Basis for Resistance to 3-Deazaaristeromycin, an Inhibitor of S-Adenosylhomocysteine Hydrolase, in Human B-Lymphoblasts*

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Clones resistant to 3-deazaaristeromycin, a potent inhibitor of S-adenosylhomocysteine hydrolase, were selected from a nucleoside kinase-deficient derivative of the WIL-2 human B-lymphoblastoid cell line. The resistant clones took up 3-deazaaristeromycin and showed no alteration in the level of S-adenosylhomocysteine hydrolase activity or in the sensitivity of the enzyme to inhibition by 3-deazaaristeromycin. However, they displayed markedly elevated S-adenosylmethionine content during growth in 3-deazaaristeromycin and, following prolonged selection, enhanced export of S-adenosylhomocysteine. As a result they maintained a high ratio of S-adenosylmethionine to S-adenosylhomocysteine and thus were resistant to the inhibition of S-adenosylmethionine turnover and transmethylation caused by 3-deazaaristeromycin. Expanded S-adenosylmethionine pools declined over several weeks of nonselective growth, suggesting a metabolic adaptation rather than a mutational mechanism.

No alterations in S-adenosylmethionine synthetase activity were found in the 3-deazaaristeromycin-resistant clones. S-Adenosylhomocysteine export appeared to be carrier-mediated and largely unidirectional. The resistant clones showed a 5-fold increased rate of S-adenosylhomocysteine export compared with parental cells, but a similar \( K_m \) for intracellular S-adenosylhomocysteine, estimated to be \(~1 \text{mM}\). Our results highlight the opposing effects of S-adenosylmethionine and S-adenosylhomocysteine on transmethylation and suggest that the ability to elevate S-adenosylmethionine pools and to export S-adenosylhomocysteine may provide for homeostatic control of transmethylation in lymphoid cells when S-adenosylhomocysteine hydrolase activity is limited.

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*S-Adenosylmethionine (AdoMet)\(^1\) as substrate and S-adenosyl-L-homocysteine (AdoHcy) as a product and competitive inhibitor of all transmethylation reactions are potentially regulators of methylation-dependent cellular processes (1, 2).

**AdoHcyase is inhibited by Ado (7–9) and inactivated by dAdo (10–12). Thus, impaired catabolism of AdoHcy may contribute to the failure of lymphoid development caused by inherited deficiency of adenosine deaminase (EC 3.5.4.4). AdoHcyase activity is significantly decreased in erythrocytes and marrow cells of patients with adenosine deaminase deficiency (13–15), and AdoHcy accumulates to levels capable of inhibiting methylation reactions in the lymphoblasts of leukemia patients treated with the adenosine deaminase inhibitor 2'-deoxycoformycin (16, 17). Studies of AdoMet utilization in vitro suggest that lymphocytes may have a greater requirement for transmethylation than other cell types (18), so that diminished AdoHcyase activity might be particularly detrimental to immune function.

In order to dissociate their AdoHcyase-dependent and nucleotide-dependent actions, we have previously studied Ado and dAdo in adenosine deaminase-inhibited mutants of the WIL-2 human B-lymphoblastoid cell line that lack Ado kinase and deoxycytidine kinase (9, 11, 19). To more specifically investigate the consequences of selective AdoHcyase inhibition, we have isolated clones of Ado kinase-deoxycytidine kinase-deficient WIL-2 cells resistant to 3-deazaaristeromycin (C \(^{3}\) Ari), a potent inhibitor of AdoHcyase that undergoes only limited deamination and phosphorylation (20, 21). C \(^{3}\) Ari-resistant clones exhibited an unusual, long-term adaptation in AdoMet metabolism, and enhanced cellular export of AdoHcy.

These findings highlight the opposing roles of AdoMet and AdoHcy as regulators of transmethylation and suggest a basis for homeostatic control of this essential process in lymphoid cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Selection of C \(^{3}\) Ari-resistant Cells**—The WIL-2 human splenic B-lymphoblastoid cell line (22) and its Ado kinase- and deoxycytidine kinase-deficient derivative have been described (19). The Ado kinase- and deoxycytidine kinase- double mutant is referred to as K2B in this report. Cells were cultured under 5% \( \text{CO}_2 \) in air at 37 °C in RPMI 1640 medium (Gibco) supplemented with nonessential amino acids, 1 mM sodium pyruvate, and 10% horse serum (Gibco). Cells were maintained at \(~1 \times 10^6 \text{cells/ml} \) by dilution with fresh medium every 2–3 days. Cultures were checked periodically.
for mycoplasma by determining the ability of culture medium to catalyse the conversion of adenosine to adenine in the presence of an inhibitor of adenosine deaminase (23). Isolation of cells resistant to C'Ari is described under "Results."  

Effect of Nucleosides on Cell Growth—Cells (initially 10^6 cells) in log phase growth for 3-5 days in the absence (control) or the presence of nucleoside analogs, and cell titer was determined daily with a Coulter Model ZBI particle counter (Coulter Electronics, Hialeah, FL). After subtracting the initial cell density from the final cell density (day 3 or 4), "relative growth" was calculated as the ratio of the final cell density in wells containing nucleosides to this value for the control. Doubling time was estimated from plots of linear growth (semilog plots of cell density versus time) over the course of these experiments.  

