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S-Adenosylhomocysteine toxicity in normal and adenosine kinase-deficient lymphoblasts of human origin

(*S*-adenosylmethionine/methylation/adenosine deaminase/combined immunodeficiency disease)

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ABSTRACT **The human lymphoblast line WI-L2 is subject to growth inhibition by a combination of the adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4.) inhibitor *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) and adenosine. Although adenosine-induced pyrimidine starvation appears to contribute to this effect, uridine only partially reverses adenosine toxicity in WI-L2 and not at all in strain 107, an adenosine kinase-(ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) deficient derivative of WI-L2. Treatment of both cell lines with EHNA and adenosine leads to striking elevations in intracellular *S*-adenosyl-L-homocysteine (AdoHcy), a potent inhibitor of *S*-adenosyl-L-methionine (AdoMet)-dependent methylation reactions. The methylation *in vivo* of both DNA and RNA is inhibited by concentrations of EHNA and adenosine that elevate intracellular AdoHcy. Addition of 100 μM L-homocysteine thiolactone to cells treated with EHNA and adenosine enhances adenosine toxicity and further elevates AdoHcy to levels approximately 60-fold higher than those obtained in the absence of this amino acid, presumably by combining with adenosine to form AdoHcy in a reaction catalyzed by *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1). In the adenosine kinase-deficient strain 107, a combination of ADA inhibition and L-homocysteine thiolactone markedly increases intracellular AdoHcy and inhibits growth even in the absence of exogenous adenosine. These results demonstrate a form of toxicity from endogenously produced adenosine and support the view that AdoHcy, by inhibiting methylation, is a mediator of uridine-resistant adenosine toxicity in these human lymphoblast lines. Furthermore, they suggest that AdoHcy may play a role in the pathogenesis of the severe combined immunodeficiency disease found in most children with heritable ADA deficiency.**

Heritable deficiency of adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) is usually associated with severe combined immunodeficiency disease (1–3). Several theories concerning the necessity for ADA in normal lymphoid cell function have originated from studies on cultured cell lines, mitogen-stimulated lymphocytes, and tissues of patients. The central focus of most investigations has been on mechanisms of adenosine toxicity, although more recently evidence has been presented suggesting that failure to deaminate 2′-deoxyadenosine may be an important factor in the pathogenesis of the immune dysfunction found in ADA-deficient patients (4–7).

The toxic effects of adenosine in ADA-inhibited cells have been attributed to several different processes, including pyrimidine starvation (8–11), stimulation of adenylate cyclase (12), and inhibition of *S*-adenosylmethionine (AdoMet)-mediated methylation. The latter phenomenon has been described in cultured mouse lymphoblasts (13) and is caused by the intracellular accumulation of *S*-adenosylhomocysteine (AdoHcy), both a product and potent inhibitor (14–19) of AdoMet-mediated methylation reactions (Fig. 1). Ordinarily AdoHcy is catabolized by hydrolysis to adenosine and L-homocysteine in a reaction catalyzed by *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1) (20). However, the reversibility of this reaction and a K_{eq} of only 1.4 μM in the hydrolytic direction (20) can result in the accumulation of AdoHcy in adenosine treated, ADA-inhibited cells.

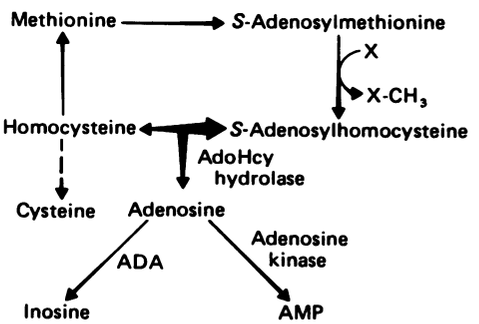

FIG. 1. Pathway of *S*-adenosylhomocysteine metabolism. Because of the reversibility of AdoHcy hydrolase and an equilibrium constant favoring synthesis, the net hydrolysis of AdoHcy requires the efficient metabolism of either adenosine or L-homocysteine.

In this communication we present evidence that AdoHcy accumulation with resultant inhibition of methylation is a major cause of adenosine toxicity in cultured WI-L2 human lymphoblasts. Furthermore, using an adenosine kinase-(ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20)-deficient mutant derivative of WI-L2, we show that a combination of ADA inhibition and L-homocysteine thiolactone leads to both cytotoxicity and AdoHcy accumulation from endogenously produced adenosine.

