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Analytical DNA Flow Cytometric Analysis of Deoxyadenosine Toxicity in Cultured Human Leukemic Lymphoblasts

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SUMMARY

Human T leukemic lymphocytes are highly susceptible to growth inhibition by dAdo. We have investigated this phenomenon using analytical DNA flow cytometry. By using (a) bromodeoxyuridine quenching of Hoechst 33342 fluorescence and (b) the drug ICRF-159, a selective G_2 – M-blocking agent, we show that dAdo causes a G_1 block in cultured T leukemic cells and that cells in the S phase exposed to dAdo are able to complete that S phase, pass through G_2 + M, and return to the G_1 phase. Centrifugal elutriation was used to enrich cells for various phases of the cell cycles. dAdo caused elevation of the dATP pool to a similar extent in G_1, S, and G_2 – M-enriched cell fractions, but did not cause a fall in the dCTP pool. These findings indicate that dAdo induces a G_1 block via elevation of dATP pools, apparently independently of inhibition of ribonucleotide reductase.

INTRODUCTION

Developments in flow cytometry have proved invaluable in determining the cell cycle specificity of anticancer drugs, in particular antimetabolites. Generally, these studies have used a variety of fluorescent dyes that bind stoichiometrically to DNA to measure drug-induced changes in distributions of DNA content. This type of analysis, however, provides only a static measurement of cellular DNA content. It does not show, in a temporal sense, whether a cell is actively replicating its DNA or is capable of division. The fluorescent benzimidazole compound, Hoechst 33342, which specifically binds to thymine bases in DNA, offers unique advantages in studying cell cycle-specific phenomena. When cells are grown in media containing BrdUrd,[1] this is incorporated into DNA as a thymine substitute, and the newly synthesized DNA strands containing bromouracil bases, which are larger than thymidine, will not bind Hoechst 33342. Cells undergoing a complete replication cycle in the presence of BrdUrd will therefore have their DNA-tagged benzimidazole fluorescence reduced to 50% of original intensity. Such cells will contain a thymine-containing parental DNA strand staining with Hoechst 33342 and a non-stainable bromouracil-containing daughter strand (1). This technique, therefore, appears to offer a valuable method by which to study antimetabolite drugs capable of interfering with the progression of cells through specific phases of the cell cycle. In particular, it offers an unambiguous method to study the fate of cells in a phase of the cell cycle beyond the apparent site of a metabolic block. Similarly, the drug ICRF-159 (Razoxane), which induced a selective G_2 – M block via prophase inhibition, can be used to complement such studies (2).

The availability of drugs inhibiting adenosine deaminase (EC 3.5.4.4), and in particular deoxycoformycin, used in the treatment of certain lymphocytic leukemias, has focused interest on the mechanism of lymphocytotoxicity of dAdo (3). As well, the association of inborn adenosine deaminase deficiency with human combined immunodeficiency disease has further emphasized the importance of nucleoside metabolism and toxicity in the human lymphoid system (4). The sensitivity of cultured human T leukemic lymphocytes to growth inhibition by dAdo and the insensitivity of EBV-transformed B cell lines have provided model systems in which to investigate dAdo metabolism and toxicity (5, 6).

It is generally believed that the selective toxicity of dAdo for T cells reflects the ability of these cells to phosphorylate dAdo and to accumulate high intracellular concentrations of dATP. We have studied dAdo-induced toxicity in cultured T leukemic and EBV-transformed B cell lines and have demonstrated that exposure to dAdo results in an accumulation of T cells in G_1. By contrast, B cell growth inhibition was associated with an S phase block (7). Similarly, we have shown that dAdo (in the presence of adenosine deaminase inhibition) kills resting (i.e., G_0) lymphocytes from human peripheral blood. This G_0 toxicity was associated with elevated dATP pool levels (8).

By using BrdUrd quenching of Hoechst 33342 fluorescence and the G_2 – M blocking agent, ICRF-159, in flow cytometry, we now show that dAdo specifically causes a G_1 block in cultured T leukemic cells and that cells in the S phase exposed to dAdo are able to complete that S

[1] The abbreviations used are: BrdUrd, bromodeoxyuridine; EBV, Epstein-Barr virus; EHNA, *erythro*-9-(3,2-hydroxynonyl)adenine; dNTP, deoxyribonucleoside triphosphate; C.V., coefficient of variation.

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phase, pass through G_2 + M, and return to the G_1 phase. In contrast, the dAdo-induced block in B cells is in the S phase. This finding indicates that, apart from the differing ability of cultured T and B cells to phosphorylate dAdo and accumulate dATP, these cells clearly differ in their response to an elevated dATP pool. The exact biochemical mechanisms responsible for these cell cycle-specific phenomena remain to be determined.

MATERIALS AND METHODS

Cell lines. The cultured human leukemic T lymphocyte lines CCRF-CEM, HSB, and HPB-ALL were provided by Dr. J. Minowada (Roswell Park Memorial Institute, Buffalo, N. Y.). The origin and characteristics of these lines have been summarized previously by Minowada (9). RD-G and JP are EBV-transformed B-lymphocyte lines provided by Dr. I. Jack (Royal Children's Hospital, Melbourne, Australia). The murine T cell lymphoma lines S49, EL-41, and WEHI-7 were kindly given by Dr. A. Harris (Walter & Eliza Hall Institute, Melbourne).

The cell lines were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum, with the exception of the S49 line, which was grown in Dulbucco's modified Eagle's medium with 10% fetal calf serum. The human lines had doubling times of 24–30 hr; the murine lines, 12 hr. Cells were studied in the exponential phase of growth, and all dAdo experiments were carried out in the presence of 5 μM EHNA, an inhibitor of adenosine deaminase.

Cell counts were performed manually using a hemocytometer, and viable cells were distinguished from dead cells by trypan blue exclusion.