Enzyme Assays—0.4-1 x 10^6 cells in log phase growth were harvested by centrifugation, washed with cold phosphate-buffered saline (0.01 M NaCl, pH 7.0, 0.15 M NaClO containing 1 mg/ml bovine serum albumin, and the cell pellet was stored at -70°C. Cell pellets were lysed by three cycles of freezing and thawing in 0.1 ml of 10 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA. Extracts were centrifuged for 2-3 min in a microcentrifuge at 4°C, and supernatants were run over a small Sephadex G-25 column equilibrated with 25 mM Tris-HCl, pH 7.4, 15 mM KCl, 1 mM dithiothreitol, 1 mM EDTA. AdoHcyase was assayed as described, except that adenine deaminase was inhibited with 1 μM Z'-deoxycoformycin instead of ethylen-9-(2-hydroxy-3-nonyl)adenine (10). AdoMet synthetase was measured as described (24) except that 80 mM Tris-HCl, pH 7.4, was the buffer. The nucleophile phosphorylase was assayed as described (25). Protein was determined (26) with bovine serum albumin as standard.  

Assessment of Nucleoside Analogs as Substrates for AdoHcyase—Nucleosides (200 μM) were incubated with 6 μg of purified human placental AdoHcyase (27) and 2.5 mM homocysteine, in 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 1 μM Z'-deoxycoformycin at 37°C for 60 min. Reactions were terminated by addition of 2 mM HClO4 to give 0.25 M, and placed on ice. Extracts were centrifuged and supernatants analyzed by high pressure liquid chromatography (HPLC) as described. HPLC analysis of AdoMet, AdoHcy, and Polyamines—Cell pellets were extracted on ice with 0.25 M HClO4. Medium samples were extracted on ice by addition of 2 M HClO4 to 0.25 M. Extracts were centrifuged at 4°C in a microcentrifuge and analyzed directly by HPLC as described (28) with minor modifications. Briefly, 50-100 μl of acid extract was fractionated on a C18 Novapak column (Waters Associates) at ambient temperature with a complex gradient elution at a flow rate of 1 ml/min, using the following mobile phases: mobile phase A = 100 mM NaH2PO4, pH 2.55, 8 mM heptanesulfonic acid, 0.2 M NaEDTA, 10 μl of acetonitrile was added to 1 liter of this buffer to give the final composition. Mobile phase B = 200 mM NaH2PO4, pH 3.10, 11.4 mM heptanesulfonic acid; 700 μl of this buffer was mixed with 300 μl of acetonitrile to give the final composition. From the initial conditions (80% A, 15% B) a linear gradient was run in 15 min to 58% A and 42% B, followed by a second linear gradient to 25% A, 75% B. The final conditions were maintained for 3 min, followed by the initial conditions over 3 min. The column was then reequilibrated for 9 min before the next injection. For some analyses a C18 guard column (Waters Associates) was substituted and the flow rate was adjusted to 1.5 ml/min. The column effluent was monitored at 260 and 280 nm and adenine-containing compounds were quantified by comparing their peak areas to those of external standards. The AdoMet standard (Sigma) was purified by chromatography on SP-Sephadex (29). Polyamines were measured as described (26).  

For determination of the specific radioactivity, as well as the concentration, of AdoMet, AdoHcy, and polyamines, and methionine in cells incubated with [3H]methionine (see below) a portion of the HPLC effluent was mixed continuously with scintillation fluid (Floscint II, Radiomatic Instruments, or Scintiverse LC, Fisher Scientific) and radioactivity associated with each component was measured with an on-line scintillation counter (Floscint/ONE/Beta, Radiomatic Instruments). [3H]Methionine in medium samples was converted enzymatically to [3H]AdoMet with purified AdoMet synthetase from mouse liver (kindly provided by Dr. N. M. Kredich, Duke University) (18). Briefly, reaction mixtures contained 0.1 M Tris-HCl, pH 8.5, 0.1 M KCl, 10 mM MgCl2, 1 mM dithiothreitol, 2.5 mM ATP, 10^-2 units of AdoMet synthetase, and 0.1 ml of medium in a final volume of 0.2 ml. After incubation for 80 min at 37°C, the mixtures were extracted with acid and the specific radioactivity of the AdoMet produced was determined by radiochemical HPLC analysis as described.  

Turnover and Utilization of Cellular AdoMet—Cells were suspended at 0.8-3 x 10^6/ml in RPMI 1640 medium containing 10% horse serum, 20 mM HEPES, pH 7.4, and adjusted to 10 μM methionine. After equilibration at 37°C for 4 hr, L-[3,4,5^-3H]methionine (57 mCi/mmol; Research Products International) was added to give 10 μCi/ml (final methionine concentration was 20-30 μM including contribution from serum). Incubation was continued at 37°C. Aliquots were removed immediately (t = 0) and at various times (see Fig. 7, "Results"), chilled in an isopropanol-ice slurry (−15°C) for 20 s, and centrifuged for 45 s. Medium and cell suspensions were filtered onto GF/C filters (Whatman). After three washes with 10% trichloroacetic acid followed by 95% ethanol, filters were dried and counted in a toluene-based scintillation fluid. Aliquots of medium were also counted directly to follow methionine uptake by cells.  

Previous studies of AdoMet turnover and consumption in WIL-2 lymphoblasts demonstrated that 80% equilibration between extracellular and intracellular methionine occurs in ~1 min, and the rate of methionine turnover is >10-fold the rate of AdoMet turnover (18). In estimating AdoMet turnover we have made the simplifying assumption that this equilibration is a "buffered" system so that outside the cells, the rate of turning over of radiolabeled methionine. The present studies used [3H]methionine and a final concentration of methionine in medium of 20-30 μM, while German et al. (18) added tracer amounts of [3H]methionine to medium containing 100 μM methionine. The conditions used in the present studies permit a reliable estimation of AdoMet turnover since: 1) WIL-2 maintains a normal doubling time for 3 days as little as 10 μM methionine; 2) >75% of medium methionine remained at the end of labeling; 3) AdoMet concentration in cells did not change during labeling; and 4) the specific radioactivity of AdoMet approached a constant value, i.e. equilibrium was established between the AdoMet pool and extracellular methionine.  

