S-Adenosylhomocysteine Hydrolase Inhibition in Deoxyadenosine-treated Human T-Lymphoblasts and Resting Peripheral Blood Lymphocytes

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ABSTRACT

The pattern of inhibition and recovery of intracellular S-adenosylhomocysteine hydrolase (AdoHcyase) activity induced by cytotoxic concentrations of 2'-deoxyadenosine (dAdo) in the presence of the adenosine deaminase inhibitor erythro-9-[3-(2-hydroxynonyl)]adenine was studied in cultured human T-lymphoblasts (CCRF-CEM and HSB), Epstein-Barr virus-transformed B-lymphoblasts (JP and RDG), and resting peripheral blood lymphocytes. In the presence of 5 μM erythro-9-[3-(2-hydroxynonyl)]adenine the AdoHcyase activity was inhibited 50% after 1 hr of incubation by 3, 10, and 30 μM dAdo for B-lymphoblasts, T-lymphoblasts, and peripheral blood lymphocytes, respectively, while cytotoxicity (to 50% of controls) was effected by 600, 3, and 1 μM dAdo, respectively. Cytostatic concentrations of dAdo, which induce G1-phase arrest in T-lymphoblasts, inhibited AdoHcyase to 30% of control at 4 hr, after which recovery to normal levels occurred over 24 hr. Coincubation with 50 μM 2'-deoxycytidine, which protects these cells from both G1-phase arrest and growth inhibition by dAdo, had no effect on the pattern of inhibition of AdoHcyase activity. Epstein-Barr virus-transformed B-lymphoblasts exposed to the same concentrations of dAdo were not growth inhibited but displayed patterns of AdoHcyase inhibition closely similar to those seen in T-lymphoblasts. Coincubation with 20 μM 2'-deoxycytidine, despite its protective effect against dAdo cytotoxicity, had no effect on the pattern of inhibition of AdoHcyase in resting peripheral blood lymphocytes induced by 10 μM dAdo and 5 μM erythro-9-[3-(2-hydroxynonyl)]adenine over a 72-hr period. The lack of correlation between cytotoxicity and AdoHcyase inhibition in human lymphoid cells suggest that other mechanisms may account for the toxic effects of dAdo in these cells.

INTRODUCTION

Inhibitors of ADA2 (EC 3.5.4.4) have been introduced into clinical trial in the management of T-cell acute lymphoblastic leukemia. The most potent of these drugs, deoxycoformycin, has induced remissions in a number of patients (10, 19, 26, 31–34, 37, 39). Inherited deficiency of ADA is associated with severe immunodeficiency disease in children (9, 25). The presence of high levels of dATP in ADA-deficient erythrocytes and lymphocytes (5, 15) and the restoration of immune function to normal, paralleling a reduction in erythrocyte dATP levels after transfusion of some ADA-deficient patients with normal ADA-containing erythrocytes (30), implicated this metabolite as an effector of dAdo cytotoxicity. It was proposed that lymphocyte toxicity might be the result of allosteric inhibition of ribonucleotide reductase by accumulated dATP, with a consequent depletion of other deoxynucleotide triphosphates and inhibition of DNA synthesis (3, 36). This hypothesis was supported by studies performed in the presence of ADA inhibitors, which correlated the sensitivity of cultured human T- and null cell leukemic lymphoblasts to μM concentrations of dAdo with the capacity of these cells to elevate their dATP pools. Moreover, in EBV-transformed B-lymphoblasts, elevated dATP pools and growth inhibition occurred only at mM dAdo concentrations in the presence of ADA inhibitors (2, 4, 7, 8, 27, 38).

However, it has been shown recently that, in the presence of ADA inhibitors, nondividing PBL from normal donors and from patients with B-cell chronic lymphocytic leukemia also elevate dATP pools and are lysed by μM concentrations of dAdo (17, 18). At low dAdo concentrations, protection against cytotoxicity is effected by coincubation with dCyd (18). Furthermore, on exposure to cytostatic concentrations of dAdo in the presence of ADA inhibitors, human leukemic T-cells arrest in the G1 phase of the cell cycle, while cells in S phase, after exposure to dAdo, complete S phase (6). This G1-phase arrest is associated with a rise in the dATP pool, without a fall in the dCTP pool, yet both these phenomena are prevented by coincubation with dCyd. Clearly, dAdo cytotoxicity in nondividing cells and G1 arrest in cycling cells are not compatible with ribonucleotide reductase inhibition.