Chemicals. dAdo, BrdUrd, and dCyd were purchased from Calbiochem (La Jolla, Calif.). EHNA was a gift from Dr. C. Nichol, Burroughs Wellcome Ltd. (Research Triangle Park, N. C.). Hoescht 33342 was obtained from Calbiochem-Behring, Australia, and ICRF-159 was from Imperial Chemical Industries, United Kingdom. ^{3}H-Labeled dNTPs (dTTP, dCTP, dGTP, and dATP) were purchased from the Radiochemical Centre, (Amersham United Kingdom). Unlabeled dTTP, dCTP, dATP, and dGTP were obtained from Sigma Chemical Company (St. Louis, Mo.). *Micrococcus luteus* DNA polymerase and templates for the DNA polymerase assay for dNTPs, poly(dA-dT), and poly(dI-dC) were purchased from Miles Laboratories (Elkhart, Ind.).

Flow cytometry. The cellular DNA content was measured by techniques and staining procedures previously described (1, 10). Calculations of percentages of cells in various phases of the cell cycle and of coefficients of variation of the G_1 DNA peak were made using a curve-fitting method of analysis (11).

Growth of cells in the presence of BrdUrd. The cell lines were grown in a wide range of BrdUrd concentrations (1–50 μM) for 72 hr. Above 5 μM BrdUrd, the growth of both the human CCRF-CEM line and the mouse S49 line were inhibited. At 10 μM, growth was 80% of control, and at 50 μM 10% and 0% of control, respectively. The growth inhibition was prevented by dCyd. The EBV-transformed B lymphocyte lines were less sensitive to BrdUrd growth inhibition. At 50 μM BrdUrd, growth was 75% of control.

The concentrations of BrdUrd used in these studies, therefore, were 5 μM for the T cell lines and 20 μM for the B cell lines. These concentrations did not inhibit cell growth over 72 hr. dCyd was not used in these studies, as it competes with dAdo for phosphorylation (7).

Cell separation by centrifugal elutriation. Centrifugal elutriation, which separates cells according to size and thus position in the cell cycle, was used as previously described (12). CCRF-CEM cells (3×10^8) were suspended in medium (RPMI 1640 plus 5% fetal calf serum, 10^6 cells/ml) and introduced into the chamber of a Beckman JE-6 elutriator rotor (flow rate 4 ml/min, 1500 rpm, room temperature). Four 100-ml fractions were collected at flow rates of 8, 10, 12, and 14 ml/min, with the rotor speed kept constant. The position in the cell cycle of cells in each fraction was determined by flow cytometric analysis of cell DNA content as above. Prior to elutriation, cells were incubated with dAdo (3 μM) and EHNA (5 μM) for 4 hr, as in the above studies.

dNTP pool assay in elutriated cells. Following elutriation centrifugation, the fractions collected above were harvested and the dNTP pools were measured by the DNA polymerase assay, using 5×10^6 cells for each determination (13, 14).

RESULTS

Hoechst 33342 flow cytometry of dAdo-induced cytostasis in CCRF-CEM cells. CCRF-CEM cells were incubated with 3 μM dAdo (in the presence of 5 μM EHNA). This concentration of dAdo is cytostatic, and the number of viable cells remains constant over 24 hr. After this time, cells recommence to grow, with a normal doubling time at least to 96 hr. DNA analysis, using the Hoechst stain, was carried out at various time intervals (4–24 hr) following incubation with dAdo. At 4 hr after addition of dAdo, disappearance of cells with an early S phase content of DNA was apparent. After 8 hr, the majority of cells were in G_1, with residual late S phase and G_2 – M cells. By 16–24 hr, the G_1 cells and only a small percentage of G_2 – M cells remained (Fig. 1). Similar findings were observed with other human T cell lines (HSB and HPB-ALL).

We interpreted these data to indicate that cytostatic concentrations of dAdo create an immediate block in G_1 but do not prevent cells in the S phase from replicating DNA for that cycle. Identical findings were made using ethidium/mithramycin stain (7).

Influence of BrdUrd on Hoechst 33342 fluorescence distribution of exponentially growing CCRF-CEM cells. DNA fluorescence distribution was measured, at various times after addition of BrdUrd (5 μM) to the culture

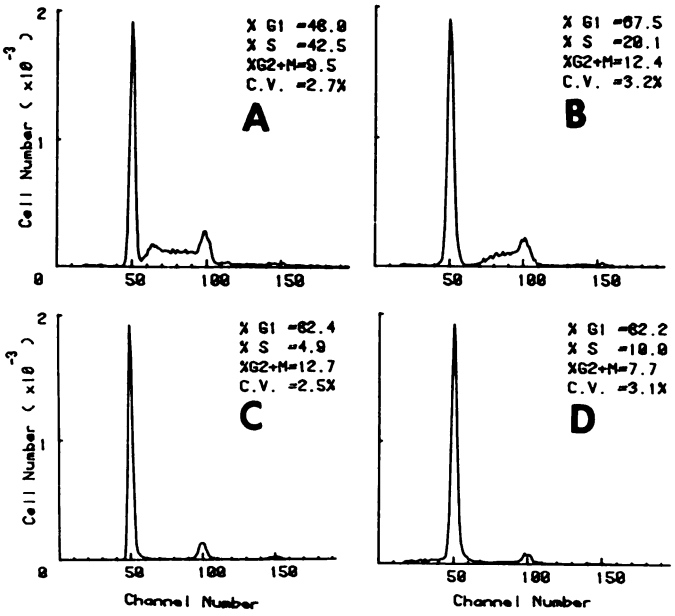

FIG. 1. *Changes in the DNA distribution of CCRF-CEM cells with time of exposure to 3 μM EHNA)*