Based on the above considerations (for a more detailed discussion, see Ref. 18), the relationship between radio-label in AdoMet and extracellular methionine is given by  

SA AdoMet = SA methionine − e^-kt[SA AdoMet = 0 at t = 0] where SA AdoMet and SA methionine are, respectively, the specific radioactivities of AdoMet and extracellular methionine, t is time, and k is the fractional turnover rate of the AdoMet pool. As noted previously (18), the specific radioactivity of AdoMet in lymphoblasts sometimes plateaus at 70-85% of the specific radioactivity of extracellular methionine. This approach is not used, but the AdoMet pool is then substituted for the experimentally measured specific radioactivity of extracellular methionine, yielding  

SA AdoMet = SA AdoMet_{new} = e^{-kt} where SA AdoMet_{new} are, respectively, the specific radioactivities of the AdoMet pool at time t and at a time when the
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specific radioactivity of AdoMet has reached a constant value. AdoMet consumption was estimated by multiplying the turnover rate \((k)\) and the Adomet pool size (the mean of five to seven measurements of AdoMet content made at the beginning, during, and end of each experiment).

RESULTS

Characteristics of Adenosine Analogs and 3-Deazaaristeromycin-resistant (C3Arir) Clones—C3Arir and the related compounds 3-aziridinocytosine, aristeromycin (Ari) and 3-deazaadenosine (C3Ado) all inhibit AdoHcyase, but differ in their ability to undergo deamination, phosphorylation, and AdoHcyase-catalyzed condensation with L-homocysteine. We examined these aspects of metabolism in relation to their growth inhibitory effects on WIL-2 lymphoblasts. Ari and C3Ado were not deaminated by adenosine deaminase, while Ari was deaminated slowly (data not shown), as reported previously (30). C3Arir was converted to an S-homocysteinyl derivative by human placental AdoHcyase at a rate of 0.037 pmol/min/mg, about twice the rate with Ari and ~1% the rates with Ado and C3Ado (nucleosides were 200 \(\mu\)M and homocysteine of 2.5 mM). S-Nucleosidylhomocysteine derivatives were detectable in HPLC analyses of extracts of lymphoblasts incubated with Ari and C3Arir, but never exceeded 10% of AdoHcy in these cells. Metabolism by adenosine deaminase and AdoHcyase does not appreciably influence the cytotoxicity of Ari and C3Arir. Thus, their toxicity was not enhanced by either an adenosine deaminase inhibitor or homocysteine thiolactone, both of which potentiate Ado toxicity to WIL-2 (9); the toxicity of C3Ado, an active substrate for AdoHcyase but not adenosine deaminase, was potentiated by homocysteine thiolactone but not 2'-deoxycoformycin (Table I).

Both C3Arir and Ari inhibited the growth of WIL-2 by 50% at concentrations between 1 and 10 \(\mu\)M; the EC50 of C3Ado was 30-45 \(\mu\)M (Fig. 1, Table I). C3Arir and C3Ado were about equally toxic to WIL-2 and K2B, its Ado kinase- and deoxycytidine kinase-deficient derivative, while Ari was more toxic to WIL-2 than K2B (Fig. 1). These results suggest a phosphorylation-dependent component of Ari toxicity in the wild type cells, while growth inhibition by C3Arir and C3Ado depends on neither Ado kinase nor deoxycytidine kinase. We decided to use K2B for isolating C3Arir-resistant cells in order to minimize the possibility of nucleotide-dependent selection pressure and to permit the eventual use of resistant cells for studies of nucleotide-independent effects of analogs that might undergo adenosine kinase- or deoxycytidine kinase-dependent phosphorylation in WIL-2.

Selection for C3Arir resistance was carried out by exposing K2B over several months to increasing concentrations of C3Arir in mass culture, followed by cloning in 75 \(\mu\)M C3Arir. Several resistant clones able to grow in the presence of 350 \(\mu\)M C3Arir were then maintained in medium containing 100 \(\mu\)M C3Arir. A clone designated C3Arir"1 (clone 1) was selected

| TABLE I | Toxicity of Ado analogs in WIL-2 |
|---------|---------------------------------|
| ~1 \times 10^6 cells/ml were grown in medium containing various concentrations of Ado analogs in the absence or presence of 5 \(\mu\)M 2'-deoxycoformycin (dCF) or 100 \(\mu\)M homocysteine thiolactone (HTL), as indicated. Cell densities were determined at 0, 24, 48, and 72 h. EC50 was determined graphically from plots of relative growth calculated at 72 h as described under “Experimental Procedures.” |
| Additions | EC50 (\(\mu\)M) |
|-----------|----------------|
| None      | 1.2            | 1.5           | 1.6 |
| Ari       | 1.4            | 0.7           |     |
| C3Ado     | 42             | 5.5           |     |

Fig. 1. Toxicity of adenosine analogs in B lymphoblasts. Cells (~1 \times 10^6 cells/ml) were incubated in medium containing the indicated concentration of nucleoside. After 3-4 days, cell number was determined with a Coulter particle counter on aliquots of each culture. Relative growth is expressed as described under “Experimental Procedures.” Data presented are from a single representative determination for each cell line and nucleoside. Experiments were repeated at least three times. A, C3Arir; B, Ari; C, C3Ado.
for study; data are also presented for C3’Ari’-9 (clone 9) with similar properties. Both C3’Ari’ clones were ∼25-fold less sensitive to C’Ari than parental cells (Fig. 1, Table II); the doubling time of the resistant clones was prolonged by <2-fold in the presence of ∼500 μM C’Ari (Fig. 2). Compared with K2B, the C3’Ari’ clones were also ∼25-fold less sensitive to Ari, and ∼3-fold less sensitive to Ado and to adenine arabinoside, an inactivator of AdoHcyase (10); neither clone was appreciably resistant to C’Ado (Fig. 1, Table II). These growth characteristics have been stable over a 3-year period of observation and have been documented in cells maintained under nonselective conditions for from 10 to 50 doublings.

