ABSTRACT

The mechanism of the cytotoxic action of 2-chlorodeoxyadenosine in mouse FM3A cells was investigated. Imbalance of the dNTP pools occurred within 3 h of treatment with 20 μM 2-chlorodeoxyadenosine; the dATP and dGTP pools were depleted and the dTTP pool increased. The dTTP pool increase was probably important in the cell death caused by 2-chlorodeoxyadenosine.

INTRODUCTION

2-Chlorodeoxyadenosine, an adenosine-deaminase resistant 2'-deoxypurinenucleoside analogue, is toxic to cultured cells (1–7). Carson et al. reported that 2-chlorodeoxyadenosine had similar cytotoxicity toward resting T-cells (3, 5). The cytotoxicity of 2-chlorodeoxyadenosine may be explained by the breaks in the double strands of DNA, which can be lethal to cells. Here, we measured the levels of the intracellular dNTP pool, DNA strand breaks, and cell viability in FM3A cells treated with 2-chlorodeoxyadenosine, and compared the results with those found for cells treated with dAdo. The dNTP imbalance caused by 2-chlorodeoxyadenosine is followed by breaks in the double strands of DNA and later cell death.
ES medium containing 1.2 μM [2-14C]thymidine (55.2 Ci/mol, New England Nuclear Corp., MA). Then the cultures were incubated for 24 h without the label so that all radioactivity would be incorporated into DNA of high molecular weight. Cells (density, 2 x 10^6/ml) with [14C]-DNA were incubated at 37°C in 5% CO₂ with 20 μM 2-chlorodeoxyadenosine in ES medium containing serum. At 18 h, portions containing about 5 x 10^6 cells were rinsed with 10 ml of PBS and put onto polycarbonate filters (diameter, 25 mm; pore size, 2.0 μm; Nucleopore Corp., CA) in a Swinnex funnel (Millipore Corp., MA) as described by Kohn et al. (16, 17) and Bradley and Kohn (18). Then 1.5 ml of lysis solution (50 mM Tris, 50 mM glycine, 25 mM Na₂EDTA, 2% sodium dodecyl sulfate, 0.5 mg/ml proteinase K), adjusted to pH 9.6 with NaOH was added to the funnel and the mixture was left for 60 min at 25°C. The lysis solution was removed from the funnel; the mixture was allowed to pour into a scintillation vial. The exit tube of the funnel assembly was then connected to a tube leading to a peristaltic pump, and then elution was done with a tetrapropylammonium hydroxide-EDTA-solution (pH 12.0, as described by Kohn et al. (17)) at 25°C in the dark. Fractions were collected for 90-min periods (flow rate, 2 ml/h) for 15 h. Neutral elution was done as described elsewhere (18) with an eluent of 50 mM Tris, 50 mM glycine, 25 mM Na₂EDTA, and 2% sodium dodecyl sulfate, adjusted to pH 9.6 with NaOH. Cells were irradiated with a γ-ray irradiator with cesium-137 as the source (500 Ci) at 4°C. Irradiated cells were studied immediately by alkaline or neutral elution.

The ratio of SSB versus DSB in DNA was calculated from the following equation (19, 20):

\[ \frac{s}{d} = \left( \frac{k_{SSB}}{k_{DSB}} \right) \left( \frac{[SSB]}{[DSB]} \right) - 2 \]

where \( s/d \) is the ratio of the actual numbers of SSB and DSB, \([SSB]/[DSB]\) is the ratio of rad equivalents found by alkaline elution for SSB and by neutral elution for DSB, \( k_{SSB} \) is the frequency of SSB produced per unit of X-ray dose, as measured by alkaline assay, and \( k_{DSB} \) is the frequency of DSB produced per unit of X-ray dose. The ratio of actual numbers of SSB to DSB for X-rays, \( k_{SSB}/k_{DSB} \), is between 10 and 40 (19, 20). If only DSB were produced by a drug, \( s \) would equal zero, and the measured \( [SSB]/[DSB] \) would be 0.05-0.2.

Orthogonal-Field-Alternation Gel Electrophoresis. OFAGE apparatus used were described previously (21). Agarose blocks containing intact chromosomal DNA from Saccharomyes cerevisiae B0133-3B were prepared as described elsewhere (22). Agarose blocks containing DNA from FM3A cells growing asynchronously and treated with 2-chlorodeoxyadenosine were prepared by a slight modification of the procedure of Schwartz and Cantor (22). The 2-chlorodeoxyadenosine was added when the cell density reached 2 x 10^6/ml. At certain times, cells were harvested and washed once with PBS at 4°C; the cells (about 2 x 10^6) were then placed in 0.6 ml of 0.6% low-melting agarose (Bio-Rad Laboratories, CA). The cells were lysed in an aqueous solution of 0.5 M EDTA, pH 9.0, 1% lauroyl sarcosine, and proteinase K at 1 mg/ml for 2 days at 50°C. Fractions of DNA were washed three times in 0.2 M EDTA, pH 8, before storage at 4°C. OFAGE was done as described by Carle and Olson (21); electrophoresis was performed with 0.6 x TBE (50 mM Tris, 50 mm boric acid, 1.2 mM EDTA, pH 8.3) at 13°C for 18 h including linear gradient pulsing lasting 50 to 100 s. The buffer was recirculated and changed at the midpoint of the run for optimum resolution of the DNA samples. The gel was stained with ethidium bromide.