Cells were stained with Hoechst 33342 dye. Channel numbers correspond to relative fluorescence intensity (DNA content). Numbers of cells are shown on the ordinate. A, 4 hr; B, 8 hr; C, 16 hr; and D, 24 hr. Zero-time cell distributions were as follows: G_1, 38.8%; S, 50.0%; and G_2 + M, 11.2%. The C.V. was 2.7%.

medium, through one cell cycle time. Cells that undergo a complete replication cycle in the presence of BrdUrd should have their G_1 peak Hoechst 33342 fluorescence reduced to 50% of original intensity. The "new" G_1 peak should appear in channel 25 rather than channel 50. In initial experiments, BrdUrd quenching of Hoechst 33342 fluorescence was less than expected; i.e., the new G_1 peak moved to channel 35 rather than channel 25. This appeared to reflect failure of BrdUrd to compete completely with thymidine (produced via endogenous dTTP synthesis) for incorporation into DNA. After increasing the concentration of BrdUrd to 50 μM and co-incubating with 50 μM dCyd (to prevent growth inhibition), the new G_1 peak shifted to channel 25.

It also was possible to inhibit endogenous dTTP synthesis by growing the cells in HAB medium (100 μM hypoxanthine, 5 nM methotrexate, and 5 μM BrdUrd). Growth was not inhibited, and after Hoechst 33342 staining, the new G_1 peak shifted to channel 25 (i.e., fluorescence was reduced by 50%) (Fig. 2B).

BrdUrd-quenched Hoechst 33342 flow cytometry of dAdo growth inhibition in CCRF-CEM cells. Studies identical with those described above were carried out with the addition of BrdUrd (5 μM) to the culture medium at the same time as dAdo. At 24 hr, cells with fluorescence in channels 25 and above had appeared, whereas the "original" G_1 cells remained in channel 50. The broad distribution of the new G_1 peak (i.e., channels 25 and above) is explained by the variation in the extent of BrdUrd incorporation that would be expected in cells originally in early to late S phase (Fig. 2C). A progressive disappearance of cells from early to late S phase was not seen in the presence of BrdUrd, but rather an over-all lowering of S phase fluorescence. As cells progress through the S phase, their Hoechst 33342 fluorescence will not increase, owing to BrdUrd incorporation, and they will not appear to progress through the S phase. However, their passage from G_2 – M and into G_1 is detected by the subsequent appearance of cells with an apparent less than G_1 DNA content. This confirms the interpretation above that dAdo, at this cytotostatic concentration, has induced a G_1 block and that those cells in the S phase are capable of continuing that round of replication, passing through G_2M, and reaccumulating in G_1. These findings were confirmed with the T cell lines HSB and HPB-ALL.

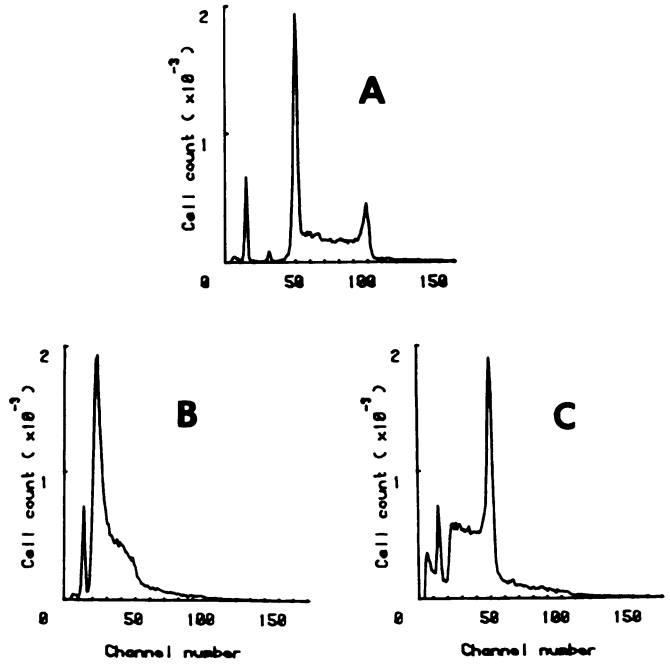

FIG. 2. *Changes in the DNA distribution of CCRF-CEM cells after 24-hr exposure to 3 μM dAdo (in the presence of 5 μM EHNA)*

Cells were cultured in RPM1 1640 with 10% fetal calf serum to which was added 5 nM methotrexate and 100 μM hypoxanthine. BrdUrd (5 μM) was used to quench Hoechst 33342 dye fluorescence. Channel numbers correspond to relative fluorescence intensity (DNA content). Numbers of cells are shown on the ordinate. A. Zero-time control. The G_1 peak is in channel 50, and the G_2 – M peak is channel 100; i.e., it contains twice as much DNA as the G_1 peak. B. cells stained with Hoechst 33342 dye after 24 hr of growth in BrdUrd. DNA fluorescence was reduced by 50% owing to incorporation of BrdUrd into newly replicated DNA, and thus the new G_1 appears in channel 25. C. Cells stained with Hoechst 33342 dye after 24 hr of growth in BrdUrd and dAdo. The cells originally in G_1 (i.e., channel 50) did not change their fluorescence because of the dAdo-induced G_1 block, but those cells originally in the S phase incorporated BrdUrd, completed S, and passed through G_2 – M, and thus appeared to have a lower fluorescence; i.e., they appear in channels below 50.

Hoechst 33342 flow cytometry of dAdo-induced cell killing. DNA flow cytometry studies also were carried out using a dose of dAdo (17.5 μM) capable of killing CCRF-CEM cells. Studies were carried out with and without BdUrd quenching. Using Hoechst 33342 staining alone, DNA profiles at 4–24 hr after addition of dAdo indicated the lethal arrest of growth to be non-cell cycle-specific; i.e., all phases of the cycle were affected. There was a small increase in the proportion of cells in G_1 but also an apparent block in the S phase. The appearance of cells with an apparent content of DNA less than G_1 (channels 20–49) is characteristic of dead cells undergoing lysis (Fig. 3).

