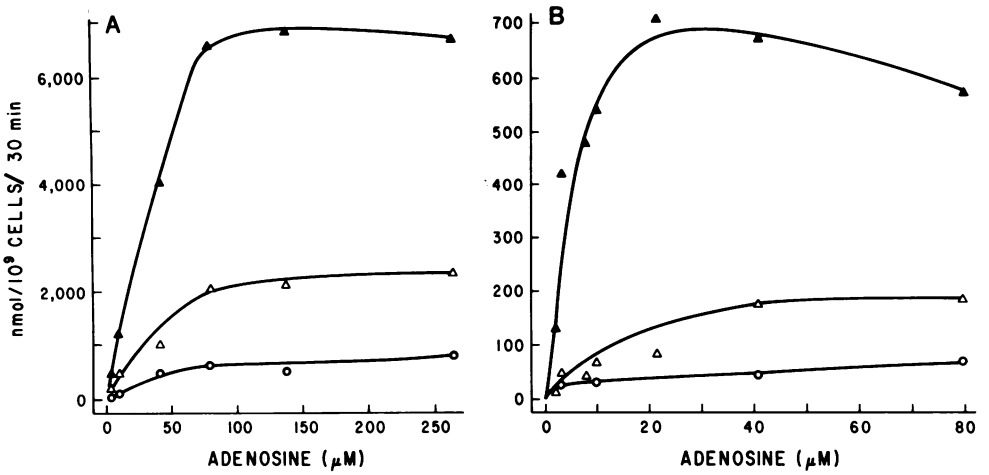

FIGURE 5 Effect of adenosine concentration on rate of deamination (A) and phosphorylation (B) of adenosine. Freshly prepared cell suspensions (○) or cells incubated in the presence (▲) and absence (△) of 1 μg/ml phytohemagglutinin for 68 h were resuspended in fresh medium and incubated for 30 min with varying concentrations of [^{14}C]adenosine. Radioactivity in the products of deamination (A), inosine plus hypoxanthine, and phosphorylation (B), acid-soluble nucleotides, were measured, and the results are the average of duplicate cultures.

sine cleavage: adenosine kinase: adenosine deaminase, was 0.0015 : 0.12 :1.0 at the initiation of cultures.

Accumulation of extracellular adenosine deaminase activity. Extracellular adenosine deaminase activity accumulated in the lymphocyte culture medium during the 72-h culture (Fig. 3). As much as 38% of the initial cellular adenosine deaminase activity could be found in the medium of cultures exposed to phytohemagglutinin, compared to approximately 10% of total cellular activity for control cultures. Extracellular adenosine deaminase activity could reflect a greater loss in viability of stimulated than unstimulated cells, but there was more than 90% viability in both cultures by criterion of dye exclusion. Further studies were performed with lymphocytes cultured in the presence of 10% autologous human serum rather than horse serum. The human serum was heated for 2 h at 60°C to substantially inactivate serum adenosine deaminase activity. Lymphocytes cultured in human serum also exhibited the accumulation of extracellular adenosine deaminase activity, with release of up to 30% of total cellular activity from phytohemagglutinin-stimulated lymphocytes after 72 h of culture.

Adenosine metabolism in intact lymphocytes. The relative rates of deamination and phosphorylation of adenosine may be simultaneously measured in intact cells during short-term incubations with [^{14}C]adenosine (9), and these methods are applied here to the metabolism of adenosine in human lymphocytes. To prevent extracellular metabolism of [^{14}C]adenosine by adenosine deaminase activity accumulated in the medium, lymphocytes were resuspended in fresh prewarmed medium 30 min before incubation with radiolabeled nucleoside. The 72-h incubation with phytohemagglutinin produced approximately a 12-fold increase over the initial rate of deamination of adenosine, and the increase was apparent as early as 3 h after addition of mitogen (Fig. 4). The phosphorylation of adenosine exhibited similar stimulation in response to phytohemagglutinin (Fig. 4), the overall increase being about six-fold. Both deamination and phosphorylation of adenosine appeared to be approaching a maximal rate by 48 h of incubation, the change during the last 24 h being smaller than during the previous 48-h interval. These studies were performed at a concentration of 85 μM adenosine, which gave near-optimal rates for both adenosine phosphorylation and deamination (Fig. 5).