Incorporation of Labeled Thymidine into DNA. In a study of the effects of 20 μM 2-chlorodeoxyadenosine on thymidine incorporation into DNA, FM3A cells at the density of 2 x 10^6/ml were incubated at 37°C in a medium containing both 2-chlorodeoxyadenosine and [methyl-3H]thymidine (1 μCi/ml, 20 Ci/mmol). At times, duplicate samples were removed and washed with cold PBS twice. Then 100% trichloroacetic acid was added to the suspension at 4°C to the final concentration of 10%. The mixture was kept for 15 min at 0°C and filtered through a nitrocellulose filter (diameter, 0.45 μm; Gelman). After the mixture was filtered, the disks were washed five times with 5 ml of cold 5% trichloroacetic acid. Disks were dried and dissolved in 0.8 ml of dimethyl sulfoxide, and the radioactivity in Triton X-100 scintillation fluid was counted.

In a study of the effects of 2-chlorodeoxyadenosine on protein and RNA synthesis, incubation was done in the same way except that 1- [4,5-3H]leucine (1 μCi/ml, 5 Ci/mmol) or 5- [3H]uridine (1 μCi/ml, 28.5 Ci/mmol) was used in place of the labeled thymidine.

Studies of Cell Kinetics. Samples containing about 5 x 10^6 cells were centrifuged at 100 x g for 5 min. The medium was discarded, and the pellet was washed with PBS and suspended in 1 ml of a solution of 4 mM sodium citrate, 0.1% Triton X-100, 500 units/ml RNase, and 50 μg/ml propidium iodide (23). After incubation of 20 min at 37°C, NaCl was added to the final concentration of 0.15 M. Fluorescence at wavelength longer than 630 nm emitted from the complexes of propidium iodide and DNA in cell nuclei was measured with a flow cytometer (CS-20, Showa Denko Co., Tokyo), with excitation provided by an argon laser, adjusted to deliver 400 mW at 488 nm. The relative fluorescence intensity of each cell was recorded in a frequency histogram based on a linear scale divided into 255 channels. The fluorescence of chicken RBC as the external standard was adjusted on the 15th channel. The DNA-specific fluorescence of about 10000 cells was measured for each histogram. Data were transferred directly to a computer (CTU-II; Showa Denko), and the percentages of cells in the G₁, S, and G₂/M phases of the cell cycle were estimated by the modification by Fukuda et al. of Fried's method (24).

RESULTS

Cytotoxicity of 2-Chlorodeoxyadenosine. The inhibition by 2-chlorodeoxyadenosine of the growth of FM3A cells is shown in Fig. 1. The EC₅₀ was 0.43 μM. The toxic effect of 2-chlorodeoxyadenosine was partially prevented by the addition of dC, and the apparent EC₅₀ of 2-chlorodeoxyadenosine in the presence of 1.0 and 10 μM dC was 0.90 and 8.6 μM, respectively. dA, dG, and dT at the concentration of 10 μM did not affect the toxic effect of 2-chlorodeoxyadenosine. EC₅₀ values for dA, dC, dG, and dT on FM3A cells were 160, >2000, 25, and 160 μM, respectively (9). When cells were exposed to 20 μM 2-chlorodeoxyadenosine for 24 h, washed twice, and incubated 2 days in fresh ES medium, they did not grow.

Cycloheximide can prevent death of thymidylate synthetase-deficient mutants of FM3A cells in the absence of thymidine (25) and dNTP imbalance death (9, 10), so we examined the effect of cycloheximide on cell death caused by 2-chlorodeoxyadenosine. Growth of the cells was arrested by the addition of 2 μg/ml cycloheximide (Fig. 2). In cells treated with 20 μM 2-chlorodeoxyadenosine alone, viability did not change during the first 8 h, and then decreased to 95% at 12 h, 66% at 16 h, and 17% at 24 h. Cell death caused by 2-chlorodeoxyadenosine was almost completely prevented by the addition of cycloheximide at 2 μg/ml.