MATERIALS AND METHODS

Materials. AutoPow minimal essential medium and fetal calf serum were from GIBCO and Irvine Scientific Sales (Fountain Valley, CA), respectively; the fetal calf serum was heated at 62°C for 4–5 hr to inactivate most of its ADA and then filtered prior to use. Calf intestine alkaline phosphatase (type VII, 355 units per mg) was purchased from Sigma, and various nucleic acid bases and nucleosides were products of P-L Biochemicals. *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) was a gift from Wellcome Research Laboratories (Research Triangle Park, NC), while radiolabeled uridine and other materials were obtained as described (13, 21).

Cell Culture. The cells used were the human splenic lymphoblast line WI-L2 (22, 23) and its adenosine kinase-deficient derivative 107 (21). The medium and methods used for cell growth and quantification of cytotoxicity (21), and the preparation of acid-soluble extracts (13) have been described.

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Abbreviations: ADA, adenosine deaminase; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine.

Purification and Hydrolysis of Nucleic Acids. A pellet of approximately 10^7 washed [6-^{3}H]uridine-label cells was treated with 0.2 ml of cold 1 M perchloric acid, and after centrifugation the supernatant was neutralized with KOH for assay of AdoHcy and AdoMet by high-performance liquid chromatography. The perchloric acid-insoluble material, containing nucleic acids, was washed with 70% (vol/vol) ethanol, dried in a N_2 stream, and suspended in 0.5 ml of 0.1 M Tris·HCl, pH 7.6, containing 0.5% sodium dodecyl sulfate, 20 mM Na_2EDTA, and nuclease-free Pronase at 1 mg/ml (24). After 1–2 hr at 37°C, 0.3 mg of salmon DNA was added as carrier, and the solution was extracted three times with phenol. Excess phenol was extracted from the final aqueous layer with ether, and nucleic acids were precipitated with 2 vol of cold ethanol. The precipitate was collected by centrifugation, washed with 70% and then absolute ethanol, dried in a N_2 stream, and dissolved in 0.3 ml of 0.25 M NaOH. After incubation for 18 hr at 37°C to hydrolyze RNA, DNA was precipitated by adding 0.08 ml of cold 5 M perchloric acid. This precipitate was washed with 70% and then absolute ethanol, dried, and hydrolyzed by heating in 0.2 ml of 90% (wt/vol) formic acid at 180°C for 30 min in a sealed tube. The residue obtained after removal of formic acid with a stream of N_2 was dissolved in a small volume of water and analyzed for radiolabeled pyrimidine bases by high-performance liquid chromatography as described below.

The perchloric acid solution of ribonucleotides obtained from alkaline hydrolysis was neutralized with KOH, and insoluble $KClO_4$ was removed by centrifugation. A 0.2-ml portion of the supernatant was added to 0.1 ml of 0.2 M glycine adjusted to pH 9.2 with 10 M NaOH, which contained 1.4 units of alkaline phosphatase, and the mixture was incubated at 37°C for 1 hr. This material was then analyzed for radiolabeled pyrimidine ribonucleosides by high-performance liquid chromatography.

High-Performance Liquid Chromatography. Analyses were performed by using a Waters Associates (Milford, MA) model 6000A solvent delivery system and a model 440 detector monitoring absorbance simultaneously at 254 and 280 nm. DNA bases were separated on a Whatman Partisil-10 SCX column (0.46 × 25 cm), eluting isocratically with 45 mM $NH_4H_2PO_4$ adjusted to pH 2.3 with 2 M H_3PO_4 at a flow rate of 1.0 ml/min (25). Fractions of the eluate were collected and assayed for radiolabel in a toluene-based scintillation fluid containing Triton X-100 (26). AdoHcy was analyzed by using the same system and was eluted at 8 ml. AdoMet was assayed on the same column, using 0.2 M $NH_4H_2PO_4$ adjusted to pH 2.6 with 2 M H_3PO_4 at a flow rate of 1.0 ml/min and was eluted at 7 ml. Results are expressed as nmol per 10^9 cells; 10^9 cells have a packed volume of approximately 1.4 ml.

