Deoxyadenosine Metabolism and Toxicity in Cultured L5178Y Cells[1]

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SUMMARY

The growth of cultured L5178Y cells is inhibited by relatively low concentrations of deoxyadenosine in the presence of deoxycoformycin, an inhibitor of adenosine deaminase. Cell viability is reduced, presumably as a consequence of the induced state of unbalanced growth which is characterized by inhibition in DNA synthesis, accumulation of cells in G_1 or early S phase, a continuation in RNA synthesis, and increasing cell volume. The intracellular concentrations of purine and pyrimidine ribonucleoside phosphates remain essentially unchanged. The significant changes in the intracellular deoxynucleoside triphosphate pools are an increase in deoxyadenosine triphosphate and a decrease in deoxycytidine triphosphate.

INTRODUCTION

dAdo[3] has been reported to inhibit DNA synthesis in Ehrlich ascites tumor cells (6, 14), in chick embryo (11), in Alcaligenes faecalis (9), in HeLa S-3 cells (5), and in a cultured line of bovine liver cells (19). In all of these studies millimolar concentrations of dAdo were required to demonstrate this inhibition due to the ubiquitous, very active adenosine deaminase. In such instances it is always difficult to be sure that the observed effects are solely related to the agent in question and not dependent in part or in whole on a catabolite or a minor impurity. The recent availability of adenosine deaminase inhibitors such as 2'-deoxycoformycin (13, 15, 20) has allowed us to examine the toxicity of much lower concentrations of dAdo on cultured L5178Y cells. A preliminary communication describing the increased toxicity of adenosine and 2'-deoxyadenosine toward L-cells in the presence of adenosine deaminase inhibitors has been reported by Lapi and Cohen (8). It should be pointed out that these adenosine deaminase inhibitors are not without additional metabolic effects so that their judicious use is essential; for instance, we have observed that in Ehrlich cells concentrations of deoxycoformycin in the range of 20 to 50 μM exhibit inhibitory effects on adenosine uptake, on hypoxanthine utilization, and possibly on AMP deaminase (3).

MATERIALS AND METHODS

Purine and pyrimidine nucleosides and propidium iodide were obtained from the Sigma Chemical Co., St. Louis, Mo. The deoxyribonucleoside triphosphates were from P-L Biochemicals, Milwaukee, Wis. [methyl-^{3}H]Thymidine and [5-^{3}H]uridine were supplied by New England Nuclear Canada, Lachine, Quebec, Canada, while [8-^{3}H]dAdo, [8-^{3}H]dATP, and [methyl-^{3}H]dTTP were from Schwarz/Mann, Orangeburg, N. Y. All labeled compounds were purified by thin-layer chromatography on either cellulose or polyethyleneimine cellulose. 2'-Deoxycoformycin [(R)-3-(2-deoxy-β-D-erythropentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-d]-[1,3]diazepin-8-ol)] (NSC 218321) was obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md.

The mouse leukemia L5178Y cells were routinely grown in suspension cultures with Fischer's medium supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, N. Y.). Stock cultures were maintained in logarithmic growth with an average doubling time of 11 hr by subculturing to 2.0×10^4 cells/ml every 48 hr. Cell counts and all volume distributions were determined with a Coulter Model Z_F electronic particle counter equipped with a Coulter Channelyzer II which is a 100-channel pulse-height analyzer. The latter was calibrated by using 9.69-μm-diameter polystyrene microspheres (Coulter Electronics, Inc., Hialeah, Fla.).

Cell viability was measured by a semisolid agar cloning method similar to that described for these cells by Chu and Fischer (1). Cloning mixtures of 3 ml in 100- x 17-mm plastic culture tubes contained Fischer's medium, 10% horse serum, 10% 5-day conditioned medium, 0.14% agar, and sufficient cells to give between 10 and 50 colonies/tube. Colonies were counted after incubation for 7 to 10 days in a humidified, 4% CO_2 atmosphere at 37°. The cloning efficiency of untreated L5178Y cells during these experiments was between 60 and 70%.