The substrate dependence of deamination and phosphorylation on adenosine concentration was examined in freshly purified lymphocytes and after 68 h incubation in the presence or absence of phytohemagglutinin (Fig. 5A and B). Approximately equivalent adenosine concentrations produced half-maximal rates of deamination and phosphorylation in stimulated and unstimulated lymphocytes. As seen in Fig. 5A and B, a significant increase in adenosine metabolism was observed in control cultures at 68 h versus 0 h control lymphocytes, suggesting a degree of stimulation that was not mitogen-specific and may be attributable to incubation of lymphocytes with 10% horse serum.

The metabolism of nucleosides by the intact lymphocyte is potentially regulated not only by the relative activities of each enzyme, but also by the uptake of the nucleoside by the cell. Nitrobenzylthioinosine is a po-

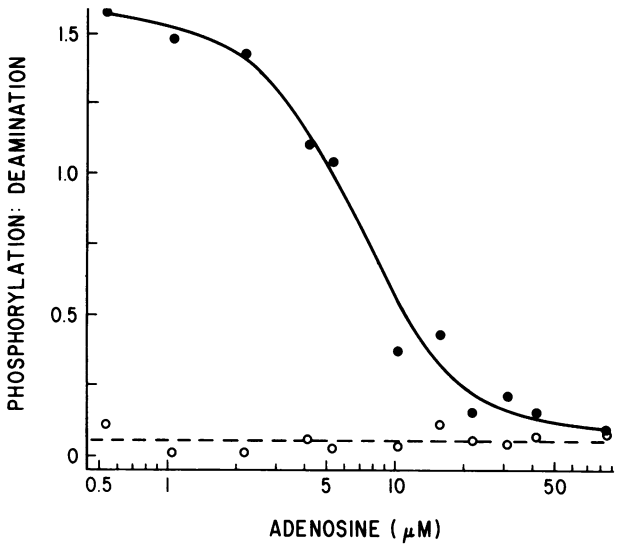

FIGURE 6 Effect of phytohemagglutinin on the ratio of adenosine phosphorylation to deamination in intact lymphocytes as a function of adenosine concentration. Lymphocytes cultured in the presence (●) and absence (○) of 1 μg/ml phytohemagglutinin for 72 h were incubated for 30 min with various concentrations of [^{14}C]adenosine. Radioactivity in the products of phosphorylation and deamination were measured as described for Figs. 2 and 3, and the ratio of phosphorylation to deamination is given as a function of adenosine concentration.

tent inhibitor of nucleoside transport (26, 27), with an apparent dissociation constant of 1.1 nM for the human erythrocyte (28). Nitrobenzylthioinosine inhibited both the deamination and phosphorylation of adenosine to the same respective basal level in intact lymphocytes previously incubated in the presence and absence of phytohemagglutinin (Table II). The residual uninhibitable activity may reflect an uptake mechanism for which nitrobenzylthioinosine lacks specificity or may be due to a loss of viability in a small portion of the cells, since 5–10% of the lymphocyte population gave positive trypan blue staining after 72 h of culture. The highest concentration of nitrobenzylthioinosine employed (3 μM) inhibited adenosine deaminase activity by less than 1% in lymphocyte extracts. These findings indicate the increased rates of deamination and phosphorylation of adenosine principally result from increased entry of the nucleoside into stimulated lymphocytes.