Pyrimidine bases and nucleosides from RNA digests were resolved on a Waters Associates μBondapak C_{18} column (0.4 × 20 cm), using isocratic elution with 20 mM $(NH_4)H_2PO_4$ adjusted to pH 5.5 with 3 M NH_4OH containing 2% (vol/vol) methanol at a flow rate of 1.0 ml/min. Elution volumes were as follows: cytosine, 4.5 ml; uracil, dihydrouridine, pseudouridine, 5.5 ml; cytidine, 7.0 ml; 5-methylcytosine, 7.9 ml; uridine, 9.5 ml; 2′-deoxycytidine, 10.4 ml; thymine, 11.7 ml; 5-methylcytidine, 13.3 ml; 2′-*O*-methylcytidine, 16.4 ml; 5-methyluridine, 20.2 ml; 4-thiouridine, 25.5 ml; 2′-*O*-methyluridine, 28.2 ml; thymidine, 34.2 ml. Fractions of the eluate were assayed for radiolabel in cytidine, uridine, 5-methylcytidine, and 5-methyluridine, as described above. The same system was used for adenosine analyses except the methanol concentration was increased to 10%, giving an elution volume of 20 ml for this nucleoside.

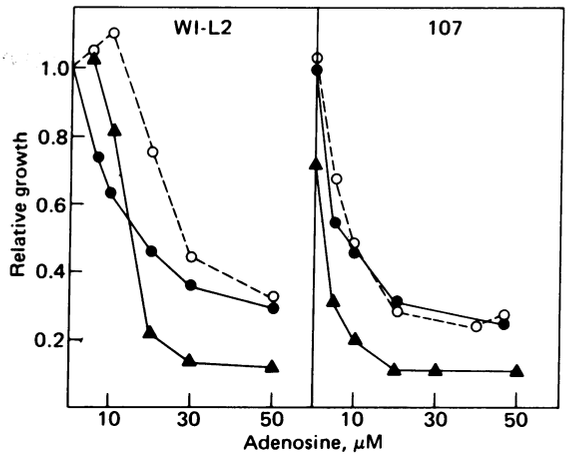

FIG. 2. Growth inhibition of WI-L2 and strain 107 by adenosine. Cells at a density of 1 × 10^5 per ml were grown in medium containing 5 μM EHNA and various concentrations of adenosine with: no other additions (●); 10 μM uridine (○); or 10 μM uridine plus 100 μM L-homocystein thiolactone (▲). Growth after 72 hr is relative to a control lacking adenosine.

RESULTS

Nucleotide-Independent Growth Inhibition by Adenosine. Both WI-L2 and its adenosine kinase-deficient derivative 107 are sensitive to the cytotoxic effects of adenosine when ADA is inhibited by 5 μM EHNA (Fig. 2). The results are similar to those reported previously for these cell lines (21) and demonstrate the existence of a form of adenosine toxicity that does not require phosphorylation of this nucleoside. The addition of 10 μM exogenous uridine provides some protection against adenosine toxicity for WI-L2, but not for 107 (Fig. 2), confirming earlier reports (21, 27, 28) that adenosine-mediated pyrimidine starvation is phosphorylation-dependent in WI-L2. Similar results in a mouse lymphoma line have been reported (10, 11).

Uridine-resistant adenosine toxicity is markedly enhanced in both WI-L2 and 107 by 100 μM L-homocysteine thiolactone (Fig. 2). Similar results have been obtained with L-homocystine in S49 cells (13), but we prefer to use the thiolactone in such experiments because of its greater solubility and lack of reactivity with thiols and disulfides present in medium. We assume that the thiolactone is readily converted to free homocysteine by an intracellular thiolactonase. The adenosine kinase-deficient strain is even more sensitive to adenosine plus L-homocysteine thiolactone than is its parent. Furthermore, while this concentration of L-homocysteine thiolactone by itself does not affect the growth of EHNA-treated WI-L2, it inhibits the adenosine kinase-deficient strain by about 30%. The basis for the greater sensitivity of 107 is addressed below. First we will show that uridine-resistant adenosine toxicity is related to the accumulation of AdoHcy in ADA-inhibited cells.

