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Treatment of Cancer Cells with Methioninase Produces DNA Hypomethylation and Increases DNA Synthesis[1]

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ABSTRACT

Methionine depletion in the human cell line CCRF-CEM through the action of recombinant methioninase (rMETase), a methionine-cleaving enzyme, was previously demonstrated to produce a strong cytotoxic synergistic effect with fluorouracil (FUra) throughout a broad range of concentrations of FUra and rMETase, including subcytotoxic levels of rMETase. Potentiation was associated with a decrease in free thymidylate synthase from preexisting levels. To further investigate the action of rMETase on CCRF-CEM cells, in the present study we explored the effects of rMETase as a single agent on DNA methylation levels and DNA synthesis, which may be changed as a result of deprivation of methionine. Cells treated with rMETase under subcytotoxic conditions contained significantly lower levels of genomic methylated DNA than did control cells, as demonstrated by incorporation of the methyl radical of [*methyl*-^{3}H]*S*-adenosylmethionine in DNA and by use of methylation-sensitive arbitrarily primed PCR. DNA hypomethylation produced by rMETase was of similar magnitude as that produced with the DNA methyltransferase inhibitor 5-azacytidine. Cells exposed to rMETase synthesized significantly more DNA than did untreated cells. Incorporation of [6-^{3}H]thymidine and [6-^{3}H]2′-deoxyuridine in these cells was augmented over that in control by mean factors of 1.78 and 2.36, respectively. Increased ^{3}H nucleoside incorporation resulted in greater numbers of nuclear grains as demonstrated by autoradiography. The increase in DNA synthesis induced by rMETase is likely to result from enhancement of DNA repair because it was not accompanied by differences in cell cycle phase distribution or in total DNA content as determined by flow cytometry. We hypothesize that potentiation of FUra cytotoxicity by rMETase may result from increased inhibition of thymidylate synthase, together with DNA hypomethylation and enhanced DNA repair that could be involved in cell responses to drug-induced damage.

INTRODUCTION

In a previous study performed in the human leukemic cell line CCRF-CEM, we demonstrated a strong synergistic cytotoxic effect between FUra[3] and rMETase, an enzyme that cleaves methionine, over a broad range of drug concentrations including subcytotoxic levels of rMETase (1). Synergism was associated with lower levels of free TS in cells treated with FUra and rMETase relative to that in cells exposed to FUra alone. In the present report, we further explore the effects of rMETase by studying changes in DNA that may be the consequence of cellular depletion of methionine.

Tumor cells can synthesize methionine provided folate, cobalamin, and homocysteine are supplied (2, 3). However, most tumor cells require larger amounts of methionine than they are able to produce, and in the absence of an exogenous supply, they experience growth inhibition or die (4–6). Methylation changes in regions of DNA containing CpG islands are frequently found in cancer cells (6–9). Altered DNA methylation has been associated with abnormal expression of genes involved in various functions, including cell proliferation control, DNA damage detection and repair processes, and apoptosis (9–15). These abnormal DNA methylation patterns, which are accompanied by the increased methionine requirement found in cancer cells, contribute to malignancy and may be involved in sensitivity to cytostatic agents (6–8, 16–18).

In the present study, we explored the effects of subcytotoxic concentrations of rMETase as a single agent on CCRF-CEM cells by measurement of genomic DNA methylation levels and by estimation of the magnitude of DNA synthesis; changes in genomic DNA methylation and DNA synthesis may be associated with the cytotoxic synergism described previously with FUra (1, 16–20).

MATERIALS AND METHODS

Cell Line and Culture Conditions

The CCRF-CEM human T-lymphoblastic leukemia cell line was grown in suspension culture in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics (50 μg/ml streptomycin and 50 units/ml penicillin) at 37°C in an atmosphere containing 5% CO_2. The doubling time in the exponential phase of growth was 22 h.