Biochemical Correlates of the 3-Deazaaristeromycin-resistant Phenotype—The AdoMet and AdoHcy pools of K2B under standard growth conditions (basal levels) were 214 ± 60 and 17.4 ± 5.4 pmol/10^6 cells, respectively (mean ± S.D. of 21 separate determinations, Fig. 3). The ratio of intracellular AdoMet to AdoHcy for K2B was 12.5 ± 4.8. A fall in the AdoMet/AdoHcy ratio, or methylation index (MI), correlates with the degree of inhibition of methylation reactions, and cell growth, in lymphoblasts exposed to various conditions that elevate AdoHcy (7,9,31). Our initial studies in the period after cloning suggested that the C3’Ari’ phenotype was due to an ability to maintain very high levels of AdoMet, and hence a near normal MI, rather than to a diminished inhibitory effect of C3’Ari’ on AdoHcy hydrolysis.

In five experiments carried out after removal to drug-free medium for periods from several days to several months, exposure to C’Ari (5–125 μM) caused a similar dose- and time-dependent accumulation of AdoHcy in K2B and the C3’Ari’ clones. The AdoMet content and MI of the clones remained 2–4.5-fold higher than the parent during reexposure to C’Ari. A representative experiment involving a 4-h incubation with 5–80 μM C’Ari is shown in Fig. 4. At the highest drug concentration, AdoHcy in K2B and clone 1 increased from basal levels of 9–12 to 230 and 210 pmol/10^6 cells, respectively; AdoMet in K2B increased from 200 to 315 pmol/10^6 cells, compared with an increase from 515 to 745 pmol/10^6 cells in clone 1. In this experiment, the MI of K2B fell from −16 in the absence, to 4.5 in the presence of 5 μM C’Ari (roughly the EC50), and to 1.4 with 80 μM C’Ari; in clone 1 the MI fell from a basal value of >20 to 3.5 at 80 μM C’Ari.

The level of AdoMet in lymphoblasts appears to increase in response to increases in the level of AdoHcy, but the relationship involves a degree of chronic adaptation. Upon short-term exposure to C’Ari, AdoMet pools in WIL-2 and K2B expanded by 1.2–2-fold, rarely approaching 3-fold, over 1–3 h of exposure to C’Ari, after which AdoMet remained stable or fell slightly over 24–48 h (Fig. 4, Table III, and data not shown). During chronic growth in 100 μM C’Ari AdoMet in the resistant clones rose to much higher levels (Fig. 24, arrow). For example, on one occasion AdoHcy levels in clones 1 and 9, respectively, were 131 and 197 pmol/10^6 cells; their AdoMet levels at the time were 1400 and 1440 pmol/10^6 cells (about one-half the level of ATP), yielding MI values of 10.7 and 7.3. After transfer to drug-free medium, AdoHcy in the C3’Ari’ clones fell to normal within a few hours, but AdoMet and the MI remained 2–4-fold higher than in K2B for several weeks, after which these values were variably elevated and often in the parental range (Fig. 3).

In preliminary experiments we found <2-fold difference in AdoMet synthetase activity, measured at saturating or sub-saturating substrate (methionine, ATP) concentrations, in extracts of K2B, clone 1 and clone 9. There was no difference

| Table II |
| Compounds were evaluated as described in the legend to Table I. 5 μM 2’-deoxycoformycin was included when Ado, dAdo, and adenine arabinoside (Ara-A) were evaluated. 100 μM homocysteine thiolactone (HTL) was included when tested. ND, not determined. |
| K2B | Clone 1 | Clone 9 |
| C’Ari | 5 | >600 | 290 |
| Ari | 3 | 200 | 290 |
| C’Ado | 41 | 50 | 60 |
| Ado | 45 | 160 | ND |
| Ara-A | 130 | 340 | ND |
| dAdo | >600 | >600 | ∼500 |
| C’Ari + HTL | 10 | >600 | 250 |
| C’Ado + HTL | 7 | 5 | 6 |
| Ado + HTL | 4 | 12 | ND |

Fig. 2. Effect of C’Ari on growth. Cells (~1 x 10^6 cells/ml) were incubated in medium containing the indicated concentrations of C’Ari. Data presented are from a single representative determination. Experiments were repeated at least three times. Concentration of C’Ari (μM): 0 (□); 1 (■); 5 (○); 25 (□); 125 (△); 475 (▲). A, WIL-2; B, K2B; C, C3’Ari’ clone 1.
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A clone

B clone

FIG. 3. AdoMet and AdoHcy pools in C'Ari-resistant clones. C'Ari clones 1 and 9 were grown in the presence of increasing concentrations of C'Ari, up to 350 µM for 1–4 months after cloning. They were then transferred to C'Ari-free medium, and the intracellular levels of AdoMet (A) and AdoHcy (B) were determined after nonselective growth for the number of days indicated on the horizontal axis. The arrows to the left of the vertical axes represent the mean of four random determinations of AdoMet (A) or AdoHcy (B) levels in clones growing chronically in 100 µM C'Ari. Lines drawn across graphs represent ±1 standard deviation of the mean AdoMet (A) or AdoHcy (B) pools of unselected K2B cells.