We sought to determine if dAdo inactivation of AdoHcyase, described as a putative mechanism for dAdo lymphotoxicity in human leukemic lymphoblasts in vitro and in vivo (11–14), might account for these "ribonucleotide reductase-independent" effects of dAdo. We show that dAdo-resistant B-lymphoblasts and dAdo-sensitive T-lymphoblasts have a similar pattern of inhibition of AdoHcyase by dAdo. The inhibition of AdoHcyase induced by cytostatic concentrations of dAdo in cultured T-lymphoblasts and by lympholytic concentrations in resting PBL is not prevented by dCyd, despite the protective effect of this nucleoside against the observed cytotoxicity. The lack of correlation between cytotoxicity and AdoHcyase inhibition suggests that other mechanisms may account for the toxic effect of dAdo against human lymphoid cells.

MATERIALS AND METHODS

Reagents

Deoxynucleosides were obtained from Sigma Chemical Co., St. Louis, Mo. PEI-cellulose chromatography plates were from Merck, Darmstadt, Germany. EHNA was supplied by Dr. C. Nichol (Burroughs Wellcome Co., Research Triangle Park, N. C.). RPMI 1640 and fetal calf serum were from Flow Laboratories, North Ryde, New South Wales.
Wales. Radiochemicals were from the Radiochemical Centre, American, United Kingdom. Ficoll-Paque was from Pharmacia Fine Chemicals, Uppsala, Sweden. L-Homocysteine thiolactone (Sigma) was converted to L-homocysteine immediately before use by incubation at 37°C for 30 min in 0.05 N NaOH, followed by neutralization with HCl.

Cell Preparations

Human Lymphocytes. PBL were obtained from healthy blood donors. Monocyte-depleted mononuclear cell suspensions were obtained after Ficoll-Paque gradient centrifugation as described previously (17). PBL (1 x 10^6/ml) were incubated at 37°C in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered RPMI 1640, pH 7.4, supplemented with glutamine (0.2 mM), penicillin (100 μg/ml), streptomycin (100 units/ml), and 10% extensively heat-inactivated fetal calf serum (61°C, 4 hr) in 500-ml air-tight polystyrene tissue culture flasks (100 to 300 ml/flask). A 16-hr recovery period was allowed between lymphocyte isolation and the commencement of experiments. All cultures were routinely checked by DNA cytfluorimetry at 24, 48, and 72 hr to exclude mitogen-induced proliferation.

Cultured Human Lymphocyte Lines. Cultured human leukemic lymphocytes derived from patients with T-cell acute lymphoblastic leukemia were provided by Dr. J. Minowada (Roswell Park Memorial Institute, Buffalo, N. Y.). These lines were CCRF-CEM, HPB-MLT, 8402, and CCRF-HSB. The origin and characteristics of these cell lines have been described previously and summarized by Minowada (24).

Cultured diploid B-lymphoblasts transformed by EBV were provided by Dr. H. Lazarus (Sidney Farber Institute, Boston, Mass.), I. Jack (Royal Children’s Hospital, Melbourne, Victoria, Australia), and H. Zola (Findlers Medical Centre, Adelaide, South Australia). These cell lines were JP, RDG, WIL, Je-Tg, GK, and LAZ-007. All cell lines were grown in suspension culture in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered RPMI 1640 (pH 7.4), glutamine (0.2 mM), penicillin (100 μg/ml), streptomycin (100 units/ml), and 10% fetal calf serum. The lines had approximately similar doubling times (24 to 30 hr) and were studied in the log phase of growth.