![Fig. 1. Effect of 2-chlorodeoxyadenosine on growth of FM3A cells in the presence and absence of deoxyribonucleosides. Culture was at 37°C for 48 h. The datum points are the means of duplicate assays. Incubation with 2-chlorodeoxyadenosine only (○), with 1 μM dC (□), with 10 μM dC (◇), with 10 μM dA (△), with 10 μM dG (●), or with 10 μM dT (●).](image-url)
When FM3A cells in culture were exposed to 2-chlorodeoxyadenosine for 12 h, [3H]thymidine incorporation into DNA during this period was inhibited to 8% of the control rate. After 12 h, incorporation of leucine into protein and of uridine into RNA were decreased by 20 μM 2-chlorodeoxyadenosine to 61% and 67% of the control, respectively.

Effect of 2-Chlorodeoxyadenosine on dNTP Pools in FM3A Cells. Fig. 3 shows the dNTP pool changes in the presence of 2-chlorodeoxyadenosine. After 3 h of treatment, the dATP pool was lower than 10% of the zero-time control. At 12 h dTTP had increased, dGTP had decreased, and dCTP had not changed. At 24 h, dATP, dCTP, and dGTP were below the level for the zero-time control. Intracellular rNTP pools did not change significantly with 2-chlorodeoxyadenosine treatment (data not shown).

To investigate the changes in the size of dNTP pools with various concentrations of 2-chlorodeoxyadenosine, cells were incubated with 0.5–20 μM 2-chlorodeoxyadenosine at 37°C for 8 h (Fig. 4). The dATP pool decreased depending on the concentration of 2-chlorodeoxyadenosine. dTTP increased, dGTP decreased, and there was little change in dCTP. Table 1 shows formation of 2-CldAdoTP over time as a metabolite of 2-chlorodeoxyadenosine.

DNA Strand Breaks in Cells Treated with 2-Chlorodeoxyadenosine. SSB and DSB in the DNA were detected by trypan blue exclusion. Incubation was with 2-chlorodeoxyadenosine. Asynchronous growing FM3A cells were cultured with 2-chlorodeoxyadenosine. SSB and DSB in the DNA were detected at 18 h after treatment with 20 μM 2-chlorodeoxyadenosine (Fig. 5).

The cell viability was 53% with the treatment. The numbers of SSB were approximately equal to the SSB that would be caused by γ-irradiation at 130 rads, the numbers of DSB were approximately equal to the DSB that would be caused by γ-irradiation at 2180 rads. The ratio [SSB]/[DSB] found was 0.060. The ratio [SSB]/[DSB] found in human CCRF-HSB2 lymphoblastic cells treated with 1 μM 2-chlorodeoxyadenosine for 19, 22.5, 23, and 23.5 h was 0.064 ± 0.021 [mean ± SD, N = 4, part of results has been published (26)]. Therefore, the DSB were not the result of the accumulation of SSB, but were formed by direct cuts of double strands. The breaks in DNA strands, induced by 2-chlorodeoxyadenosine were abolished by the addition of 2 μg/ml cycloheximide, which inhibits protein synthet-
cell block preparation of Saccharomyces cerevisiae (Y).
The gel contains a treatment. Agarose blocks containing cells treated with 2-chlorodeoxyadenosine at 12 and 24 h were analyzed by OFAGE (Fig. 6). In untreated cells, DNA remained at the origin of the gel. After 12 h treatment with 2-chlorodeoxyadenosine, fragmented DNA bands were observed. At 20 h of treatment, the number of DSB caused by 2-chlorodeoxyadenosine dose-dependent at concentration of 0.5–20 μM according to results of OFAGE: a fragmented DNA band was observed at the concentration of 5 μM of 2-chlorodeoxyadenosine (data not shown). The band was 100–200 kilobase pairs long. We used DNA from λ phage (48.5 kilobase pairs), T4 phage (166 kilobase pairs), herpes simplex virus type I (150 kilobase pairs), and yeast (260–2000 kilobase pairs) as size markers.

Effect of 2-Chlorodeoxyadenosine on Cell Cycle Kinetics. During exposure of FM3A cells to 20 μM 2-chlorodeoxyadenosine for up to 24 h, 2-chlorodeoxyadenosine caused a redistribution in the percentage of cells in the different phases of the cell cycle. The percentage of cells in the S phase, and particularly in the earlier part of the S phase, increased. With 0, 12, and 24 h of incubation time, the percentage of viable cells were 98, 85, and 31%, respectively. At these times, the percentages of cells in the G1 and the S phase (G1/S) were 42/43, 22/70, and 15/76. Analysis by computer simulation showed that the major population was in the early S phase, not the G1 phase. The fluorescence intensities of the major peaks observed in the cells treated with 2-chlorodeoxyadenosine at 12 and 24 h were slightly stronger than the intensity of the major peak for the control cells.