The relative DNA content of dAdo-treated L5178Y cells was determined by microfluorometry after propidium iodide staining. Cells were collected by centrifugation and stained for 15 min at a density of 4.0×10^5 cells/ml with propidium iodide (0.05 mg/ml) in 0.1% sodium citrate (7). The cellular fluorescence intensities were recorded with a Bio/Physics Model 4800 A cytofluorograph equipped with a Bio/Physics Model 2100 pulse-height analyzer (Bio/Physics Systems Inc., Mahopac, N. Y.). The output from the pulse-height analyzer was through a standard teletype.

The incorporation of [methyl-³H]thymidine (2 Ci/mmole and [5-³H]uridine (25 Ci/mmole) into the acid-insoluble fraction was measured as previously described (4). [³H]dAdo metabolites were examined by thin-layer chromatography. L5178Y cells at a cell density of approximately 2.5×10^5 cells/ml were exposed to [8-³H]dAdo. Fifteen-ml aliquots of cell cultures were centrifuged, and the cell pellet was extracted with 0.15 ml of 0.4 M perchloric acid. After neutralization with KOH, the acid-soluble fraction was chromatographed on Macherey-Nigal Polygram Cel 300 polyethylene-imine thin layers (Brinkman Instruments Canada Ltd., Toronto, Ontario) in either 1 dimension to separate purine nucleoside mono-, di-, and triphosphates (2), or in 2 dimensions to separate the ribonucleoside phosphates from deoxyribonucleoside phosphates (16). Eastman Kodak unsubstituted cellulose thin layers ((160 μm) were used to purify the ³H-nucleosides and monitor the metabolism of [³H]dAdo in the cell culture media (2). The polyethylene-imine cellulose or unsubstituted cellulose from ³H-containing chromatograms was scraped from the plastic backing and wrapped in filter paper, and the samples were oxidized with a Packard Model 305 oxidizer before liquid scintillation counting. Oxidation of thin-layer samples containing ³H resulted in a 6- to 8-fold increase in counting efficiency.

Concentrations of deoxyribonucleoside triphosphates were measured enzymatically as originally described by Solter and Handschumacher (18) and as modified by Lowe and Grindey (11). The Micrococcus luteus DNA polymerase (P-L Biochemicals) was used at a concentration of 0.3 unit/ assay tube. $MnCl_2$ at 0.1 mM was used as the divalent cation. Concentrations of acid-soluble ribonucleoside phosphates were measured by liquid chromatography of neutralized perchloric acid cell extracts with a Varian Aerograph LCS-1000 chromatograph with a Reeve Angel Paritisil-10 SAX column (4.6 mm x 25 cm) essentially as described previously (17).

RESULTS

Concentrations of dAdo up to a least 100 μM have no significant effect on the growth rate of logarithmically growing L5178Y cells. In contrast, Chart 1 shows that, in the presence of the adenosine deaminase inhibitor 2'-deoxyco-formycin at 1.0 μM, dAdo is much more toxic, with 10 μM causing a slight inhibition of growth and 50 μM effecting essentially a cessation of growth. The 2'-deoxycoformycin itself has no growth-inhibitory effect at the concentration used. In the absence of deoxycoformycin, thin-layer chromatography of media showed that the L5178Y cells had deaminated in excess of 90% of 50 μM [³H]dAdo (20 mCi/ mmole) within 1 hr, with hypoxanthine being the major catabolite, whereas when 1 μM 2'-deoxycoformycin was added, greater than 98% of the 50 μM [³H]dAdo was still present after 24 hr. The consequences of this deamination of dAdo were seen when the cellular metabolites of [³H]dAdo were examined after a 15-min incubation in the presence or absence of deoxycoformycin. In the absence of deoxycoformycin over 85% of the radioactivity was in either acid-soluble purine ribonucleotides or RNA. Of this radioac-

tivity, some 35% was in either acid-soluble or acid-insoluble guanine. Although total cellular uptake of dAdo in the presence of deoxycoformycin was reduced to less than one-eighth of that in its absence, over 90% of the radioactivity was in either dATP or DNA adenine.