AdoHcyase Inhibition in Whole Cells

To flasks containing PBL (1 x 10^6/ml) or cultured lymphoblasts (2 x 10^5/ml), EHNA was added to a final concentration of 5 μM. After 30 min at 37°C, dCyd and dAdo were added at the concentrations indicated and the incubation was continued. At standard time points, cells were sampled and chilled on ice for 5 min. In PBL experiments, the dead cells were removed by Ficoll-Paque centrifugation (16). Cells (1 x 10^6 cultured lymphoblasts or 5 x 10^5 PBL) were harvested and washed once in 5 ml phosphate-buffered saline containing, in g/liter: NaCl, 0.8; KCl, 0.02; NaHPO₄, 0.115; KH₂PO₄, 0.02 (pH 7.2) at 4°C. The cell pellets were taken up in 0.5 to 1.0 ml 25 mM potassium phosphate: 1 mM disodium EDTA:1 mM β-mercaptoethanol, pH 7.0. Cells were disrupted by 3 cycles of freezing and thawing, and 25 or 50 μl of the 10,000 x g supernatant were incubated in duplicate in a reaction mixture (100 μl final volume) containing 25 mM potassium phosphate (pH 7.0), 1 mM disodium EDTA, 1 mM β-mercaptoethanol, 5 mM L-homocysteine, 100 μM [U-¹⁴C]adenosine (1.0 μCi/ml), and 10 μM EHNA. Enzyme activity was not inhibited by 10 μM EHNA. Following incubation for various times at 37°C, reaction products were separated by thin-layer chromatography on PEI-cellulose plates in butanol:meanol:H₂O:NH₄OH (60:20:20:1) using unlabeled adenosine, inosine, 5′-adenosylhomocysteine, and hypoxanthine as markers. The UV-fluorescing spots were cut out and counted in a scintillation counter. Protein was measured by the method of Lowry et al. (22) with bovine serum albumin as standard.

DNA Cytfluorimetry

Cellular DNA content was measured using an ICP22 flow cytometer (Ortho Instruments, Westwood, Mass.), cells were stained with ethidium bromide and mithramycin, and computer-determined DNA histograms were obtained as described previously (23, 35).

RESULTS

AdoHcyase Activity in Lymphoid Cells. The AdoHcyase activity for various human lymphoid cells is shown in Chart 1. Cultured T-leukemic lymphoblasts had similar enzyme activities to EBV-transformed B-lymphoblasts. AdoHcyase activity in resting PBL from several different individual donors is also shown in Chart 1. The phosphorylated derivatives of adenosine and dAdo were tested for their inhibitory effect on AdoHcyase. There was no inhibition of AdoHcyase activity when AMP, dAMP, ADP, dADP, ATP, or dATP was added to the reaction mixture over a range of concentrations from 0.1 to 100 μM (data not shown).

Ado Inactivation of AdoHcyase in Cultured Lymphoblasts. After 1 hr of incubation of cells in various concentrations of dAdo in the presence of 5 μM EHNA, the AdoHcyase in extracts of dAdo-resistant EBV-transformed B-lymphoblast line was inhibited at lower concentrations than that of dAdo-sensitive T-lymphoblast line and PBL (Chart 2). The AdoHcyase of EBV-transformed lymphoblasts was inhibited to 5% of control by 50 μM dAdo, a concentration some 60-fold lower than the 50% growth-inhibitory concentration, which, in the presence of 5 μM EHNA, is 600 μM (data not shown). Conversely, the AdoHcyase activity of CCRF-CEM cells, which are 50% growth inhibited by 3 μM dAdo in the presence of 5 μM EHNA, required 10 μM dAdo to achieve 50% inhibition of AdoHcyase.