DISCUSSION

Incubation of FM3A cells with 2-chlorodeoxyadenosine caused intracellular dNTP imbalance, DSB, formation of DNA fragments, and hence loss of cell viability. Huang et al. (6), and Seto et al. (5) have compared the cytotoxicity of dAdo (plus deoxycoformycin as an adenosine deaminase inhibitor) with those of 2-chlorodeoxyadenosine, and found a close similarity. Treatment of FM3A cells with dAdo causes an dNTP imbalance, followed by DSB and cell death; the DSB seem to be important in the process of the cell death caused by dAdo, and the intracellular dNTP imbalance probably triggers these events (9). FrdUrd causes dNTP pool imbalance, DSB in DNA and cell death (10). The cytotoxic effects of 2-chlorodeoxyadenosine are similar to those of dAdo and FrdUrd except for the pattern of the dNTP imbalance. The dNTP pool imbalance with 2-chlorodeoxyadenosine involves decreases in dATP and dGTP, an increase in dTTP, and a little change in dCTP within 3 h of the start of treatment. Blakley et al. (27) found that 2-CldAdoTP inhibited ribonucleotide reductase in vitro. These authors also described preliminary results concerning dNTP pool imbalance in human CCRF-CEM lymphoblastic cells caused by 2-chlorodeoxyadenosine (27). Studies on Escherichia coli enzyme systems in vitro has shown that intracellular dNTP pools are controlled by the allosteric regulation of ribonucleotide reductase (28). Conceivably, 2-CldAdoTP represses the reduction of ADP and, as a result, decreases the level of dATP in FM3A cells.

2-Chlorodeoxyadenosine inhibits DNA synthesis more than RNA or protein synthesis, as is the case for 2-chlorodeoxyadenosine and dAdo in CCRF-CEM cells and lymphocytes (1, 6, 27, 29). 2-CldAdoTP is a poor substrate for Klenow enzyme from E. coli, and it is not incorporated into DNA in vitro. Formation of 2-CldAdoTP and depletion of dATP caused by 2-chlorodeoxyadenosine might inhibit DNA synthesis in the cells. However, it is also possible that formation of 2-CldAdoTP leads to mistaken incorporation of 2-chloroadenine residues into DNA; excision of the residues by repair mechanisms later may result in fragmentation of the DNA, so the 2-chlorodeoxyadenosine-induced depletion of the intracellular NAD level causes strand breaks in DNA as found for lymphocytes by Seto et al. (5). However, this mechanism can produce only SSB, but not the DSB found here.

Our hypothesis (Fig. 7) about the mechanism of cell death caused by 2-chlorodeoxyadenosine is similar to hypotheses in studies of FrdUrd and dAdo (9, 10). 2-Chlorodeoxyadenosine, after conversion to 2-CldAdoTP, may interfere with the regulation of ribonucleotide reductase; the resultant formation of 2-CldAdoTP may cause dNTP pool imbalance by further effects on ribonucleotide reductase. The dNTP pool imbalance would be a signal for the production of an endonuclease that would cause DSB in DNA. Large numbers of DSB are lethal for cells (30–32). We investigated the DSB that occurred when 2-chlorodeoxyadenosine was added to a culture of FM3A cells. The results suggest that the DSB were not due to the accumulation of SSB, but involved direct cuts of double strands. The DSB induced by dNTP imbalance were not repaired; both the DNA breakage and the cell death induced by 2-chlorodeoxyadenosine were prevented by addition of cycloheximide, an inhibitor of protein synthesis. Endonuclease activity toward double-stranded DNA is detectable in cells treated with FrdUrd (10) or dAdo (9). Such activity was also found when FM3A cells were treated with 2-chlorodeoxyadenosine. On treatment of...
cells with 2-chlorodeoxyadenosine for 12 h, a fragmented DNA band was observed by OFAGE. The band was 100–200 kilobase pairs long, close to the size postulated for replication units in mammalian cells (33). The DNA breaks must have occurred in specific regions of the genome, as no further breakage in the DNA fragments was observed after 24 h of 2-chlorodeoxyadenosine treatment by OFAGE (data not shown).

Flow cytometric studies of 2-chlorodeoxyadenosine-induced cytostasis in FM3A cells showed that this compound brought about accumulation of cells in the S phase, especially the early S phase. Events crucial for the regulation of cell growth seem to occur in the G1 phase (34). These seem to be a commitment point about accumulation of cells in the S phase, especially the early S phase. Moreover, 2-chlorodeoxyadenosine probably pass through the commitment point, and at the earlier part of the S phase, the mechanism causing cell death from dNTP imbalance goes into action.

We propose a new kind of mechanism by which cancer chemotherapy agents act: dNTP-imbalance death.

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REFERENCES


Imbalance of Deoxyribonucleoside Triphosphates, DNA Double-Strand Breaks, and Cell Death Caused by 2-Chlorodeoxyadenosine in Mouse FM3A Cells

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