Cytotoxicity Studies of rMETase

rMETase from *Pseudomonas putida* was from AntiCancer Inc. (San Diego, CA). It catalyzes the α,γ-elimination of methionine to α-ketobutyrate, methanethiol, and ammonia in the presence of pyridoxal 5′-phosphate. One unit of enzyme is the amount that produces 1 μmol α-ketobutyrate/min (21). Exposure to rMETase decreases the levels of methionine in the supernatant of CCRF-CEM cell cultures, and the effect is augmented with increasing concentrations of the enzyme, as demonstrated previously (1).

The effect of rMETase as a single agent on cytotoxicity in CCRF-CEM cells was assessed over concentration ranges of 0.02–0.225 unit/ml. Cell viability was measured after exposure to rMETase for 3, 6, and 72 h with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay, as described previously (1, 22).

Estimation of DNA Methylation Levels

The effect of exposure to rMETase as a single agent on DNA-(5-methyl) cytosine levels was assessed with two different methods in CCRF-CEM cells growing exponentially under subcytotoxic conditions. The tests used were the measurement of the incorporation of the methyl radical from L-[*methyl*-^{3}H]SAM in DNA (23) and MS.AP-PCR (24).

Incorporation of the Methyl Radical of L-[*methyl*-^{3}H]SAM in DNA. The assay was performed on cells exposed to rMETase at 0.2 unit/ml for 6 h and

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[3] The abbreviations used are: FUra, 5-fluorouracil; rMETase, recombinant methioninase; SAM, *S*-adenosylmethionine; dThd, thymidine; dUrd, 2′-deoxyuridine; TS, thymidylate synthase; 5-AzaCr, 5-azacytidine; MS.AP-PCR, methylation-sensitive arbitrarily primed PCR; PI, propidium iodide.

at 0.02 unit/ml for 72 h. It was also performed on cells exposed for 48 h to 0.3 μM 5-AzaCr (Sigma Chemical Co., St. Louis, MO), a cytotoxic substance that, once it is incorporated into DNA, inactivates the DNA(5-cytosine) methyltransferases, thereby preventing methylation of the 5-carbon of the cytosines, resulting in progressively increasing amounts of unmethylated DNA (7, 8, 25, 26).

The experimental procedure was adapted from the assay reported by Balaghi and Wagner (23). The assay is based on the specific methylation by *Sss*I CpG methylase of the unsubstituted 5-carbon of the cytosines on DNA within the dinucleotide sequence 5′-CG-3′. It allows estimation of the methyl(5-cytosine) levels of DNA by measurement of the radioactivity incorporated in DNA upon release of the ^{3}H *methyl* radical from L-[*methyl*-^{3}H]SAM, the methyl donor for CpG methylase. In this assay, DNA-bound radioactivity increases with increasing levels of unmethylated DNA.

Genomic DNA from 10^7 cells obtained from each culture condition and from untreated control cells was treated with proteinase K (Roche Diagnostics, Meylan, France) and extracted as described previously (27). The reaction mixture contained 0.5 μg of DNA, 1 μCi of L-[*methyl*-^{3}H]SAM (78 Ci/mmol; New England Nuclear Life Science Products, Inc., Boston, MA), 3 units of recombinant *Sss*I CpG methylase (New England BioLabs, Inc., Beverly, MA), and buffer [10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT]. After incubation at 30°C for 1 h to enable DNA(5-cytosine) methylation, DNA was isolated on an ion-exchange filter (Whatman DE81), washed in PBS, and then washed in ethanol. The dried filter was assayed for radioactivity with Pico-Fluor 40 (Packard Instruments, Groningen, the Netherlands) scintillation mixture in a Rackbeta (LKB Wallac, Turku, Finland) counter. Methylation was expressed in dpm/0.5 μg DNA. Comparisons were done with Wilcoxon's test.