In order to determine if the pattern of inhibition and recovery of AdoHcyase activity correlated with cytotoxic effects of dAdo...
over longer incubation periods, T- and B-lymphoblasts were incubated in 2 concentrations of dAdo in the presence of EHNA, and AdoHcyase activity was compared with dAdo-induced changes in cell growth and DNA profile (Charts 3 and 4). In the presence of 5 µM EHNA, 3 µM dAdo is cytostatic to T-lymphoblasts, which arrest in G1 phase. This effect is demonstrable at 4 hr and persists for 30 to 36 hr (6). Cytostasis and G1-phase arrest are both prevented by coinubcation of the cells in 50 µM dCyd (6). At a concentration of 17.5 µM dAdo (in the presence of 5 µM EHNA), T-lymphoblasts die and lyse. Coincubation with 50 µM dCyd partially prevents this toxicity, and the cells arrest in G1, without loss of viability (6). At 3 µM, dAdo inhibited AdoHcyase in T-lymphoblasts to 30% of initial activity at 4 to 8 hr. By 24 hr, the enzyme activity had recovered to approximately 90% of the initial level (Chart 3, A and C). At 17.5 µM, dAdo caused more complete inhibition of AdoHcyase, with no recovery over the 24-hr period. Coincubation with 50 µM dCyd had no significant effect on the inhibition of AdoHcyase induced by either the high or low dose of dAdo (Chart 3, B and D). Thus, T-lymphoblasts grown in 50 µM dCyd, 5 µM EHNA, and 3 µM dAdo grow normally and display a normal DNA profile, despite a marked, although transient, inhibition of AdoHcyase activity.

The inhibitory effect of 3 µM dAdo on AdoHcyase activity in EBV-transformed B-lymphoblasts had a pattern similar to that seen in T-lymphoblasts, despite the fact that the 50% growth-inhibitory concentration of dAdo is 100-fold higher for these cells (Chart 4). Neither 3 nor 17.5 µM dAdo affected the DNA profile of EBV-transformed B-lymphoblasts at 24 hr (Chart 4). AdoHcyase activity recovered over a longer time course than that in T-lymphoblasts and had not returned to initial values by 24 hr. This may reflect the apparent greater sensitivity of...
AdoHcyase from EBV-transformed B-lymphoblasts to inhibition by dAdo. At 24 hr, dAdo concentrations in the growth medium from T-lymphoblasts and EBV-transformed B-lymphoblasts are 50% of commencing concentrations, despite the presence of 5 μM EHNA as an ADA inhibitor (6). Thus, while AdoHcyase from EBV-transformed B-lymphoblasts may remain inhibited by these lower concentrations of dAdo at 24 hr, that in T-lymphoblasts recovers. Yet, despite a persisting inhibition of AdoHcyase to 10% of control values, EBV-transformed B-lymphoblasts show unperturbed growth and normal DNA histograms (Chart 4). The presence of 5 μM EHNA alone in the medium of control flasks had no effect on growth, DNA profile, or AdoHcyase activity for either T-lymphoblasts or EBV-transformed B-lymphoblasts over the period of incubation (data not shown). AdoHcyase from EBV-transformed B-lymphoblasts to inhibition by dAdo. At 24 hr, dAdo concentrations in the growth medium from T-lymphoblasts and EBV-transformed B-lymphoblasts are 50% of commencing concentrations, despite the presence of 5 μM EHNA as an ADA inhibitor (6). Thus, while AdoHcyase from EBV-transformed B-lymphoblasts may remain inhibited by these lower concentrations of dAdo at 24 hr, that in T-lymphoblasts recovers. Yet, despite a persisting inhibition of AdoHcyase to 10% of control values, EBV-transformed B-lymphoblasts show unperturbed growth and normal DNA histograms (Chart 4). The presence of 5 μM EHNA alone in the medium of control flasks had no effect on growth, DNA profile, or AdoHcyase activity for either T-lymphoblasts or EBV-transformed B-lymphoblasts over the period of incubation (data not shown).

dAdo Inactivation of AdoHcyase in PBL. The toxic effect of dAdo on resting PBL has been described previously (17, 18). At a concentration of 1 μM dAdo (plus 5 μM EHNA), resting PBL undergo progressive cell death after the first 24 hr of exposure and 50% are killed (on the basis of dye exclusion) after 96 hr. Coincubation with 50 μM dCyd is almost completely protective against the cytotoxicity of 10 μM dAdo (Chart 5). However, despite its protective effect against cytotoxicity, coincubation with 50 μM dCyd had no effect on the inhibition of PBL AdoHcyase activity induced by 10 μM dAdo (Chart 5). While 5 μM EHNA alone had little effect on AdoHcyase activity over the 72-hr incubation period, 10 μM dAdo, in the presence and absence of 50 μM dCyd, inhibited the enzyme to 25% of control activity at 4 hr, after which there was a gradual recovery to 50 to 70% of control values at 72 hr. The cell viability of resting PBL treated with 10 μM dAdo, 50 μM dCyd, and 5 μM EHNA was equivalent at 96 hr to that of control cells treated with the ADA inhibitor alone (approximately 85% of initial viability), despite a sustained period of AdoHcyase inhibition in the cells incubated with dAdo and dCyd.