Within 3 hr of exposure to 50 μM dAdo in the presence of 1 μM 2'-deoxycoformycin, L5178Y cells begin to exhibit an increase in volume. Chart 2 shows the volume distribution after 6 hr exposure as compared to untreated
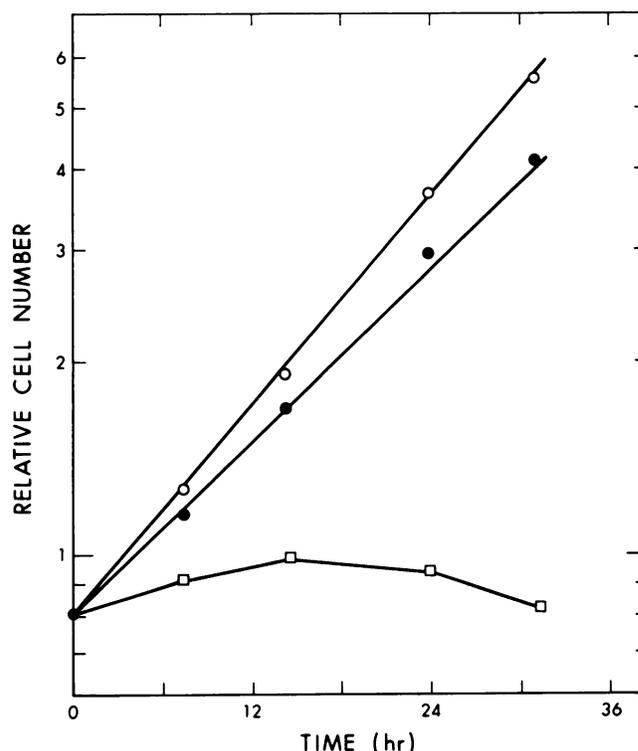

Chart 1. Growth inhibition of L5178Y cells by dAdo. Cell cultures were set up at 20,000 cells/ml, and after approximately 20 hr there was either no addition (O) or an addition of 10 μM dAdo (●) or 50 μM dAdo (□). All cultures contained 1.0 μM deoxycoformycin.
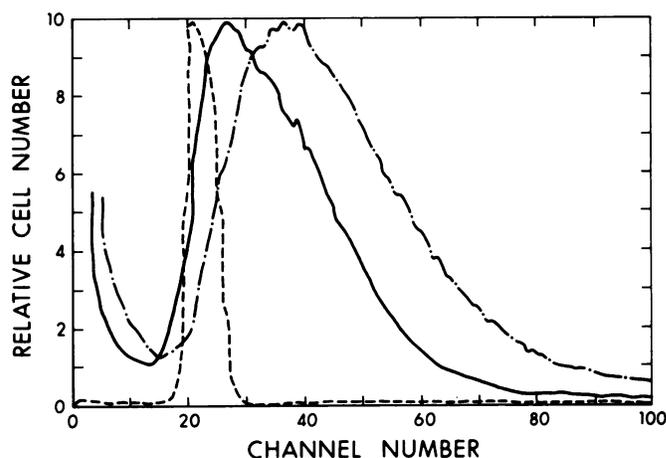

Chart 2. Volume distribution of L5178Y cells. Shown are the volume distribution for control cells (——), for cells treated 6 hr with 50 μM dAdo and 1 μM deoxycoformycin (·—·), and for the 9.69-μM polystyrene microspheres (- - -). In all cases the peak channel contains 4000 particle counts.

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L5178Y cells. The modal volume has increased from 610 to 840 cu μm. The particle counts below Channel 10 represent cell debris, and this is seen to increase with time of exposure to dAdo, indicating that cell lysis is occurring.