***S*-Adenosylhomocysteine Accumulation in WI-L2.** Treatment of WI-L2 cultures for 3 hr with a combination of 5 μM EHNA and various concentrations of adenosine caused appreciable increases in intracellular AdoHcy from a level of approximately 5 nmol per 10^9 cells in control cultures to 45 and 124 nmol per 10^9 cells at 25 μM and 100 μM adenosine, respectively (Fig. 3). Inclusion of 100 μM L-homocysteine thiolactone in such experiments stimulated adenosine-dependent AdoHcy accumulation approximately 60-fold with a level of 8300 nmol per 10^9 cells obtained at 100 μM adenosine. At fixed concentrations of exogenous adenosine, intracellular AdoHcy content was almost linearly proportional to L-homocysteine thiolactone added over a range of 10–200 μM of this amino acid (data not shown).

AdoMet concentrations varied in direct proportion to

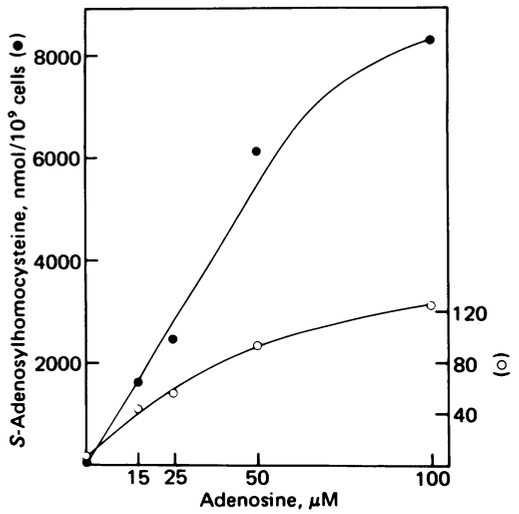

FIG. 3. *S*-Adenosylhomocysteine levels in WI-L2 cells cultured for 3 hr in the presence of 5 μM EHNA and various concentrations of adenosine. Cell densities were approximately 2 × 10^5 per ml. ○, EHNA and adenosine alone; ●, EHNA, adenosine, and 100 μM L-homocysteine thiolactone. Note the difference in scales.

AdoHcy levels and ranged from between 100 and 180 nmol per 10^9 cells in control cultures to as high as 550 nmol per 10^9 cells where AdoHcy was 9900 nmol per 10^9 cells. This response presumably reflects inhibition of methylation (see below) and a resultant accumulation of AdoMet.

Inhibition of Methylation. Cultures of WI-L2 (2 × 10^5 cells/ml) were treated for 90 min with 5 μM EHNA and various concentrations of adenosine and L-homocysteine thiolactone to elevate intracellular AdoHcy and then were given a 2-hr pulse of [6-^{3}H]uridine [24 Ci/mmol; 1 μCi/ml culture (1 Ci = 3.7 × 10^{10} becquerels)] to label nucleic acids. The cells were harvested and their DNA and RNA were purified and analyzed as described in *Materials and Methods*. The extent of DNA methylation during the pulse was estimated as the percentage of total cytosine radiolabel that was present as 5-methylcytosine in a DNA hydrolysate. Because methylation of cytosine residues occurs within a few minutes after the residues are incorporated into eukaryotic nascent DNA (29), this method of expressing results largely eliminates effect due to variations in the size of pyrimidine pools and corrects for changes in absolute rates of DNA synthesis *per se*.

RNA methylation was quantified in these same experiments by measuring the radiolabel incorporated into 5-methylcytidine and 5-methyluridine, two ribonucleosides known to occur in eukaryotic tRNA (30, 31). An alkaline hydrolysate of unfractionated RNA was treated with alkaline phosphatase, and nucleosides were separated by high-performance liquid chromatography in a system that gives good separation between 5-methylcytidine, 5-methyluridine, and other pyrimidine nucleosides and bases. Results are expressed as the percentage radiolabel in the methylated nucleoside compared to that in its immediate precursor—i.e., cytidine or uridine. As in the case of our DNA methylation assays, the use of such ratios gives methylation data that are independent of changes in the specific activities of nucleotide precursor pools. Differential effects on rates of mRNA, rRNA, and tRNA synthesis could themselves vary such ratios, however, because eukaryotic mRNA and rRNA contain very little or no 5-methylcytidine and 5-methyluridine (32–34).