**DISCUSSION**

Various types of human lymphoid cells, with differing sensitivity to dAdo in the presence of ADA inhibitors, have been studied to explore the possible contribution of dAdo-mediated AdoHcyase inhibition (1, 11) to dAdo-induced growth inhibition and cytotoxicity. In the human EBV-transformed B-lymphoblast line WIL-2, AdoHcyase was 94% inactivated in 80 min by 5 μM dAdo in the presence of EHNA, and when this inactivation was interrupted, AdoHcyase activity recovered at a rate of 0.07 hr⁻¹ (12). The inactivation of AdoHcyase by dAdo has been
proposed as a potential mechanism for dAdo cytotoxicity independent of dATP, the elevation of which correlates closely with sensitivity to dAdo in lymphoid cells. Support for this mechanism came from the finding of depressed AdoHcyase activities in erythrocytes from patients with congenital ADA deficiency (14) and in lymphocytes from patients with acute T-cell lymphoblastic leukemia (13) and chronic B-cell lymphocytic leukemia (18) treated with the ADA inhibitor deoxycoformycin. Furthermore, AdoHcyase-inhibited cells have a decreased capacity for transmethylation reactions, the proposed end result of the accumulation of S-adenosylhomocysteine which follows AdoHcyase inhibition (20). We sought to determine if such a mechanism might account for the toxicity of dAdo in nondividing lymphoid cells and the G1 arrest induced by dAdo in T-lymphoblasts, effects which could not be explained by the allosteric inhibitory effects of dATP on ribonucleotide reductase and DNA synthesis. In 2 types of experiments, no correlation has been shown between AdoHcyase inhibition and dAdo lymphocytotoxicity and growth arrest.

(a) On short-term exposure of dAdo-sensitive cultured human T-lymphoblasts and nondividing PBL and dAdo-resistant EBV-transformed B-lymphoblasts to dAdo in the presence of an ADA inhibitor, there is an inverse relationship between growth-inhibitory concentrations of dAdo and those concentrations which induce 50% inhibition of AdoHcyase. (b) When these cells are studied over the period during which dAdo-induced cytotoxic effects are seen, coinubcation with concentrations of dCyd (which prevent these cytotoxic effects) fails to have any protective effect against AdoHcyase inhibition. Furthermore, on exposure to cytostatic concentrations of dAdo, AdoHcyase activity in T-leukemic lymphoblasts recovers to 90% of normal levels by 24 hr, despite a continued cytostatic effect.

The protective effect of dCyd against dAdo lymphotoxicity cannot be explained by replenishment of a lowered dCTP pool, since this pool is not depleted by cytostatic dAdo concentrations in T-lymphoblasts (6). At low dAdo concentrations, phosphorylation to dAMP is performed by dAdo-dCyd kinase which appears to be a single enzyme (3, 21). Hence, dCyd appears to compete with dAdo for its initial phosphorylation and prevent potentially toxic elevations in dATP pools. Protection is not complete at higher dAdo concentrations at which adenosine kinase also accepts dAdo as a substrate (3). Competition between dCyd for dAdo transport is also possible, as a single facilitated diffusion system may transport all nucleosides across the cell membrane (28, 29).

The experiments reported here suggest that inhibition of AdoHcyase is unlikely to play a major role in dAdo-induced lymphotoxicity in T-lymphoblasts or PBL. The mechanism of dAdo toxicity in nondividing human lymphoid cells or T-lymphoblasts is not clear but correlates with elevation of the dATP pool. We are investigating the possibility that dATP may interfere with ATP-dependent processes in resting PBL or T-lymphoblasts which are not involved in DNA replication.

REFERENCES

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