The question of whether dAdo treatment is cytotoxic or cytostatic was assessed by both cloning and outgrowth experiments. Chart 3 shows an example of a cloning experiment in which L5178Y cells were exposed to 50 μM dAdo and 1 μM deoxycoformycin for the indicated times when they were collected by centrifugation, washed with warm medium, and cloned in soft agar. As evidenced by this type of experiment, L5178Y cells are killed relatively rapidly by the 50 μM dAdo. In the outgrowth experiments the cells were treated for up to 10 hr and washed as before, but they were set up in static suspension cultures at 5.0×10^4 cells/ml instead of in the soft agar. After 24 to 36 hr the doubling time of the treated cultures stabilized, and back-extrapolation indicated the actual number of cells capable of undergoing cell division after the dAdo exposure. The outgrowth experiments again indicated that dAdo was killing cells, the extent of kill being within a factor of between 2 and 3 of that observed in the cloning experiments.
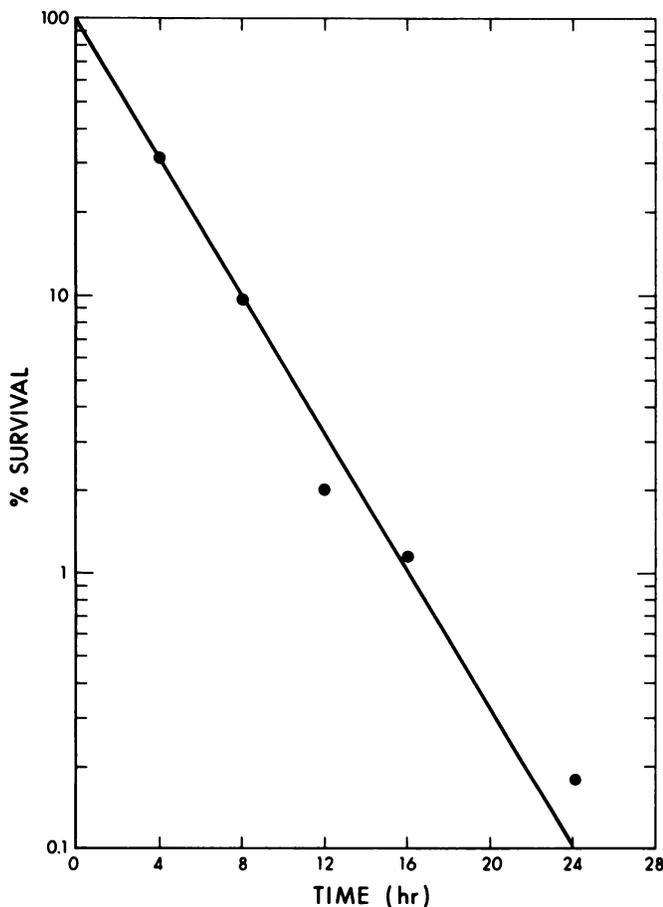
The effect of dAdo on the progression of L5178Y cells through the cell cycle was examined by flow microfluorometry. Cultures of L5178Y cells were grown to about 2.5×10^5 cells/ml at which time 50 μM dAdo and 1 μM deoxycoformycin were added. At various times thereafter, samples of control and treated cells were collected and stained with propidium iodide, and the DNA distributions were determined with the Bio/Physics cytofluorograph. Chart 4 shows a typical experiment in which cells were exposed to dAdo for up to 8 hr. It appears that dAdo treatment results in an accumulation of cells in either G_1 or early S phase. In the 5 experiments performed the major peak appeared to shift between 2 and 4 channels to the right, suggesting that cells were entering into S phase where they were blocked. It is also apparent from Chart 4 that cells in G_2, M, and perhaps even late S phase can divide in the presence of dAdo. This is evidenced by the decrease in numbers of cells in the G_2-M portion of the DNA distribution and an increase in the major peak, since in all cases 50,000 pulses were counted.

The dAdo-induced increase in cell volume and the accumulation of cells at or near the G_1-S boundary suggested a state of unbalanced growth. The data in Table 1 are less consistent with this. Thymidine incorporation is reduced to less than 20% of control, whereas RNA synthesis is unaffected as evidenced by the rate of uridine incorporation into acid-insoluble material.