AdoHcy Efflux—After 6–7 months of continuous growth of clone 1 in 100 µM C'Ari, a second basis for resistance to C'Ari became apparent. During chronic growth in drug, clone 1 continued to maintain very high levels of AdoMet. However, upon short-term (up to 48 h) incubation with C'Ari (after growth from several days to several months in drug-free medium) the resistant cells now consistently accumulated less AdoHcy than parental cells. For example, in the experiment presented in Table III, AdoHcy levels in K2B exposed to 5–100 µM C'Ari rose 4–15-fold, while in clone 1 AdoHcy rose to only about one-third of these levels. AdoMet pools in K2B and clone 1 differed by <25% throughout this experiment.

We addressed some possible mechanisms for the diminished ability of C'Ari to elevate intracellular AdoHcy in chronically selected clone 1 cells. Uptake of C'Ari was not grossly altered (Table IV). The level of AdoHcyase activity, and the sensitivity of AdoHcyase to inhibition by C'Ari, were similar in extracts of parent and resistant cells (Table V), and they contained similar amounts of AdoHcyase protein detectable by immunoblot analysis with monoclonal antibodies to placental AdoHcyase (data not shown).

Because these results suggested that AdoHcy hydrolysis should be inhibited to the same degree in the resistant and parental cells, we measured the level of AdoHcy in culture medium of cells exposed to C'Ari. For the experiment described in Table III, the total AdoHcy production (AdoHcy in cells plus AdoHcy in culture medium) was indeed greater for clone 1 than for K2B, but the resistant clone excreted a greater fraction of the AdoHcy at all concentrations of C'Ari (Fig. 5A). Fig. 5B shows that, for this experiment and for another, similar experiment, clone 1 released AdoHcy to the medium at lower intracellular AdoHcy levels than K2B. In experiments not presented we found similar AdoHcy excretion by K2B and WIL-2 (detectable only in the presence of an inhibitor of AdoHcyase). Since AdoHcy efflux was not
examined in experiments conducted immediately after cloning, it is unclear when increased AdoHcy export began to occur, or whether the rate of export increased gradually or as a single-step event in the course of selection.

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**TABLE III**

Effect of C₃Ari on intracellular AdoMet, AdoHcy, and MI

Cells were incubated in medium containing C₃Ari as indicated for 4 and 24 h. The clone 1 cells used in this experiment had been maintained in 100 μM C₃Ari for >6 months but had been cultured in C₃Ari-free medium for 8 days before the experiment.

| C₃Ari in medium | Cellular C₃Ari (pmol/10⁶ cells) | AdoHcyase (nmol/min/mg protein) |
|----------------|---------------------------------|---------------------------------|
|                | K2B                             | Clone 1                         | Clone 2 |
| **Experiment 1** |                                 |                                 |         |
| 20 μM          | 19                              | 17                              | ND      |
| **Experiment 2** |                                 |                                 |         |
| 50 μM          | 47                              | 57                              | 45      |
| **Experiment 3** |                                 |                                 |         |
| 20 sM          | 15                              | 8                               | ND      |
| 80 μM          | 69                              | 73                              | ND      |

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**TABLE IV**

Uptake of C₃Ari by K2B and C₃Ari-resistant cells

3–5 × 10⁶ cells/ml were incubated in medium containing the indicated concentrations of C₃Ari. After 3 or 4 h, cells were harvested, washed and acid-extracted, and C₃Ari was determined by HPLC as described under "Experimental Procedures." ND, not determined.

| C₃Ari in medium | Cellular C₃Ari (pmol/10⁶ cells) | AdoHcyase (nmol/min/mg protein) |
|----------------|---------------------------------|---------------------------------|
|                | K2B                             | Clone 1                         | Clone 2 |
| **Experiment 1** |                                 |                                 |         |
| 4 h            |                                 |                                 |         |
| K2B            | 0.29                            | 0.027                           | 10.5    |
| 5              | 0.40                            | 0.096                           | 4.2     |
| 25             | 0.45                            | 0.220                           | 2.0     |
| 100            | 0.48                            | 0.370                           | 1.3     |
| Clone 1        | 0.37                            | 0.020                           | 18.8    |
| 5              | 0.47                            | 0.038                           | 12.4    |
| 25             | 0.44                            | 0.057                           | 7.5     |
| 100            | 0.50                            | 0.120                           | 4.2     |
| 24 h           |                                 |                                 |         |
| K2B            | 0.22                            | 0.031                           | 7.1     |
| 5              | 0.25                            | 0.103                           | 2.4     |
| 25             | 0.29                            | 0.230                           | 1.3     |
| 100            | 0.28                            | 0.360                           | 0.8     |
| Clone 1        | 0.25                            | 0.016                           | 15.7    |
| 5              | 0.33                            | 0.037                           | 8.9     |
| 25             | 0.35                            | 0.065                           | 5.3     |
| 100            | 0.37                            | 0.090                           | 3.7     |

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**TABLE V**

AdoHcyase activity and sensitivity to inhibition by C₃Ari and Ari

Cell extracts were prepared and AdoHcyase activity was measured as under "Experimental Procedures." Numbers in parentheses represent percent of control activity. ND, not determined.

| Condition | AdoHcyase (nmol/min/mg protein) |
|-----------|---------------------------------|
|           | K2B                             | Clone 1                         | Clone 2 |
| **Experiment 1** |                                 |                                 |         |
| Control (no analog) | 9.35                          | 4.74                            | 7.0     |
| **Experiment 2** |                                 |                                 |         |
| Control     | 6.25 (100)                      | 5.95 (100)                      | 6.85 (100) |
| C₃Ari (10 μM) | 1.76 (28)                      | 1.43 (24)                      | 2.07 (30) |
| C₃Ari (50 μM) | 0.45 (7)                       | 0.39 (7)                       | 0.57 (8) |
| **Experiment 3** |                                 |                                 |         |
| Control     | 1.00 (100)                      | 11.9 (100)                      | ND      |
| Ari (1 μM)  | 1.28 (12.6)                     | 1.68 (14.1)                     | ND      |
| Ari (8.7 μM) | 0.29 (2.9)                     | 0.28 (2.3)                     | ND      |
| Ari (87 μM) | 0.16 (1.6)                     | 0.10 (1.3)                     | ND      |