In control experiments 5-methylcytosine comprised 3.08% of the total cytosine bases in DNA, while in RNA 0.32% of total cytidine residues were present as 5-methylcytidine and 0.14% of total uridine residues were present as 5-methyluridine. The data given in Fig. 4 show that conditions that increase intracellular AdoHcy cause significant inhibition of both DNA and RNA methylation in WI-L2. The methylation of cytosine is inhibited to approximately the same extent in DNA and RNA, with 50% of control values noted at about 140 nmol of AdoHcy per 10^9 cells. The methylation of uridine appears to be a less sensitive reaction, with 50% inhibition occurring at approximately 300 nmol of AdoHcy per 10^9 cells. Fifty micromolar adenosine, which in the presence of uridine inhibits WI-L2 growth by 70%, increased AdoHcy about 20-fold to 100 nmol per 10^9 cells and inhibited the methylation of cytosine and uridine 40% and 30%, respectively.

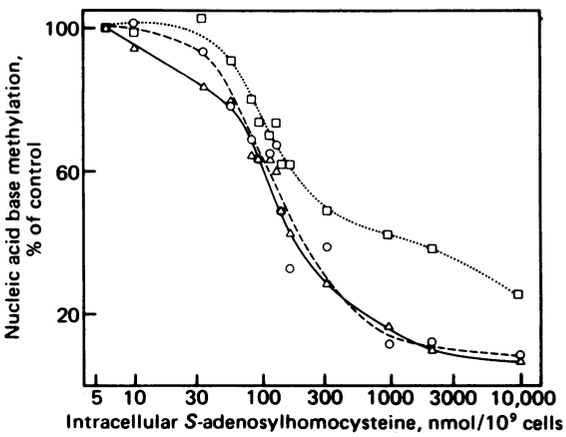

FIG. 4. Inhibition of DNA and RNA methylation *in vivo* in WI-L2 as a function of intracellular *S*-adenosylhomocysteine. AdoHcy levels were increased by various combinations of EHNA, adenosine, and L-homocysteine thiolactone, and the extent of methylation is compared to that found in cells cultured in the absence of agents that elevate AdoHcy. △, 5-Methylcytosine in DNA; ○, 5-methylcytidine in RNA; □, 5-methyluridine in RNA.

Adenosine and L-Homocysteine Thiolactone Toxicity in Strain 107. Incubation of strain 107 for a short time (3 hr) with EHNA and various concentrations of adenosine and L-homocysteine thiolactone increases AdoHcy to levels that are equal to or slightly higher than those found in similarly treated WI-L2; in addition, the sensitivity of DNA methylation *in vivo* to inhibition at any given level of AdoHcy is the same in 107 as in WI-L2 (data not shown). However, rates of adenosine metabolism differ markedly between the two strains. In the presence of 5 μM EHNA and at an initial adenosine concentration of 8 μM, WI-L2 cells metabolize 60% of the adenosine in 3 hr and 98% in 8 hr, while strain 107 metabolized less than 20% in 24 hr (Fig. 5). In the same experiment AdoHcy levels declined

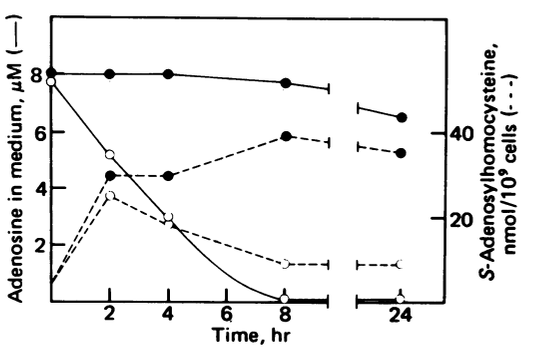

FIG. 5. Time course of intracellular *S*-adenosylhomocysteine and extracellular adenosine levels in WI-L2 (○) and the adenosine kinase-deficient strain 107 (●). Cells at an initial density of 3 × 10^5 per ml were incubated with gentle shaking at 37°C in medium to which 10 μM EHNA and 8 μM adenosine were added at *zero* time. At the times indicated, samples were removed for measurements of intracellular AdoHcy (- - -) and of adenosine remaining in the medium (—).