Purine and pyrimidine ribonucleotide concentrations determined by high-performance liquid chromatography did not significantly change over this period of exposure to
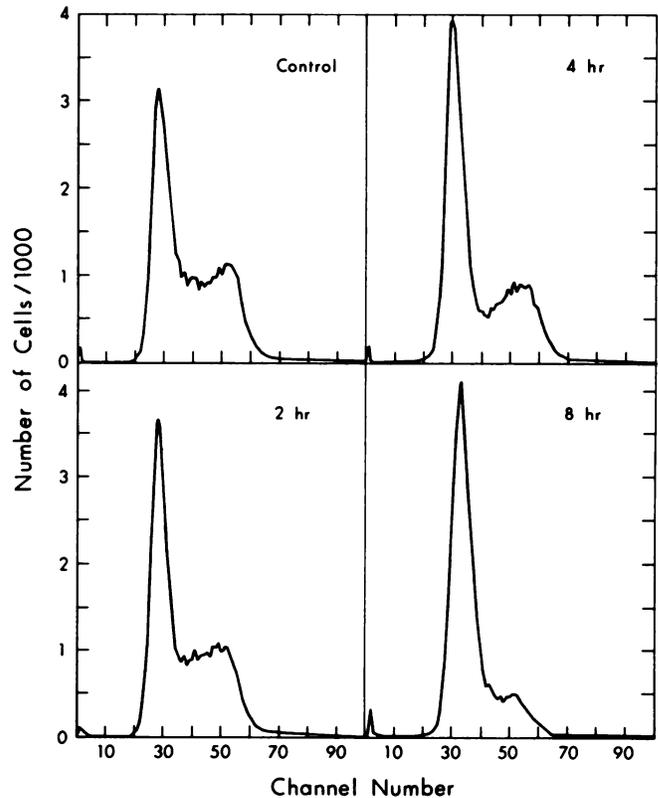

Chart 3. Survival kinetics of L5178Y cells exposed to 50 μM dAdo in the presence of 1.0 μM deoxycoformycin. After exposure for the indicated times, cells were collected by centrifugation and washed once with warm media, and the cloning procedure was carried out as described in the text. In this experiment each *time point* is the mean of triplicate cultures, and cloning efficiency of control cells was 64%.

Chart 4. Microfluorometric DNA frequency histograms of dAdo-treated L5178Y cells. After exposure to 50 μM dAdo and 1.0 μM deoxycoformycin for the indicated times, cells were stained with propidium iodide, and DNA distributions were determined as described in the text.

dAdo when the cell volume increase was taken into account. When deoxyribonucleotides were determined by the DNA polymerase assay method a significant increase in dATP was observed together with a decrease in dCTP levels as shown in Chart 5. The dGTP and dTTP concentrations did not appear to be altered, since the values from 2 experiments were well within 1 S.D. from the average values obtained in untreated L5178Y cultures (Chart 5). Again, from 2 experiments, deoxyribonucleotide pool sizes in the presence of 1 μM deoxycoformycin alone were all within 1 S.D. from the average normal values. Including 50 μM deoxycytidine along with the dAdo and deoxycoformycin resulted in dCTP levels that were of normal magnitude. However, the increase in dATP level and the growth inhibition were not altered. Addition of both deoxyguanosine and deoxycytidine at concentrations of 50 μM also had no effect on the observed growth inhibition.

DISCUSSION

The use of deoxycoformycin as an inhibitor of adenosine deaminase has allowed us to study the toxicity exhibited by

Table 1
Inhibition of precursor incorporation into DNA and RNA

L5178Y cells at 1.30×10^5 cells/ml were treated with 50 μM dAdo plus 1 μM deoxycoformycin for the times indicated. Incorporation of either [³H]deoxythymidine (2 Ci/mmole, 2 μCi/ml) or [³H]uridine (25 Ci/mmole, 2 μCi/ml) into acid-insoluble material was then measured over 20 min at 5-min intervals. The zero time control rates of [³H]deoxythymidine and [³H]uridine incorporation were 7.11×10^6 and 5.27×10^6 cpm/min/10^9 cells, respectively.