MS.AP-PCR. The experimental procedure was adapted from the procedure reported by Markl *et al.* (24). The MS.AP-PCR method is based on the comparison of the digestion of DNA with a restriction endonuclease (*Rsa*I) that reduces the size of genomic sequences and double digestion with *Rsa*I plus either an enzyme (*Hpa*II) sensitive to the methylation on the 5-carbon of the internal cytosine within the sequence CCGG (*i.e.*, a PCR amplicon is detected only when the target contains methylated *Hpa*II restriction sites) or an enzyme (*Msp*I) insensitive to the methylation of the cytosine residue within the same sequence (*i.e.*, the enzyme cleaves the bonds comprising either methylated or unmethylated cytosines). The three different digestions are then subjected to an arbitrarily primed PCR reaction with two or three GC-rich primers at a time. We used 18 different primers, for a total of 14 primer sets (Table 1).

Genomic DNA from cells exposed to rMETase (0.2 unit/ml) for 6 h and from untreated control cells was extracted with the Wizard extraction kit (Promega, Charbonnières, France) and quantitated spectrophotometrically. DNA (2 μg) was digested separately in a total volume of 40 μl with 20 units of *Rsa*I, 20 units of *Rsa*I and *Hpa*II, or 10 units each of *Rsa*I and *Msp*I (Roche Diagnostics) at 37°C for 16 h. Another 2 units of each restriction endonuclease were added for 1 additional hour to ensure complete digestion. Samples of 100 ng of restriction enzyme-digested DNA were subjected to amplification by use of sets made of a combination of two or three GC-rich primers (Table 1), at least one of which was labeled with a fluorescent dye in the 5′ position (Eurogentec, Seraing, Belgium). The PCR was performed in a mixture containing 1× buffer [10 mM Tris (pH 8.3), 1 mM $MgCl_2$, and 50 mM KCl], 200 μM of each of the four deoxyribonucleotide triphosphates (Roche Molecular Biochemicals), 0.5 μM of each primer, and 1 unit of Taq DNA polymerase (Q. Biogene, Illkirch, France) by use of a GeneAmp PCR system 2400 (Applied Biosystems, Courtabœuf, France). Conditions for amplification consisted of a primary denaturation step at 95°C for 5 min; followed by 35 cycles of 95°C for 2 min, 40°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 8 min. One μl of each PCR product was then diluted in 25 μl of final formamide with 0.5 μl of the GeneScan size standard (Applied Biosystems), denatured at 94°C for 4 min, cooled in ice, and then separated by capillary electrophoresis by use of an ABI PRISM 310 automatic sequencer (Applied Biosystems). Peak areas were measured for products obtained in the three restriction endonuclease digestions and scored to yield the percentage of DNA methylation. Peaks that were present together in the *Rsa*I, *Rsa*I + *Hpa*II, and *Rsa*I + *Msp*I digestions, indicating the absence of internal CCGG sites, were used as internal controls for migration and peak area.

The methylation level of a given amplified fragment containing CCGG sites was assessed as the ratio of the peak area of the fragment resulting from the *Rsa*I + *Hpa*II digestion, divided by the peak area of the fragment resulting from the *Rsa*I digestion alone. The variation of DNA methylation level between rMETase-treated and control cells was assessed as the difference between the peak area ratio in treated cells [(peak area *Rsa*I + *Hpa*II/peak area *Rsa*I)$_{rMETase}$] and the peak area ratio in control cells [(peak area *Rsa*I + *Hpa*II/peak area *Rsa*I)$_{control}$]. Statistical analysis for differences in peak area ratio was performed with the Student's *t* test.

Table 1 *GC-rich primers and primer sets for MS.AP-PCR*

A. Primers

Primer	Primer sequence (label)[a]
G1	5′-GCG-CCG-ACG-T-3′ (FAM)
G2	5′-CGG-GAC-GCG-A-3′ (FAM)
G3	5′-CCG-CGA-TCG-C-3′
G4	5′-TGG-CCG-CCG-A-3′ (HEX)
G5	5′-TGC-GAC-GCC-G-3′
GC1	5′-GGG-CCG-CGG-C-3′
GC2	5′-CCC-CGC-GGG-G-3′
GC3	5′-CGC-GGG-GGC-G-3′ (HEX)
GC4	5′-GCG-CGC-CGC-G-3′ (HEX)
GC5	5′-GCG-GGG-CGG-C-3′ (TET)
TIM1	5′-AGC-GGC-CGC-G-3′ (FAM)
TIM7	5′-GAG-GTG-CGC-G-3′
TIM10	5′-AGG-GGA-CGC-G-3′
TIM11	5′-GAG-AGG-CGC-G-3′ (HEX)
TIM12	5′-GCC-CCC-GCG-A-3′ (HEX)
TIM13	5′-CGG-GGC-GCG-A-3′
TIM17	5′-GGG-GAC-GCG-A-3′ (HEX)
TIM18	5′-ACC-CCA-CCC-G-3′