Further studies have examined the relative rates of adenosine phosphorylation and deamination during phytohemagglutinin stimulation, as a function of adenosine concentrations. The ratio of adenosine phosphorylation to deamination showed a dependence on adenosine concentration in phytohemagglutinin-stimulated lymphocytes but not in unstimulated lymphocytes (Fig. 6). Thus, in mitogen-stimulated cells, a greater proportion of adenosine was phosphorylated at low adenosine concentrations than at high substrate concentrations, and phosphorylation was the principal route of adenosine metabolism at less than 5 μM adenosine. In unstimulated lymphocytes, deamination was an average of 20 times greater than phosphorylation over the entire range of adenosine concentrations.

Because of the possible association of purine nucleoside phosphorylase deficiency with immunological disease, the metabolism of guanosine was also examined. A comparison of phytohemagglutinin-induced changes in deamination of adenosine and phosphorolysis of guanosine for parallel lymphocyte cultures is given in Table IV. The phosphorolysis of guanosine also increased approximately six fold after 72 h of culture with phytohemagglutinin.

Purine base metabolism in lymphocytes. Adenosine may be converted to hypoxanthine, by the sequential action of adenosine deaminase and purine nucleoside phosphorylase, and may possibly also be a precursor of adenine (Fig. 1). The phosphoribosyltransferases convert these purine bases directly to nucleotides in PP-ribose-P-dependent reactions. The activities of the phosphoribosyltransferases and of the enzyme producing PP-ribose-P were measured in cell extracts after 72 h of culture. The ratio of enzyme activities for stimulated to unstimulated cultures were: 0.78, adenine phosphoribosyltransferase; 1.71, hypoxanthine phosphoribosyltransferase; and 1.06, PP-ribose-P synthetase (Table III B); other studies have indicated no apparent change in specific activity of these enzymes (29, 30). In intact lymphocytes, the rate of nucleotide synthesis from adenine and hypoxanthine increased to 10-fold over the initial rate after 72 h of incubation with mitogen. A comparison of stimulated to unstimulated cultures after 66 h of incubation showed more than five fold increase in nucleotide synthesis from adenine and hypoxanthine (Table I). The intracellular concentration of PP-ri-

TABLE IV

Effect of Phytohemagglutinin on Rates of Purine Nucleoside Metabolism in Intact Lymphocytes

PHA	Incubation	Guanosine phosphorylase	Relative activity	Adenosine deaminase	Relative activity
		nmol/10^6 cells/30 min			
−	0	932	1.0	592	1.0
−	72	1,972	2.1	2,670	4.5
+	72	5,430	5.8	6,994	11.8

The deamination of 300 μM [^{14}C]adenosine and phosphorolysis of 200 μM [^{14}C]guanosine was measured during a 30-min incubation of lymphocytes resuspended in fresh medium before and after 72 h incubation in presence and absence of 1 μg/ml phytohemagglutinin. Radioactivity in products of adenosine deaminase (inosine plus hypoxanthine) and guanosine phosphorylase (guanine) were measured and results are the average of duplicate determinations.

bose-P, co-substrate for purine phosphoribosyltransferases, was 8.7-fold greater in stimulated than unstimulated lymphocytes after 66 h of culture (Table I); similar increases in PP-ribose-P have been reported (29).

DISCUSSION

In view of the known abnormalities of lymphocyte function in patients with adenosine deaminase deficiency, which include depressed expression of both humoral and cellular immunity (4), the pathways of adenosine metabolism were examined in normal stimulated human lymphocytes. The conversion of adenosine to adenine was very low in lymphocyte extracts (Table IIIA) and not detected in human serum (Fig. 2), showing cleavage of the glycosidic bond to be the minor route of adenosine metabolism. The rates of adenosine phosphorylation by adenosine kinase and deamination to inosine by adenosine deaminase were examined in both cell lysates and intact lymphocytes. In lysates the ratios of adenosine deaminase to adenosine kinase-specific activities were 9.4 and 4.5 for unstimulated and stimulated lymphocytes, respectively (Table IIIA).