Addition of BdUrd to the medium made little difference to the Hoechst 33342 fluorescent profiles, indicating lack of incorporation of BdUrd into DNA. This was interpreted to indicate both a G_1 block and inhibition of DNA replication at all levels of the S phase by high-dose dAdo.

ICRF-159-induced G_2 – M block in CCRF-CEM cells. The drug ICRF-159 (Razoxane), a dioxopiperazinyl, induces a selective G_2 – M block via prophase inhibition (2). This phenomenon was exploited in the study of the G_1 block induced by dAdo in the cultured human leukemic cell lines. Analysis of the DNA content of CCRF-CEM cells exposed to 400 μM ICRF-159 demonstrated that, with time (0–24 hr), there was a progressive accumulation of cells with a DNA content equivalent to that of the G_2 – M phase of the cell cycle (Fig. 4). Cells grown in the presence of both ICRF-159 and dAdo maintained a relatively constant proportion of cells with G_1 DNA and a progressive decrease in cells with an S content. The proportion of cells with a G_2 – M content increased (Fig. 5), with some accumulation of late S phase cells. Similar findings were made with the other T cell lines (HSB and HPB-ALL). These findings complement the

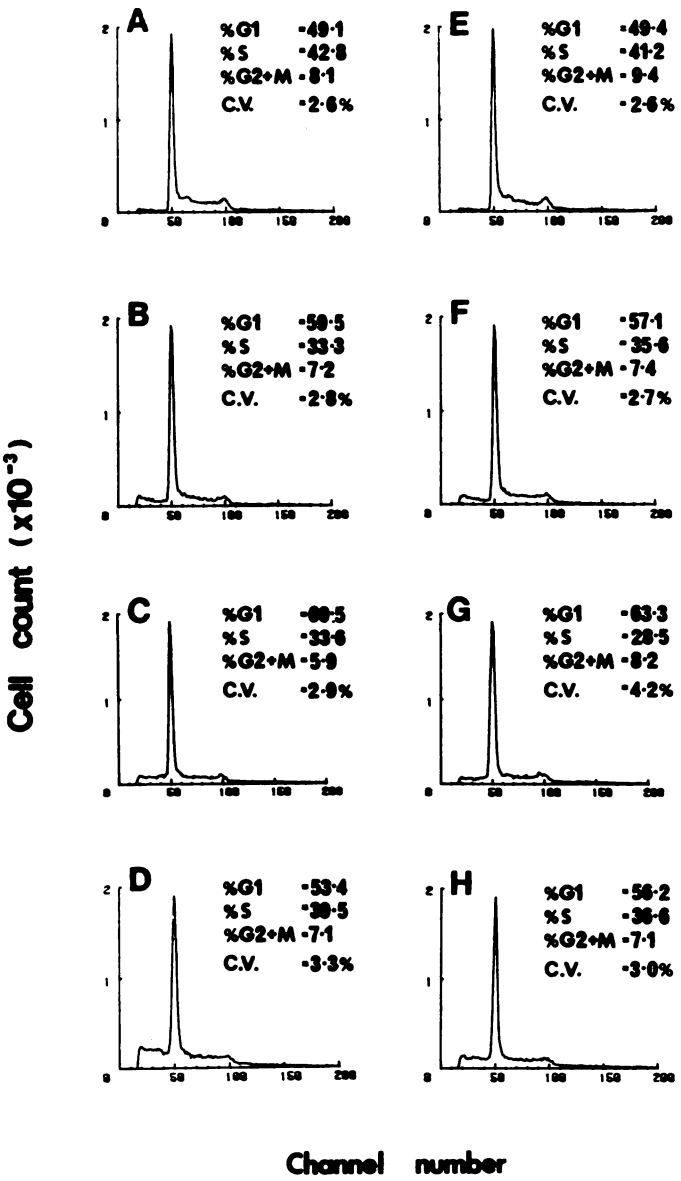

FIG. 3. *Changes in the DNA distribution of CCRF-CEM cells with time of exposure to 17.5 μM dAdo (in the presence of 5 μM EHNA), with and without 5 μM BrdUrd quenching of Hoechst 33342 dye fluorescene*

Channel numbers correspond to relative fluorescence intensity (DNA content). Numbers of cells are shown on the ordinate. A, 4 hr; B, 8 hr; C, 16 hr; and D, 24 hr. E, 4 hr (+ BrdUrd); F, 8 hr (+BrdUrd); G, 16 hr (+ BrdUrd); and H, 24 hr (+BrdUrd).