B. Primer sets

Primer set no.	Primer combination
1	G2-G4-G5
2	GC1-GC4
3	GC1-GC5
4	GC2-GC5
5	TIM1-TIM7
6	TIM1-TIM18
7	TIM11-TIM12-TIM13
8	TIM13-TIM17
9	TIM1-TIM13
10	G1-G3-G4
11	G1-G4-G5
12	G1-G2-G3
13	G4-GC4-TIM10
14	GC3-TIM17

[a] FAM, HEX, and TET are fluorescent dye labels linked to the 5′ position of primers.

Analysis of DNA Synthesis

The effect of cell exposure to rMETase on overall DNA synthesis was analyzed by measurement of the incorporation of tritiated nucleosides into DNA and by autoradiography. DNA content and cell cycle phase analysis were determined by flow cytometry. Studies were carried out in exponentially growing nonsynchronized CCRF-CEM cells exposed to rMETase as a single agent under subcytotoxic conditions and in untreated control cells.

Cellular Incorporation of [6-^{3}H]dThd, [6-^{3}H]dUrd, and [5-^{3}H]dUrd. The effect on DNA synthesis of cell exposure to rMETase was assessed by measurement of the radioactivity incorporated in DNA after incubation with [6-^{3}H]dThd (25 Ci/mmol; Amersham Life Science, Little Chalfont, United Kingdom) and with [6-^{3}H]dUrd (23.3 Ci/mmol; New England Nuclear). Incorporation of [5-^{3}H]dUrd (9.5 Ci/mmol; Moravek Biochemicals, Inc., Brea, CA), whose tritium atom is lost when converted to dTMP through the action of TS, was used to test for misincorporation of dUTP in DNA.

Cells were exposed to rMETase at concentrations of 0.02, 0.1, and 0.2 unit/ml for 3 and 6 h. One million rMETase-treated and control cells were incubated for 1 h in 0.5 ml of cell culture medium containing 1 μCi of one of the ^{3}H nucleosides. Cells were washed in PBS, filtered on a Whatman 3M filter, and dried at 37°C. Macromolecules were precipitated with trichloroacetic acid. The precipitate was washed with methanol, dried at 37°C, and assayed for radioactivity. Incorporation was expressed in dpm/10^6 cells and represented graphically as a percentage of the control. Statistical assessment was performed with Wilcoxon's test.

Autoradiography. DNA synthesis was analyzed by autoradiography after incubation with [6-^{3}H]dUrd and [6-^{3}H]dThd. Cells exposed to rMETase at 0.2 unit/ml for 6 h and control cells were incubated for 2 h with one of the ^{3}H nucleosides. After a chase phase with the corresponding cold nucleoside for 40 min, cells were cytospun and mounted on glass slides dipped in EM-1 photoemulsion (Amersham) and exposed for 1 week at 4°C. The number of grains/nucleus was analyzed by counting between 125 and 200 nuclei from morphologically intact cells, regardless of their apparent cell cycle phase. Automatic nuclear grain counts were performed with the Alcatel TITN device with Autoradio 3.09 software (20). Statistical assessment was performed with Wilcoxon's test.

Measurement of Cellular DNA Content and Cell Cycle Analysis by Flow Cytometry. DNA content and cell cycle phase distribution were assessed by flow cytometry on both fixed and fresh cells. Cells were exposed to rMETase at 0.2 unit/ml for 6 h and compared with untreated control cells.