Of the activities examined, only the specific activity of adenosine deaminase markedly decreased in lysates of phytohemagglutinin-stimulated lymphocytes (Table III). However, mitogen-stimulated cultures had a 1.7-fold net increase in adenosine deaminase activity if the extracellular activity (Fig. 3) and increase in cellular protein per culture are included in calculating total activity. Activities of other enzymes that remained at approximately constant specific activity, such as adenosine kinase and purine nucleoside phosphorylase, increased in total activity corresponding to the increase in cell protein per culture (i.e., 2.9-fold). Other studies in phytohemagglutinin-stimulated human lymphocytes showed a 7% decrease in adenosine deaminase activity, reported as a function of cell number (31). In previous work from the same laboratory (32), a 2.6-fold increase in protein content of stimulated to unstimulated cultures was also noted at 72 h, which renders their results comparable to the specific activity measurements in the present work. Decreases in the activities of adenosine deaminase have also been noted in lymphocyte preparations from patients with chronic lymphocytic leukemia (33) and in some but apparently not all cases of acute lymphocytic leukemia (33, 34). Other studies during antibody production in sheep have suggested that lymphoid tissues may be an important source of serum adenosine deaminase activity (35), and elevated levels of serum adenosine deaminase activity have been observed in cases of infectious mononucleosis (36). The present studies of lymphocyte transformation indicate adenosine deaminase is apparently subject to a different rate of synthesis or catabolism from several other enzymes.

Additional studies of adenosine metabolism were conducted with intact lymphocytes, because activities measured in cell extracts may have little correspondence to rates of metabolism in the intact cell. In lymphocytes incubated for 72 h with phytohemagglutinin, the rates of phosphorylation and deamination of exogenously added adenosine increased 6- and 12-fold, respectively, over initial rates (Fig. 4). These increases may be principally due to increased entry of adenosine into the cell. Evidence for this view includes the following observations: the increased rates of deamination and phosphorylation were not attributable to changes in total enzyme activity (Table III), since the rate of metabolism by intact lymphocytes was never greater than 25% of the activity measured in lysates; the increases in adenosine metabolism were detectable as early as 3 h and clearly established at 12 h after addition of mitogen (Fig. 4); the overall maximal velocity was increased and the apparent affinity for deamination and phosphorylation of adenosine remained essentially unchanged (Fig. 5); and the increased rates of adenosine metabolism could be reduced to that in unstimulated lymphocytes by the nucleoside transport inhibitor, nitrobenzylthioinosine (Table II). A phytohemagglutinin-induced increase in the velocity of uridine transport in bovine lymphocytes has also been reported (37).

The ratio of the amount of adenosine phosphorylation to deamination varied as a function of adenosine concentration in lymphocytes cultured in the presence of mitogen (Fig. 6). These results are consistent with the Michaelis constant for adenosine, lower with adenosine kinase, 2 μM (38, 39), than with adenosine deaminase, 25–40 μM (38, 40–42), and the relative amounts of each activity (Table III). Previous studies in other cells (9, 38) have also shown that the relative contribution of the alternate routes of adenosine metabolism depends on adenosine concentration. In unstimulated lymphocytes, however, deamination was approximately 20 times greater than phosphorylation at all concentrations of adenosine examined (0.5–250 μM). The differences in adenosine metabolism between phytohemagglutinin-stimulated and unstimulated cultures may represent a characteristic difference between transformed and nondividing lymphocytes.

The phosphorolysis of guanosine to guanine also increased approximately 6-fold in phytohemagglutinin-stimulated lymphocytes (Table IV), while there was no change in the specific activity of purine nucleoside phosphorylase in stimulated lymphocytes (Table III). Both inosine and guanosine are substrates for purine nucleoside phosphorylase, and the absence or low activity of human inosine and guanosine kinase(s) (43), when coupled with the hereditary deficiency of purine nucleoside phosphorylase activity (5), would effectively

block further metabolism of any inosine or guanosine formed. Inosine and guanosine are known inhibitors of human adenosine deaminase, having K_i's of 116 and 140 μM, respectively (40), suggesting the possibility of a relationship between the immunological disorders associated with adenosine deaminase and purine nucleoside phosphorylase deficiencies. Purine nucleoside phosphorylase is important both in purine catabolism and in facilitating the reutilization of the purine base and ribose moieties of nucleosides for nucleotide synthesis.