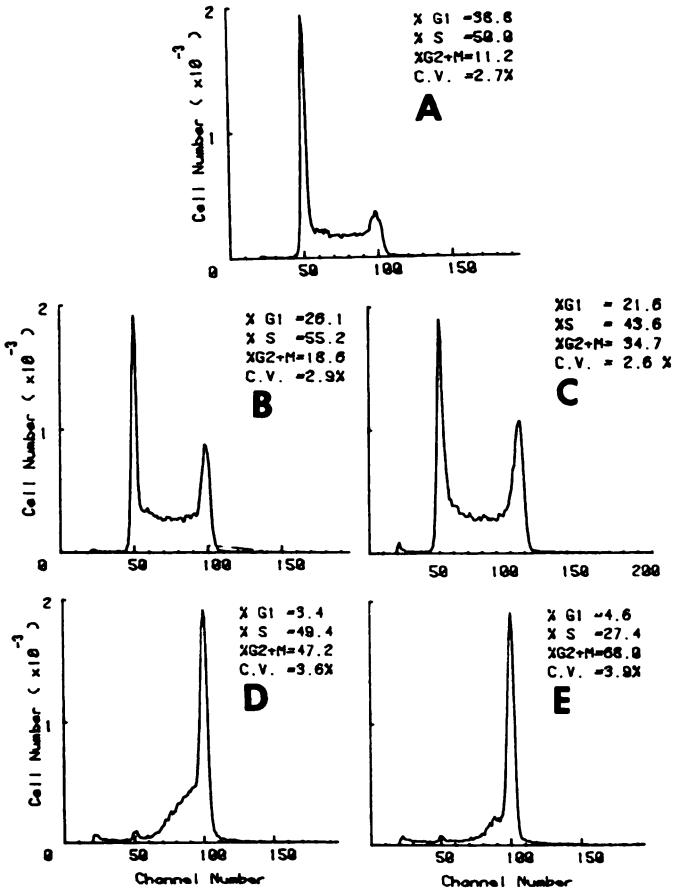

FIG. 4. *Changes in the DNA distribution of CCRF-CEM cells with time of exposure to ICRF-159 (400 μM)*

Cells were stained with Hoechst 33342 dye. Channel numbers correspond to relative fluorescence intensity (DNA content). Numbers of cells are shown on the ordinate. A, control; B, 4 hr; C, 8 hr; D, 16 hr; E, 24 hr.

studies with BrdUrd quenching of Hoechst 33342 fluorescence, confirming further that dAdo induces a G_1 block and that cells in the S phase exposed to dAdo continue to replicate their DNA and to enter $G_2 - M$.

Relationship of CCRF-CEM cell cycle phase and dATP pool changes following exposure to dAdo. A relative enrichment of cells in various phases of the cell cycle was obtained by centrifugal elutriation (Fig. 6). It was possible to obtain an almost pure population of G_1 cells and cells enriched for the S phase (but not free of G_1 cells). In the dAdo-treated cells, the dATP pools were elevated 4-fold, independent of their position in the cell cycle, as compared with cells not treated with dAdo (Table 1). This experiment was performed on three separate occasions with similar results. The variations (± standard deviations) in four replicate determinations of dNTP estimations were as follows: dATP ± 18%, dCTP ± 18%, dGTP ± 15%, and dTTP ± 37%. Similar results were seen when cells were fractionated by elutriation and then incubated with dAdo (data not shown). This dATP pool elevation, occurring equally in both G_1 and S phases, indicates that DNA replication can proceed despite the dATP pool elevation in that phase. The dCTP pool was not lowered after dAdo treatment.

Mechanism of dAdo-induced inhibition of cultured murine lymphoma T cell lines. Cultured malignant murine T cell lines are also highly sensitive to dAdo (15). The site of block in the cell cycle induced by dAdo (in the presence of EHNA) was studied in the S49 T cell lymphoma line. A range of dAdo concentrations was used, so that both cytostatic (15 μM) and cytocidal (25 μM) concentrations were studied (in the presence of 5 μM EHNA). dAdo induced a G_1 block at both cytocidal and cytostatic concentrations. These findings were confirmed in two additional murine T cell lines, EL-41 and WEH1-7 (data not shown).

The S49 cells that we studied required higher concentrations of dAdo to induce both cytostasis and cell killing

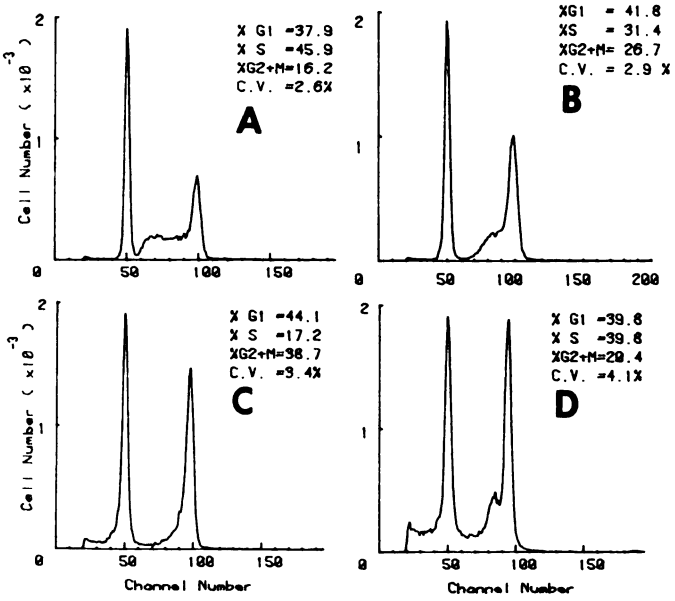

FIG. 5. *Changes in the DNA distribution of CCRF-CEM cells with time of exposure to dAdo (3 μM) (in the presence of 5 μM EHNA) and ICRF-159 (400 μM)*

Cells were stained with Hoechst 33342 dye. Channel numbers correspond to relative fluorescence intensity (DNA content). Numbers of cells are shown on the ordinate. A, 4 hr; B, 8 hr; C, 16 hr; D, 24 hr.