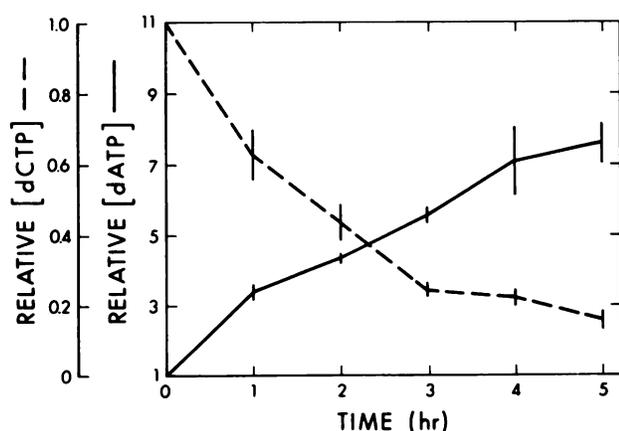| Time (hr)	Relative rates of incorporation	
	[³H]Deoxythymidine	[³H]Uridine
0	1.00	1.00
2	0.33	0.96
4	0.18	0.94
6	0.14	1.03
8	0.19	1.07

Chart 5. Changes in the dATP and dCTP levels of dAdo-treated L5178Y cells. The deoxyribonucleoside triphosphate concentrations were determined after exposure to 50 μM dAdo and 1.0 μM deoxycoformycin for the indicated times. *Vertical bars*, range of values for 2 experiments. The average control levels of dNTP's for some 8 determinations in nmoles per 10^9 cells together with the standard deviations were 14.5 ± 4.4, 8.9 ± 3.5, 43.1 ± 11.6, and 58.6 ± 19.3 for dATP, dGTP, dTTP, and dCTP, respectively.

much lower concentrations of dAdo than the millimolar concentrations that have been used previously (5, 6, 19). This capability lends somewhat more assurance that the observed effects are indeed due to dAdo and not to a combination of dAdo and such possible contaminants as adenosine or heavy metal ions. The possible effects of the large amounts of hypoxanthine produced by the deamination and cleavage of mM amounts of dAdo also complicate the interpretation of these earlier studies.

The results presented here show that, in the presence of an adenosine deaminase inhibitor, 50 μM dAdo blocks L5178Y cell proliferation and results in almost 2 orders of cell kill within an exposure time corresponding to 1 population doubling. An effect of dAdo on HeLa S-3 viability has been reported, but exposures to 2 to 8 mM for 1 population-doubling time were required (5). The dAdo-treated L5178Y cells appear to accumulate in either G_1 or early S phase and to develop a state of unbalanced growth characterized by a greatly reduced rate of DNA synthesis, a continuity in RNA synthesis, and an increase in average volume. The study of Kim *et al.* (5) with HeLa S-3 cells described an inhibition of RNA synthesis by 2 and 4 mM dAdo. It is conceivable that this RNA effect may have resulted from a pyrimidine nucleotide starvation induced by a diversion of 5-phosphoribosyl 1-pyrophosphate from pyrimidine synthesis by the hypoxanthine formed from the deamination and cleavage of dAdo.

The accumulation of dATP in L5178Y cells exposed to dAdo is much less than the massive amounts reported by Klenow (6) to be synthesized in Ehrlich ascites tumor cells. However, in another report Ehrlich cells synthesized dATP from dAdo at approximately one-tenth the rate reported by Klenow (6). Whatever the explanation for these large differences in dAdo-phosphorylating potential exhibited by various cell types, it is apparent from the work described here that if the inhibition of DNA synthesis and cell kill are related to the dATP increases, only modest increases are required. The decrease in dCTP level appears unrelated to the dAdo-induced growth inhibition, since addition of deoxycytidine prevented the dCTP decrease but did not allow growth.

The work presented suggests that the dAdo toxicity on L5178Y cells is not as related to availability of deoxyribonucleotides as to changes in the relative pool sizes. How such changes effect DNA synthesis and cell viability is not understood but may become more meaningful as additional information on mammalian DNA polymerases becomes available. We are currently examining an alternate explanation for dAdo toxicity, namely, that in the presence of an adenosine deaminase inhibitor, dAdo may be effecting *S*-adenosylmethionine metabolism through the inhibition of *S*-adenosylhomocysteine hydrolysis.

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