For analysis on fixed cells, untreated control and rMETase-exposed cell suspensions were prepared with a DNAcon3 kit (ConsulTS, Ribalta di Torino, Italy). Cells were washed in PBS, fixed, and suspended in a solution containing RNase and 50 μg/ml PI. Stained cells were kept at 4°C for 60 min in the dark, filtered, and analyzed for cellular DNA content in a Coulter Epics XL flow cytometer (Beckman-Coulter, Villepinte, France). Fresh CCRF-CEM cell suspensions were washed in PBS and incubated with the fluorochrome Hoechst 33342 (Sigma Chemical Co.) at a final concentration of 10 μg/ml during 60 min at 37°C and analyzed in a Coulter Epics Elite flow cytometer (Beckman-Coulter) with an argon ion laser. After exclusion of dead cells by light scattering measurements, 10,000 cells were analyzed from each sample for fluorescence intensity. DNA content and cell cycle phase distribution were determined with the MultiCycle AV computer program (Phoenix Flow Systems, Inc., San Diego, CA).

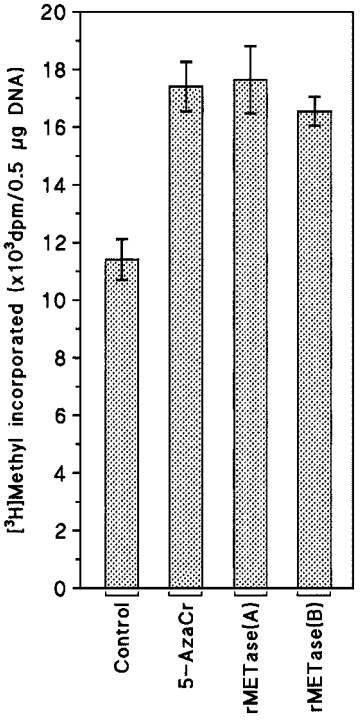

Fig. 1. Effect of rMETase on DNA methylation of CCRF-CEM cells. DNA methylation was measured by incorporation of the methyl radical from L-[*methyl*-^{3}H]SAM in genomic DNA. Histograms represent DNA methylation by *Sss*I CpG methylase in untreated control cells, in cells exposed to 0.3 μM 5-AzaCr for 48 h, and in cells exposed to rMETase at 0.2 unit/ml for 6 h [*rMETase(A)*] and 0.02 unit/ml for 72 h [*rMETase(B)*]. *Columns* represent the mean of two to six determinations, each done in duplicate. *Bars*, SE.

RESULTS

The Cytotoxic Effect of rMETase in CCRF-CEM Cells Is Concentration and Time Dependent. At concentrations of ≤0.2 unit/ml for times ≤6 h and of 0.02 unit/ml for 72 h, rMETase did not produce any cytotoxic effect. At higher concentrations for each of the exposure times, cytotoxicity appeared and was augmented with increasing concentrations of the enzyme. Therefore, rMETase at ≤0.2 unit/ml for times ≤6 h and at 0.02 unit/ml for 72 h corresponds to subcytotoxic levels on concentration-dependent cytotoxicity curves for CCRF-CEM cells (1). All experiments reported here were performed on cells exposed to rMETase as a single agent under these selected subcytotoxic culture conditions.

rMETase Decreases Genomic DNA Methylation Levels in CCRF-CEM Cells. Total genomic DNA methylation was first measured by incorporation of the methyl radical of L-[*methyl*-^{3}H]SAM in DNA through the action of *Sss*I CpG methylase (Fig. 1). Methylation rates were increased over that in control by mean factors of 1.54 and 1.45 by exposure to rMETase at 0.2 unit/ml for 6 h and at 0.02 unit/ml for 72 h, respectively. Methylation rates were increased by 5-AzaCr (0.3 μM) for 48 h by a mean factor of 1.52 over control. Statistically significant differences for methylation rates over that in control were found both in rMETase-treated cells ($P = 0.005$) and in 5-AzaCr-treated cells ($P = 0.02$). The demethylating effect of rMETase was similar to that of 5-AzaCr ($P = 0.9$).