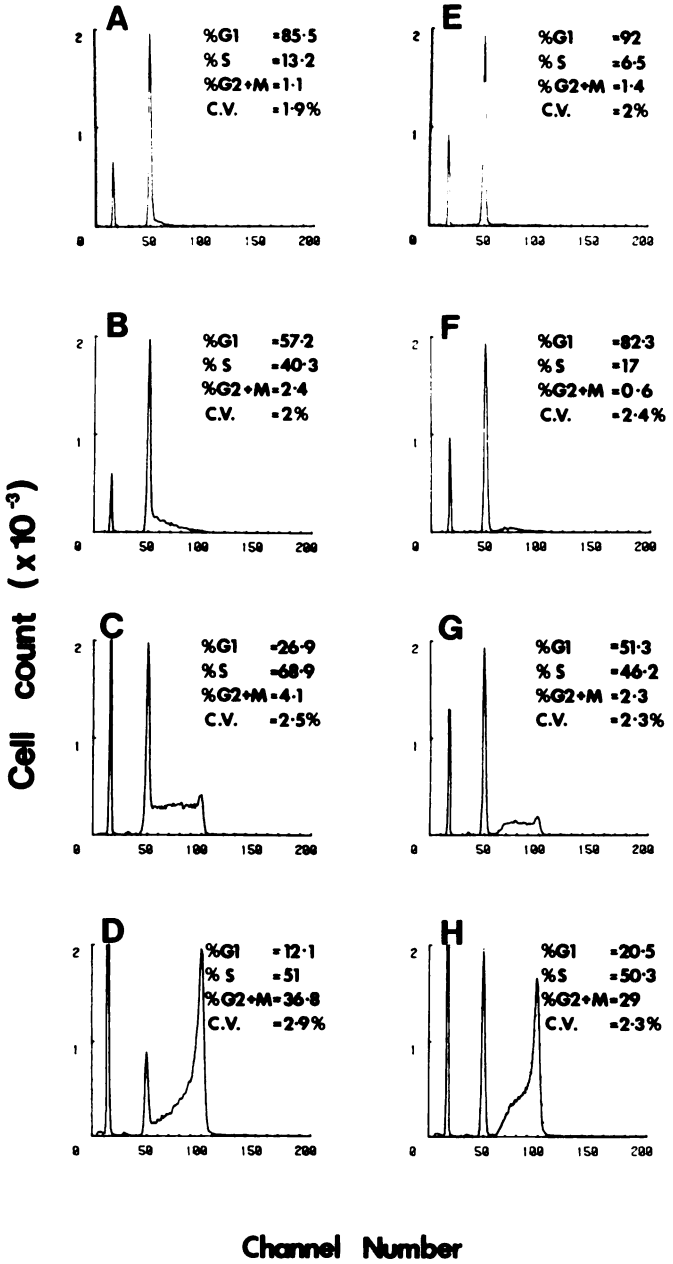

FIG. 6. *DNA distribution of CCRF-CEM cells following centrifugal elutriation*

Cells were stained with Hoechst 33342 dye. Channel numbers correspond to relative fluorescence intensity (DNA content). Numbers of cells are shown on the ordinate. A–D, fractions 1, 2, 3, and 4, respectively, of CCRF-CEM cells not exposed to dAdo plus EHNA; E–H, fractions 1, 2, 3, and 4, respectively, of CCRF-CEM cells incubated with dAdo (3 μM) plus EHNA (5 μM) for 4 hr prior to fractionation by elutriation.

than that reported by Ullman *et al.* (15). However, their studies were performed with the use of cells cultured in heat-inactivated horse serum, which has undetectable levels of adenosine deaminase. The G_1 block apparently induced by dAdo in the S49 line was further investigated by BrdUrd suppression of Hoechst 33342 fluorescence. Findings similar to those seen with the human T cell lines were seen; i.e., the progressive appearance of cells with fluorescence shifted to a new G_1 peak in channel 40 (data not shown). This confirmed that, in these murine lines also, dAdo had induced a Gi block and those cells in the S phase were able to complete that round of DNA replication, pass through G_2 – M, and reaccumulate in G_1.

Mechanism of dAdo-induced inhibition of human cultured EBV B cell lines. At 400 μM, dAdo (in the presence of 5 μM EHNA) had a cytostatic effect on the growth of the RD-G line. DNA profiles at 24 hr showed accumulation of cells in the early S phase (Fig. 7). When these cells were grown in the presence of ECRF-159 alone for 24 hr, there also was a progressive accumulation of cells in the G_2 – M phase of the cell cycle (Fig. 7B). In the presence of both ICR-159 and dAdo, cells in the G_2 – M phase failed to accumulate to the same extent, providing further evidence of an S phase block induced by dAdo in these cells (Fig. 7D). Similarly, when these cells were grown with dAdo and BrdUrd (20 μM), there was no shift of the G_1 peak to a lower channel (data not shown). This indicates that this B cell line was blocked by dAdo in the S phase and could not incorporate BrdUrd into DNA. These findings were confirmed in another EBV-transformed B cell line (JP).

DISCUSSION

Flow cytometric studies of dAdo-induced cytostasis in cultured human and murine T malignant cell lines, using BdUrd quenching of Hoechst 33342 fluorescence, unequivocally demonstrate that dAdo induces a G_1 block. The intriguing finding is that those cells in the S phase, at the time of exposure to dAdo, are capable of completing that S phase. This was further confirmed by studies using ICRF-159 demonstrating an accumulation of cells in G_2 – M. The experiments with BrdUrd quenching demonstrate further that cells in the S phase, having

TABLE 1

Effect of dAdo on cultured acute lymphoblastic leukemia cell (CCRF-CEM) deoxyribonucleotide triphosphate pools

Cells were incubated with dAdo (3 μM) and EHNA (5 μM) for 4 hr and then enriched for phases of the cell cycle by centrifugal elutriation as described under Materials and Methods. The percentage of cells in G_1 was determined by flow cytometry (Fig. 6).