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FIG. 5. Effect of C₃Ari on intracellular AdoHcy and efflux of AdoHcy. K2B and C₃Ari clone 1 cells (as described in the legend to Table III) were incubated in medium containing 0, 5, 25, or 100 μM C₃Ari for 4 h (A and B) or 24 h (C and D). The bars in A and C show the total amount of AdoHcy produced in the cultures: the solid portion represents intracellular AdoHcy and the open portion AdoHcy in medium. In B and D, these data and data from another similar experiment are replotted to show the relationship between AdoHcy in medium and intracellular AdoHcy. The symbols do not distinguish the two experiments because the results were very similar.

We explored the relationship between the intracellular concentration of AdoHcy and the rate of AdoHcy export (Fig. 6). A range of intracellular AdoHcy concentrations was first established by incubating K2B and clone 1 with an adenosine deaminase inhibitor, various concentrations of Ado, and 200 μM homocysteine thiocyanate. Then the cells were transferred to medium lacking these compounds and containing 200 μM C₃Ari (to limit AdoHcy hydrolysis) and the intracellular and extracellular concentrations of AdoHcy were followed over 30 min (AdoHcy efflux had been shown to be linear for 60 min in preliminary experiments not shown). In both cell lines intracellular concentrations of AdoHcy ranged from near 0.1 to 10 nmol/10⁶ cells after the initial incubation. As intracellular AdoHcy increased, the rate of AdoHcy efflux increased and then saturated in both cell types, indicating a carrier-mediated process. Half-maximal rates of efflux occurred at intracellular AdoHcy concentrations of approximately 2 nmol/10⁶ cells for clone 1 and 1.4 nmol/10⁶ cells for K2B. Based on the volume of WIL-2 cells (18), these values yield an estimate of the Kᵣ for AdoHcy efflux of 0.7 and 1.2 mM AdoHcy. The maximal rate of efflux for clone 1 was roughly 5-fold greater than for K2B.

An indication of the efficiency of AdoHcy export by clone 1 was the finding that in the presence of 5 μM 2'-deoxycoformycin, but in the absence of an AdoHcyase inhibitor, clone 1 excreted >1 nmol of AdoHcy/10⁶ cells in 24 h, while AdoHcy excretion by K2B was not detectable (<0.09 nmol of AdoHcy/10⁶ cells) (Table VI). Under these conditions in adenosine deaminase-inhibited, adenosine kinase-deficient cells, a low level of AdoHcy accumulation results from the effect of exogenously produced Ado on the equilibrium of the reversible AdoHcyase reaction (11). Enhanced AdoHcy efflux also enabled clone 1 to maintain lower levels of AdoHcy than K2B when the cells were incubated with Ari and C₃Ado. Clone 1 accumulated less C₃AdoHcy than C₃Ado and excreted more
by incubating suspensions of K2B (squares) and C3Arir clone 1 adenosine deaminase), 200 p~5 points (picomoles/106 cells/min) is plotted against the intracellular
we compared the effects of C3Ari on AdoMet turnover and PM utilization in cells labeled with the AdoMet precursor ~-[3,4-
AdoHcy. The rate of AdoHcy excretion was determined from the net increase in medium AdoHcy during incubation of the resuspended
to inhibit hydrolysis of AdoHcy. Immediately following resuspension (t0) and after 10 and 30 min of further incubation at 37 °C, 0.55-ml samples were centrifuged through an oil cushion and extracts of cells and medium were prepared and analyzed for AdoHcy (“Experimental Procedures”). The rate of AdoHcy efflux was determined from the concentration of AdoHcy at
inhibited cells (amount at 10 or 30 min minus the amount present at
samples were centrifuged through an oil cushion and extracts of cells
were established such that total uptake of methionine was linear for 2 h (~1 nmol/106 cells/h), and <20% of medium
methionine was used by 3 h; levels of AdoMet and AdoHcy were not altered, and incorporation of methionine into protein was linear throughout the labeling period. We measured the approach to constant specific activity of the AdoMet pool and from these data estimated the fractional AdoMet turnover rate and rate of total AdoMet utilization to be 0.045-0.081 min–1 and 7.7–9.0 pmol/106 cells/min, respectively, in reasonable agreement with values of 0.076 min–1 and 7.4 pmol/106 cells/min reported by German et al. (18), who used [35S]methionine (18). Use of L-[3,4,14C]methionine enabled us to show directly that synthesis of polyamines represented ~20% of AdoMet consumption (data not presented). This agrees with the estimate of German et al. (18) that ~80% of AdoMet utilization is for transmethylation, based on their measurement of the difference between homocysteine production and total AdoMet utilization by WIL-2 (18). C3Ari (100 μM) did

In preliminary studies with WIL-2, experimental conditions were established such that total uptake of methionine was linear for 2 h (~1 nmol/106 cells/h), and <20% of medium methionine was used by 3 h; levels of AdoMet and AdoHcy were not altered, and incorporation of methionine into protein was linear throughout the labeling period. We measured the approach to constant specific activity of the AdoMet pool and from these data estimated the fractional AdoMet turnover rate and rate of total AdoMet utilization to be 0.045-0.081 min–1 and 7.7–9.0 pmol/106 cells/min, respectively, in reasonable agreement with values of 0.076 min–1 and 7.4 pmol/106 cells/min reported by German et al. (18), who used [35S]methionine (18). Use of L-[3,4,14C]methionine enabled us to show directly that synthesis of polyamines represented ~20% of AdoMet consumption (data not presented). This agrees with the estimate of German et al. (18) that ~80% of AdoMet utilization is for transmethylation, based on their measurement of the difference between homocysteine production and total AdoMet utilization by WIL-2 (18). C3Ari (100 μM) did