Total genomic methylation was also measured by MS.AP-PCR. Of the 14 sets of primers used (Table 1), 6 yielded a total of 74 PCR amplicons that could be analyzed for DNA methylation levels. Methylated DNA levels measured with this method were also found to be decreased after short-term exposure to rMETase. Most amplified DNA fragments obtained from cells treated with rMETase had lower DNA-(5-methyl)cytosine levels than did the same fragments from control cells. The mean methylation levels in DNA fragments obtained from the peak area ratio (peak area *Rsa*I + *Hpa*II/peak area *Rsa*I) were 50.6% in rMETase-treated cells and 64.9% in control cells. The mean difference (±SE) between rMETase-treated cells and control cells was 14.3 ± 3%. The differences in peak area ratio were statistically significant ($P < 0.0001$).

Exposure to rMETase Is Accompanied by Increased DNA Synthesis. Cells exposed to rMETase for 6 h synthesized more DNA than did untreated control cells (Fig. 2). DNA synthesis was measured by mean ^{3}H nucleoside incorporation. In untreated control CCRF-CEM cells, incorporation after incubation with [6-^{3}H]dThd was 16,612 ± 2,168 dpm/10^6 cells, and after incubation with [6-^{3}H]dUrd, it was 12,814 ± 1,991 dpm/10^6 cells. Rates of overall DNA synthesis increased with increasing concentrations of rMETase. At a concentration of 0.1 unit/ml for 6 h, rMETase increased the incorporation of [6-^{3}H]dThd over that in control by a mean factor of 1.47 ($P = 0.008$) and increased the incorporation of [6-^{3}H]dUrd by a mean factor of 1.27 ($P = 0.1$). rMETase at 0.2 unit/ml for 6 h increased the incorporation of [6-^{3}H]dThd over that in control by a mean factor of 1.78 ($P = 0.008$) and increased the incorporation of [6-^{3}H]dUrd by a factor of 2.36 ($P = 0.01$). When the tracer was [5-^{3}H]dUrd, there was no significant incorporation of radioactivity in control cells (48 ± 7 dpm/10^6 cells) or in rMETase-treated cells (51 ± 9 dpm/10^6 cells), which indicates that all of the incorporation of dUrd into DNA takes place as thymidylate.

The increase in DNA synthesis induced by rMETase was an early event that could be measured after only 3 h of exposure at 0.2 unit/ml. After 3 h, the incorporation of [6-^{3}H]dThd was increased over that in control by a mean factor of 1.66 ± 0.16 ($P = 0.09$), and incorporation of [6-^{3}H]dUrd was increased by a mean factor of 1.71 ± 0.13 ($P = 0.00004$).

Cells exposed to rMETase at 0.2 unit/ml for 6 h had greater overall numbers of nuclear grains than did untreated control cells after incubation with either [6-^{3}H]dThd or [6-^{3}H]dUrd, as shown by autora-