The present studies have described increases in the rates of adenosine metabolism during lymphocyte transformation. The role of adenosine deaminase remains obscure, however, to the extent that the intracellular turnover of adenosine and the concentration of extracellular adenosine that lymphocytes encounter in vivo are not known. It is of interest that the hereditary deficiency of adenosine deaminase apparently results in a loss of this activity in all tissues (44). This enzyme may be important for the metabolism of purines ingested in the diet, since the highest levels of human adenosine deaminase activity have been found in the duodenum, stomach, and small intestine (45), and the presence of adenosine deaminase activity in plasma and erythrocytes also contributes to the metabolism of adenosine. The selective dysfunction of lymphocytes resulting in the combined immunodeficiency state, found in a disease characterized by generalized absence of adenosine deaminase activity, may reflect a unique sensitivity of the lymphoid system to adenosine.

An increasing amount of experimental evidence indicates that inhibition of adenosine deaminase activity potentiates inhibition of lymphocyte transformation and function by adenosine. High concentrations of adenosine (5 mM) were initially shown to inhibit the incorporation of thymidine into DNA of phytohemagglutinin-stimulated lymphocytes (46); however, this concentration of adenosine may inhibit thymidine incorporation at the level of a common nucleoside transport system. In studies of concanavalin A-induced lymphocyte transformation, noninhibitory concentrations of the adenosine deaminase inhibitor, EHNA, potentiated the inhibitory effects of adenosine on leucine and thymidine incorporation into acid-insoluble material (7). Adenosine (37.5 μM) inhibited mouse lymphocyte-mediated cytolysis and the inhibitory effects of adenosine were potentiated by the adenosine deaminase inhibitor EHNA (6). The supression of cytolysis by adenosine and concurrent increase in cyclic AMP concentrations were transitory, however, disappearing in hours (6). In the present study, there were no long-term effects of adenosine (50 μM) upon the response of freshly obtained human lymphocytes to phytohemagglutinin by criteria of thymidine incorporation into DNA and morphologic transformation, measured 72 h after addition of the mitogen and nucleoside. Early transitory effects were not assessed. The results also suggest that more than 95% inhibition of adenosine deaminase activity with coformycin does not block lymphocyte transformation. The combination of adenosine and coformycin, however, produced a substantial reduction in both the viable cell count and the incorporation of thymidine into nucleic acids (Table I).

The present and other results from this laboratory (7) are consistent with the hypothesis that the hereditary deficiency of adenosine deaminase may potentiate the growth-inhibitory and toxic effects of adenosine on human lymphocytes. The mechanism whereby adenosine exerts these effects is not known. Adenosine is known to rapidly increase cyclic AMP concentrations in several tissues, including lymphocytes (6), but adenosine is also an inhibitor of adenylate cyclase activity (47, 48). Adenosine also increases cellular glycogen (49, 50) and adenine nucleotide concentrations (50–52), and decreases pyrimidine nucleotide concentrations (49, 50, 53). In the present studies, incubation with adenosine resulted in a marked inhibition of the phytohemagglutinin-stimulated rise in PP-ribose-P concentrations, and with coformycin also present, PP-ribose-P levels were lower than in unstimulated cultures (Table I); the reduced availability of this co-substrate may explain the observed inhibition of nucleotide synthesis from purine bases and of [^{14}C] formate incorporation into nucleic acid purines (Table I). Recent studies with a cultured lymphoblast line indicate that an adenosine-mediated reduction in cellular PP-ribose-P concentrations may effectively inhibit PP-ribose-P-dependent reactions of nucleotide synthesis necessary for lymphocyte proliferation and function.[3]

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