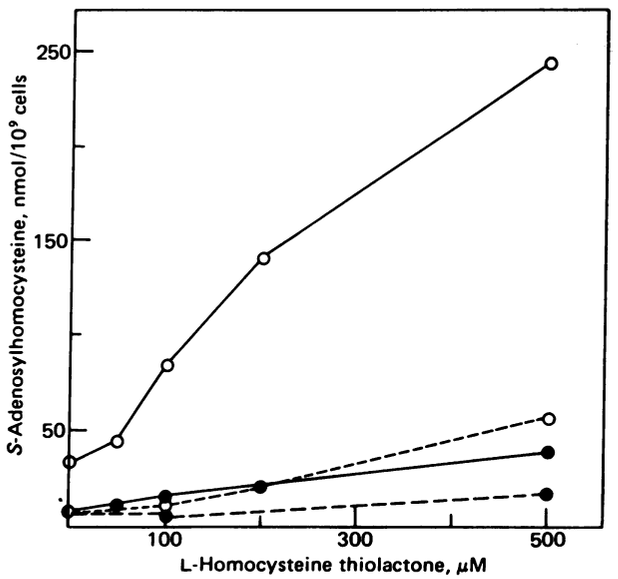

FIG. 6. Effect of L-homocysteine thiolactone on *S*-adenosylhomocysteine accumulation in WI-L2 (●) and strain 107 (○) in the absence of exogenous adenosine. Cells at 3×10^5 per ml were incubated in the presence (– –) or absence (- - -) of 5 μM EHNA and various amounts of L-homocysteine thiolactone; intracellular AdoHcy was measured after 24 hr.

to normal after 8 hr in WI-L2, but in 107 continued to increase for at least 8 hours and remained elevated even after 24 hr (Fig. 5). Thus, adenosine kinase contributes significantly to adenosine metabolism in ADA-inhibited WI-L2, and by doing so actually protects against long term AdoHcy accumulation.

The loss of both ADA and adenosine kinase activities in EHNA-treated 107 results in a greatly increased capacity for AdoHcy accumulation when cells are incubated with L-homocysteine thiolactone in the absence of exogenous adenosine. After 24 hr of incubation with 5 μM EHNA and various concentrations of L-homocysteine thiolactone, AdoHcy levels are 5- to 7-fold higher in 107 than in WI-L2 (Fig. 6). A similar difference between the two strains is also observed in the absence of EHNA, but here the absolute levels of AdoHcy are much smaller.

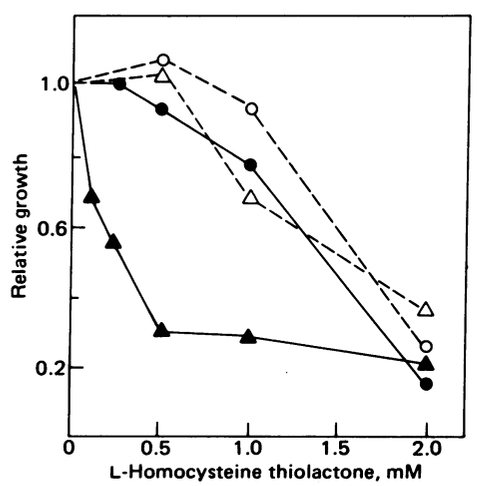

FIG. 7. Effects of EHNA and L-homocysteine thiolactone on the growth of WI-L2 and its adenosine kinase-deficient derivative 107. Cells at an initial density of 1×10^5 per ml were grown with various concentrations of L-homocysteine thiolactone in either the presence (– –) or absence (- - -) of 10 μM EHNA. Cell densities were measured after 72 hr, and the results are expressed as the fold increase in cell number compared to that obtained in the absence of L-homocysteine thiolactone. ○, ●, WI-L2; △, ▲, 107.

The notion of AdoHcy toxicity is supported by the finding that 107 is much more sensitive than WI-L2 to growth inhibition by L-homocysteine thiolactone in the absence of exogenous adenosine (Fig. 7). As is the case with AdoHcy accumulation, the toxic effects of this amino acid are largely dependent upon the presence of EHNA. Significant cytotoxicity for 107 is observed at an L-homocysteine thiolactone concentration of 100 μM, and at 500 μM growth is inhibited by 70%; WI-L2 is virtually unaffected by 500 μM L-homocysteine thiolactone. Comparing the data in Figs. 6 and 7, it is evident that in line 107 AdoHcy levels of 40–80 nmol per 10^9 cells coincide with growth inhibition of 30–45%.

DISCUSSION

In an earlier study (13) adenosine was shown to elevate intracellular AdoHcy with the coincident inhibition of DNA methylation *in vivo* in ADA-inhibited cultured mouse lymphoblasts. These results were interpreted to indicate that inhibition of AdoMet-mediated methylation is a cause of adenosine toxicity in these cells. With the work presented here, we have extended and amplified those observations by demonstrating that cytotoxic concentrations of adenosine increase AdoHcy levels in a human lymphoblast line as well, with inhibition not only of DNA methylation but also of RNA methylation.