**TABLE VI**

| Treatment | K2B | Clone 1 |
|-----------|-----|---------|
| Cells     | Medium | Cells | Medium |
| No additions | 0.022 | <0.09 | 0.012 | <0.09 |
| 5 μM dCF* | 0.043 | <0.09 | 0.026 | 1.2 |
| 100 μM C3Ari | 0.39 | 2.9 | 0.11 | 6.4 |
| 100 μM C3Ado | 0.033 (0.12) | 0.55 (2.3) | 0.020 (0.046) | 2.0 (3.1) |

*C3AdoHcy than K2B (Table VI).

Effect of C3Ari on AdoMet Turnover and Utilization—The ability to maintain higher MI despite inhibition of AdoHcy hydrolysis should permit C3Ari cells to carry out AdoMet-mediated transmethylation at concentrations of C3Ari that would inhibit these reactions in K2B. To test this hypothesis, we compared the effects of C3Ari on AdoMet turnover and utilization in cells labeled with the AdoMet precursor L-[3,4,14C]methionine.

FIG. 6. Export of AdoHcy as a function of intracellular AdoHcy. Intracellular AdoHcy was increased to varying levels (9) by incubating suspensions of K2B (squares) and C3Arir clone 1 (circles) (4–5 × 106 cells/ml) for 90 min at 37 °C in medium containing 5 μM erythro-9-(2-hydroxy-3-nonyl)adenine (a reversible inhibitor of adenosine deaminase), 200 μM homocysteine thiolactone, and 0–200 μM Ado. Aliquots (9 ml) of these suspensions were then centrifuged and the medium aspirated completely; the cells were then resuspended in 2 ml of medium lacking Ado, homocysteine thiolactone, and erythro-9-(2-hydroxy-3-nonyl)adenine, but containing 200 μM C3Ari to inhibit hydrolysis of AdoHcy. Immediately following resuspension (t0) and after 10 and 30 min of further incubation at 37 °C, 0.55-ml samples were centrifuged through an oil cushion and extracts of cells and medium were prepared and analyzed for AdoHcy (“Experimental Procedures”). The rate of AdoHcy efflux was determined from the net increase in medium AdoHcy during incubation of the resuspended cells (amount at 10 or 30 min minus the amount present at t0). The average of the AdoHcy export rate measurements for the two time points (picomoles/106 cells/min) is plotted against the intracellular concentration of AdoHcy at t0. The possibility that cell lysis contributed significantly to AdoHcy in medium was excluded since <2% of cellular purine nucleoside phosphorylase activity was released to the medium during the 30-min incubations for K2B and clone 1 cells that had been exposed to 0 or 100 μM Ado.

FIG. 7. Effect of C3Ari on AdoMet turnover. K2B (A) and C3Ari clone 1 (B) (1.6–2.1 × 106 cells/ml) were incubated at 37 °C for 4 h in medium containing no additions (control) (C), 240 μM AdoMet (B), 100 μM C3Ari (C), or both 240 μM AdoMet and 100 μM C3Ari (D), as indicated. At this point (time 0) L-[3,4,14C]methionine was added, and at the times indicated aliquots of incubations were centrifuged through oil, cells and medium extracted, and the specific activity of cellular AdoHcy and medium methionine determined as described under “Experimental Procedures.” AdoMet specific activity is plotted as a percentage of the specific activity of extracellular methionine.
not affect the rates of protein or polyamine synthesis by more than 8%. Therefore, it can be concluded that inhibition of AdoMet utilization in cells exposed to C'Ari is due entirely to inhibition of transmethylation, with negligible effect on polyamine synthesis.2

A representative study of AdoMet turnover in K2B and clone 1 is shown in Fig. 7. In the untreated cells, AdoMet was 1.8-fold higher in clone 1 than K2B, 295 versus 160 pmol/10^6 cells. Fractional turnover rates of AdoMet pools were 0.049 min^-1 and 0.027 min^-1 for K2B and clone 1, so that basal rates of AdoMet consumption for K2B and clone 1 were nearly identical, 7.4 and 7.3 pmol/min/10^6 cells, respectively. A 4-h incubation with 100 μM C'Ari increased intracellular AdoHcy from basal levels of 35 pmol/10^6 cells to 297 (K2B) and 82 (clone 1) pmol/10^6 cells; AdoMet increased by 2-fold to 326 pmol/10^6 cells in K2B, and by 37% to 404 pmol/10^6 cells in clone 1. AdoMet and AdoHcy levels were constant during the subsequent turnover studies. C'Ari did not affect the plateau value for specific activity of AdoMet pool (Fig. 7, A and B). Under these conditions consumption of AdoMet by K2B fell to 4.0 pmol/min/10^6 cells (Fig. 7A), and in clone 1 to 5.7 pmol/min/10^6 cells (Fig. 7B). Based on the considerations outlined in the previous paragraph, we estimate that transmethylation, with negligible effect on polyamine synthesis.