Fraction	Cells in G_1	Deoxyribonucleotide triphosphate pool			
		dATP	dCTP	dGTP	dTTP
	%	*pmol/10⁶ cells*			
Untreated cells					
1	86	22	10	12	34
2	57	30	18	11	60
3	27	17	17	13	43
4	12	35	28	24	71
dAdo-treated cells					
1	92	82	8	10	35
2	82	123	15	11	44
3	51	87	19	9	53
4	20	94	29	21	64

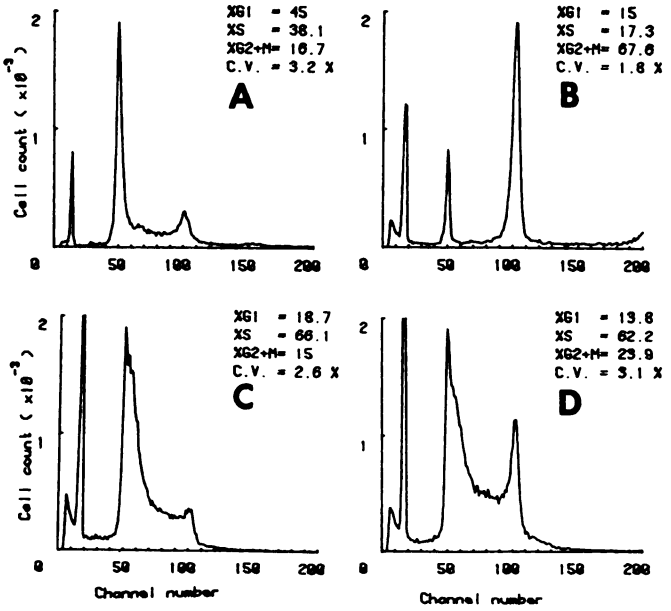

FIG. 7. *Changes in the DNA distribution of the EBV-transformed B cell line RD-G cells after exposure to 400 μM dAdo (in the presence of 5 μM EHNA) for 24 hr*

Channel numbers correspond to relative fluorescence intensity (DNA content). Numbers of cells are shown on the ordinate. A, control; B, plus 400 μM ICRF-159; C, 400 μM dAdo; D, 400 μM dAdo + 400 μM ICRF-159.

completed the S phase, are capable of passing through the G_2 − M phase of the cell cycle and re-entering G_1.

It has been considered previously that high dATP pools allosterically inhibit ribonucleotide reductase, lowering the concentration of other dNTPs with consequent cessation of DNA replication (4, 5, 6, 15). The biochemical mechanism by which dAdo induces this G_1 block in malignant T cell lines is not clear, but apparently it is independent of ribonucleotide reductase inhibition. We have previously shown (8) that (a) incubation of dAdo with CCRF-CEM lines does not induce a reduction of the dCTP pool, despite elevation of the dATP pool; (b) dCyd protection against dAdo toxicity in these cells is explained by competition of dCyd with dAdo for phosphorylation by dAdo kinase; (c) hydroxyurea, an inhibitor of ribonucleotide reductase, induces an S phase block in these cells; and (d) dAdo (in the presence of EHNA) is capable of killing resting, untransformed human peripheral blood lymphocytes. We have shown (7) that cytocidal concentrations of dAdo (e.g., 17.5 μM) cause a non-cell cycle-dependent arrest. In a previous study (7), we demonstrated this to be accompanied by very high increases in the dATP pool, with decreases in the dGTP, dCTP, and dTTP pools. The exact mechanism of cell killing in this situation is not clear; however, ATP pools were shown to be depleted (7).

It is important to note that Ullman *et al.* (15) isolated a mutant S49 line containing a ribonucleotide reductase enzyme that was insensitive to feedback inhibition by dNTPs. The sensitivities of this mutant cell line to inhibition by thymidine and deoxyguanosine were 20- and 10-fold less, respectively, than those of wild-type cells. However, this mutant cell line was only 5-fold less sensitive to dAdo; i.e., the inhibitory 50% dose was increased from 5 to 25 μM in the mutant line. This observation indicates that another mechanism of dAdo toxicity, apart from ribonucleotide reductase inhibition, must be operating in these cells to explain their growth inhibition at the relatively low concentration of 25 μM dAdo.

An alternative mechanism for dAdo-induced growth inhibition, i.e., dAdo-induced inactivation of *S*-adenosylhomocysteine hydrolase, has been demonstrated in EBV-transformed B cell lines (16, 17). This does not appear to explain the dAdo-induced G_1 block in T cell lines, since (a) the dAdo-induced block in B cell lines occurs in the S phase; (b) considerably higher levels of dAdo are required to inhibit B cell lines; and (c) the dAdo G_1 block in T cell lines is prevented by dCyd, which prevents the dATP pool elevation but does not prevent inhibition by *S*-adenosylhomocysteine hydrolase (18). We will describe elsewhere further evidence that this G_1 block in human T cells is nucleotide-dependent. CCRF-CEM cells mutant for adenosine kinase, deoxycytidine kinase, or both, require higher concentrations of dAdo to elevate their dATP pools than do wild-type cells. However, flow cytometry studies indicate that a G_1 block occurs in parallel with cytostasis and dATP pool elevation. Similarly, experiments with lower concentrations of dAdo or incubation of cells for shorter time periods (1–4 hr) with dAdo (3 μM) show a correlation between induction of the G_1 block and elevation of the dATP pool.

There are several possible reasons for the difference in the dAdo-induced block between T and B cells. First, the S phase block in B cells may reflect ribonucleotide reductase inhibition, whereas, in T cells, an expanded dATP pool (from salvage synthesis) may be compartmentalized and not able to inhibit the reductase. The G_1 block in T cells may reflect interference with the "replicase complex" of enzymes, assembly of which occurs late in G_1 in the cytoplasm, but not of the assembled replicase function in the S phase. This multienzyme complex for

metabolic channeling was described by Reddy and Pardee (19).

The ability of the dAdo, presumably via formation of dATP, to induce a G_1 block, raises many interesting questions. It is believed that vital control phenomena for growth regulation occur in the G_1 phase (20). In several cell systems, the existence of a restriction or commitment point in G_1 has been demonstrated (20). On passing this restriction point, a cell must commit itself to initiate DNA synthesis and undergo cell division. The biochemical nature of these restriction points has not been identified but may reflect synthesis of crucial amounts of a specific protein or RNA species (20). The elutriation experiments demonstrate that the dATP pool elevation is not cell cycle-specific. This indicates that S phase replication can be completed despite this 4-fold elevation of the dATP pool.