In attempting to relate a specific biochemical effect of a toxic agent to a response as complex as the inhibition of cell growth, one can rarely disprove the possibility that the agent is acting in some other, as yet unrecognized manner, which itself contributes either partially or totally to cytotoxicity. Reasonable assurance for a cause and effect relationship is obtained in those instances in which the addition of a specific metabolite both circumvents the biochemical defect and prevents cytotoxicity. Unfortunately, the thesis that uridine-resistant adenosine toxicity is due to inhibition of methylation is not readily amenable to such an approach. For this reason the sensitivity of ADA-inhibited 107 to L-homocysteine thiolactone is significant because it at least permits an evaluation of AdoHcy toxicity in the absence of other effects that might be caused by exogenous adenosine. We find that with ADA inhibition, and in the absence of exogenous adenosine, L-homocysteine thiolactone is much more toxic to 107 than to WI-L2, while increasing AdoHcy to levels that are considerably higher in the adenosine kinase-deficient strain. Furthermore, significant growth inhibition is first noted at about 40 nmol AdoHcy per 10^9 cells, both for 107 treated with L-homocysteine thiolactone and for WI-L2 treated with exogenous adenosine in the presence of uridine. Taken together, these results indicate that such levels of AdoHcy are cytotoxic and that most if not all of the previously reported (21, 27) uridine-resistant adenosine toxicity in both WI-L2 and 107 is due to the accumulation of AdoHcy. The inhibition of 107 by L-homocysteine thiolactone may also be interpreted as a demonstration of toxicity from *endogenously* produced adenosine in a cultured cell system.

While it is conceivable that one particular methylation reaction is especially sensitive to inhibition by AdoHcy or singularly crucial for normal growth, it seems more likely that AdoHcy toxicity results from the cumulative effects of partially inhibiting the methylation of many different kinds of molecules. A role for 5-methylcytosine in eukaryotic DNA has not yet been established, but considerable evidence points to a requirement for methylation in the processing and proper functioning of tRNA (35, 36), mRNA (37–40), and rRNA (41), and the undermethylation of such molecules would be expected to be detrimental to cell growth and function. Of particular interest in this regard are the findings of Vaughan *et al.* (42) and of Wolf and Schlessinger (41), who have shown that con-

ditions known to inhibit methylation prevent the normal maturation of 45S pre-rRNA to mature 28S and 18S rRNA. Furthermore, Bynum and Volkin (43) have found that adenosine selectively interferes with the accumulation of 18S rRNA in human myeloma cells, and it is very likely that this effect is secondary to AdoHcy accumulation with the resultant inhibition of pre-rRNA methylation. Other possible targets of AdoHcy toxicity include: protein carboxy-*O*-methylation (44, 45), a reaction believed to be involved in certain specialized processes such as chemotaxis (46, 47), secretion (48), and neural function (49); histone methylation (50, 51); and the metabolism of small molecules such as phospholipids (52, 53) and carnitine (54).

Studies of model cell systems have resulted in the formulation of several cogent theories pertaining to the molecular pathogenesis of the immune defect in heritable ADA deficiency. Although pyrimidine starvation due to conversion of adenosine to intracellular nucleotides is a real phenomenon in cultured cells, evidence suggests that this does not occur in ADA-deficient individuals (55). Perhaps sufficient uridine is available from diet or cell turnover to prevent pyrimidine depletion *in vivo*. Our results indicate that except for such an effect on pyrimidine metabolism, nucleotide formation from adenosine is not toxic *per se*, and in fact may prevent accumulation of AdoHcy, which we believe may be the toxic metabolite derived from adenosine that contributes to the immune dysfunction in heritable ADA deficiency.

The ADA substrate 2′-deoxyadenosine also is known to have profound effects on cellular metabolism, blocking DNA synthesis (56) through the inhibition of ribonucleotide reductase by dATP (57). Evidence favoring the notion of 2′-deoxyadenosine toxicity comes from the finding that erythrocytes of ADA-deficient patients contain large amounts of dATP (6, 7). Additional clinical studies will be required to determine the extent to which interference with deoxyribonucleotide metabolism, the inhibition of methylation, or other mechanisms participate in the pathogenesis of ADA-deficient severe combined immunodeficiency disease.

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