DISCUSSION

Resistance to C'Ari could have arisen from defective uptake of C'Ari, increased expression of AdoHcyase or a decrease in its sensitivity to inhibition by C'Ari, or from some other mechanism that alleviates toxic consequences of AdoHcyase inhibition. Considerable evidence suggests that inhibition of methyltransferases, some of which have K_i values for AdoHcy in the submicromolar range, is a major cause of AdoHcy toxicity (2, 5, 7, 9, 11, 33). However, 100 μM C'Ari apparently inhibited the growth of a mouse macrophage cell line by preventing the homocysteine-dependent regeneration of tetrahydrofolate from 5-methyltetrahydrofolate, leading to a block in de novo purine nucleotide and thymidylate synthesis (21). AdoHcy at high concentration may affect cAMP (34) and phosphoinositide metabolism (35), though the relationship of these effects to AdoHcy toxicity is unclear. We found neither a defect in C'Ari uptake nor any change in the expression or properties of AdoHcyase in C'Ari-resistant cells. WIL-2 lymphoblasts do not remethylate homocysteine (18), and in studies not presented neither homocysteine nor hypoxanthine and thymidine protected WIL-2 or K2B from C'Ari toxicity.2

We have not explored the effects of C'Ari on cAMP or phosphoinositide metabolism.

C'Ari-resistant clones displayed two properties, expanded AdoMet pools and an enhanced ability to excrete AdoHcy, that implicate inhibition of methylation as the basis for C'Ari (and AdoHcy) toxicity. Each of these metabolic alterations had the effect of maintaining a high ratio of AdoMet to AdoHcy despite a block in AdoHcy hydrolysis, diminishing the inhibition of overall cellular transmethylation by C'Ari. These results are consistent with the previous finding of an inverse correlation between the AdoMet:AdoHcy ratio (MI) and degree of inhibition of specific DNA and RNA methylation reactions in human lymphoid cells (9, 16).

AdoMet pool expansion during chronic growth in C'Ari can be partly attributed to decreased AdoMet consumption caused by AdoHcy-mediated inhibition of transmethylation (Fig. 7). Despite the rapid return of AdoHcy to normal, AdoMet levels remained elevated for several weeks after removal to C'Ari-free medium. In the absence of ongoing inhibition of AdoMet consumption this suggests that increased AdoMet synthesis may also have contributed to pool expansion. We found no increase in AdoMet synthetase activity in extracts of chronically selected cells, and neither C'Ari nor AdoHcy stimulated AdoMet synthetase or diminished its sensitivity to product inhibition by AdoHcy. Nevertheless, the enzyme from human lymphoid cells displays very complex kinetics, with some 60 terms in the reaction equation (24). The activity of the enzyme in intact cells may not be accurately reflected under conditions of in vitro assay. Stable AdoMet elevation has previously been observed in Ado-resistant mouse lymphoma cells, which were cross-resistant to C'Ari (33). Minor differences in the level and properties of AdoMet synthetase in the variant cells were reported. AdoMet pool expansion and elevated AdoMet synthetase activity were found in hamster cell lines selected for resistance to cycloheximide, a methionine analog that is both an inducer and an inhibitor of AdoMet synthetase in those cells (36, 37).

The attenuation of AdoMet pool expansion during nonselective growth of C'Ari clones in the present study argues against a simple mutational mechanism. It may be worth considering how prolonged accumulation of AdoHcy during C'Ari selection might cause an adaptation affecting AdoMet synthetase activity in intact cells, which could persist for a time after resumption of normal culture conditions. Several isozymes of AdoMet synthetase, with differing kinetic and physical properties, have been characterized (24, 38–42). The purified enzyme from human lymphocytic leukemia cells is tetrameric with an αβ2 or αα'B2 structure (24). The presence of distinct subunits and electrophoretic heterogeneity of one subunit type raised the possibility of posttranslational modification related to a regulatory function. Conceivably, prolonged elevation of AdoHcy could lead to hypomethylation, and hence activation, of a gene coding for a new AdoMet synthetase isozyme with a higher basal rate of activity. Alternatively, submaximal activity of AdoMet synthetase might normally be maintained by AdoMet-mediated methylation of amino acid residues of a regulatory subunit. Chronic AdoHcy accumulation could lead to subunit undermethylation, thus increasing basal AdoMet synthetase activity. Elimination of C'Ari would lead to a gradual remethylation of the isozyme gene or AdoMet synthetase, eventually extinguishing the elevation in AdoMet.

AdoHcy is normally hydrolyzed efficiently, and some cells depend upon the homocysteine derived from the AdoHcyase reaction for the maintenance of normal levels of methionine and folate. Thus, the need for a system to export intact
AdoHcy from the cell is not obvious. However, previous reports have demonstrated that AdoHcy was released from hepatocytes and some other cells when marked intracellular AdoHcy accumulation was induced (43, 44). We observed that plasma AdoHcy rose from undetectable to 1–2 PM in a patient who was treated with the combination of 2’-deoxycoformycin and adenine arabinoside (16). This was associated with a 12-fold increase in lymphoblast AdoHcy concentration. It has been suggested that AdoHcy export may provide a mechanism for escaping the toxic effects of AdoHcy (45). The present studies directly support this possibility. AdoHcy was released from K2B and WIL-2 when intracellular AdoHcy rose to very high levels, but efflux was insufficient to protect these cells from AdoHcy toxicity. The C3Ari’ clones, on the other hand, excreted AdoHcy at much lower cellular concentrations, effectively enough to limit AdoHcy accumulation and toxicity when AdoHcyase was severely inhibited.

The nature of the AdoHcy transport mechanism is poorly understood at present. AdoHcy does not easily enter cells (46, 47). Our studies indicate this as well: high concentrations of extracellular AdoHcy had no effect on intracellular AdoMet or it may represent a previously undefined transporter. C3Ari’ clones should provide a useful system for further biochemical and genetic analysis of the AdoHcy export system, as well as for study of the regulation of AdoMet pools and the homeostatic control of transmethylation in lymphoid cells.

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