The high levels of adenosine deaminase in T cells and the immunodeficiency syndrome associated with adenosine deficiency suggest the possibility that dAdo may have a potential role in controlling lymphocyte proliferation via control of such restriction or commitment points in G_0-G_1 lymphocytes. Further investigation of the mechanism of dAdo-induced G_1 block offers the possibility of indentifying the nature of the biochemical processes involved in such control phenomena in human lymphocytes.

REFERENCES

1. Bohmer, R. M. Flow cytometrical cell cycle analysis using the quenching of 33258 Hoechst fluorescence by BUdR incorporation. *Cell Tissue Kinet.* **12**:101–110 (1979).
2. Taylor, I. W., and N. M. Bleehan. Changes in sensitivity to radiation and ICRF-159 during the life of monolayer cultures of the EMT6 tumour line. *Br. J. Cancer* **35**:587–594 (1977).
3. Prentice, H. G., K. Ganeshaguru, K. F. Bradstock, A. H. Goldstone, J. F. Smyth, B. Wonke, G. Janossy, and A. V. Hoffbrand. Remission induction with adenosine deaminase inhibitor 2′-deoxycoformycin in Thy-lymphoblastic leukaemia. *Lancet* **2**:170–172 (1980).
4. Seegmiller, J. E., L. Thompson, H. Bluestein, R. Willis, S. Matsumoto, and D. Carson. Nucleotide and nucleoside metabolism and lymphocyte function, in *Biological Basis of Immunodeficiency* (E. W. Gelfand and H. M. Dosch, eds.). Raven Press, New York, 251–268 (1980).
5. Carson, D. A., J. Kay, S. Matsumoto, J. E. Seegmiller, and L. Thompson. Biochemical basis for the enhanced toxicity of deoxyribonucleosides toward malignant human T cell lines. *Proc. Natl. Acad. Sci. U. S. A.* **76**:2430–2433 (1979).
6. Wortmann, R. L., B. S. Mitchell, N. L. Edwards, and I. H. Fox. Biochemical basis for differential deoxyadenosine toxicity to T and B lymphoblasts: role for 5′-nucleotidase. *Proc. Natl Acad. Sci. U. S. A.* **76**:2434–2437 (1979).
7. Fox, R. M., R. F. Kefford, E. H. Tripp, and I. W. Taylor. G_1 phase arrest of cultured human leukemic T-cells induced by deoxyadenosine. *Cancer Res.* **41**:5141–5150 (1981).
8. Kefford, R. F., and R. M. Fox. Purine deoxynucleoside toxicity in non-dividing human lymphoid cells. *Cancer Res.* **42**:324–330 (1982).
9. Minowada, J. Markers of human leukaemia-lymphoma cell lines reflect haematopoietic cell differentiation, In *Human Lymphocyte Differentiation: Its Application to Cancer* (B. Serrou and C. Rosenfeld, eds.). INSERM Symposium No. 8. Elsevier/North-Holland Biomedical Press, Amsterdam, 337–344 (1978).
10. Taylor, I. W., and B. K. Milthorpe. An evaluation of DNA fluorochromes, staining techniques and analysis for flow cytometry. 1. Unperturbed cell populations. *J. Histochem. Cytochem.* **28**:1224–1232 (1980).
11. Milthorpe, B. K. FMFPAKI: a program package for routine analysis of single parameter flow microfluorimetric data on a low cost mini-computer. *Comput. Biomed. Res.* **13**:417–429 (1980).
12. Piper, A. A., M. H. N. Tattersall, and R. M. Fox. Thymidine metabolising enzymes during the cell cycle of a human lymphocyte line LAZ007, separated by centrifugal elutriation. *Biochim. Biophys. Acta* **633**:400–409 (1980).
13. Solter, A. W., and R. E. Handschumacher. A rapid quantitative determination of deoxyribonucleoside triphosphates based on the enzymatic synthesis of DNA. *Biochim. Biophys. Acta* **174**:585–590 (1969).
14. Fox, R. M., E. H. Tripp, S. K. Piddington, and M. H. N. Tattersall. Ecto-ATPase deficiency in cultured human T and null leukemic lymphocytes: a biochemical basis for thymidine sensitivity. *J. Clin. Invest.* **68**:544–552 (1981).
15. Ullman, B., S. M. Clift, L. J. Gudas, B. B. Lewison, M. A. Wormsted, and D. W. Martin. Alterations in deoxyribonucleotide metabolism in cultured cells with ribonucleotide reductase activities refractory to feedback inhibition by 2′-deoxyadenosine triphosphate. *J. Biol. Chem.* **255**:8308–8314 (1980).
16. Hershfield, M. S., and N. M. Kreditch. *S*-Adenosylhomocysteine hydrolase is an adenosine-binding protein: a target for adenosine toxicity. *Science (Wash. D. C.)* **202**:757–760 (1978).
17. Hershfield, M. S. Apparent suicide inactivation of human lymphoblast *S*-adenosylhomocysteine hydrolase by 2′-deoxyadenosine and adenine arabinoside. *J. Biol. Chem.* **254**:22–25 (1979).
18. Kefford, R. F., M. A. Helmer, and R. M. Fox. *S*-adenosylhomocysteine hydrolase inhibition in deoxyadenosine-treated human T-lymphoblasts and resting peripheral blood lymphocytes. *Cancer Res.* **42**:3822–3827 (1982).
19. Reddy, G. P. V., and A. B. Pardee. Multienzyme complex for metabolic channeling in mammalian DNA replication. *Proc. Natl Acad. Sci. U. S. A.* **77**:3312–3316 (1980).
20. Pardee, A. B., R. Dubrow, J. L. Hamlin, and R. F. Kletzien. Animal cell cycle. *Annu. Rev. Biochem.* **47**:715–750 (1978).

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