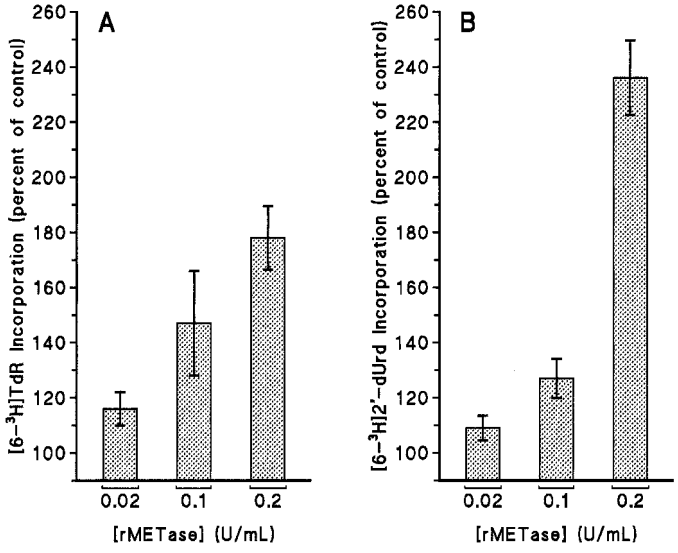

Fig. 2. Effect of rMETase on DNA synthesis of CCRF-CEM cells. Various concentrations of rMETase were tested for 6 h on cellular incorporation of [6-^{3}H]dThd (*A*) and [6-^{3}H]dUrd (*B*). Incorporation was measured in dpm/10^6 cells and expressed graphically as the percentage of that in control. *Columns* represent the mean of four to eight determinations, each done either in duplicate or in triplicate. *Bars*, SE.

diography. For cells exposed to [6-^{3}H]dThd, the median number of grains and the interquartile distribution range (*i.e.*, the numbers of nuclear grains/cell between the 25th and 75th percentiles) were 60 (4–140) and 81 (6–198) for control and rMETase-treated cells, respectively ($P = 0.026$). For cells exposed to [6-^{3}H]dUrd, the median number of grains/cell and the interquartile distribution range were 35 (3–120) and 82 (6–155) for control cells and rMETase-treated cells, respectively ($P = 0.017$).

Exposure to rMETase did not produce changes in total cellular DNA content or in cell cycle phase distribution. The cell cycle phase distribution in cells stained with PI was similar in cells treated with 0.2 unit/ml rMETase for 6 h and in control cells. There was no difference in the percentage of cells in G_0-G_1 phases ($P = 0.32$), S phase ($P = 0.45$), and G_2-M phases ($P = 0.25$) between rMETase-treated cells and control cells. Studies of DNA content and cell cycle phase distribution in fresh cell suspensions stained with the fluorochrome Hoechst 33342 yielded results similar to that obtained in cells stained with PI.

DISCUSSION

We have previously demonstrated a strong synergistic cytotoxic effect of the combination of rMETase and FUra in CCRF-CEM cells (1). The synergistic cytotoxic interaction was an early event that was already present after 48 h of exposure to the drugs and was maintained after 72 h. Synergism was accompanied by a decrease in free TS from preexisting levels. The synergistic effect was achieved with subcytotoxic concentrations of rMETase that are used in the present study to investigate its effect as a single agent on DNA methylation and DNA synthesis.

We demonstrate in the present study that CCRF-CEM cells treated with rMETase contained significantly lower levels of methylated genomic DNA than did control cells. The reduction of the mean DNA methylation level in cells treated with rMETase at 0.2 unit/ml for 6 h relative to control cells was 14.3%, as estimated by MS.AP-PCR. The decrease in DNA methylation levels measured by incorporation of the ^{3}H methyl radical of [*methyl*-^{3}H]SAM in DNA was of similar magnitude in rMETase- and 5-AzaCr-treated cells. Our results suggest that subcytotoxic levels of rMETase may be as active for reducing DNA methylation in CCRF-CEM cells as the highly cytotoxic 5-AzaCr.

Exposure to rMETase at 0.1 and 0.2 unit/ml for 6 h significantly increased the overall DNA synthesis as estimated by incorporation of ^{3}H nucleosides and by autoradiography. The increase in DNA synthesis, which was augmented with increasing concentrations of rMETase, was present as early as after 3 h of treatment. The increase in DNA synthesis is likely to result from the enhancement of DNA repair mechanisms because it was not accompanied by any detectable change in total cellular DNA content or in cell cycle phase distribution.

The increase in DNA synthesis described here could be the consequence of re-expression of previously silenced DNA repair genes through reduction of DNA methylation. Functional DNA repair has been shown to participate in the cytotoxic effect produced by fluoropyrimidines (16, 17, 19). Reactivation of these processes by rMETase may contribute, in part, to the cytotoxic synergism in combination with FUra that we reported previously (1).

The present results suggest that, in addition to DNA repair genes, rMETase may also affect the expression of genes involved in cell proliferation control, tumor suppression, differentiation, and apoptosis, a number of which have been associated with silencing through DNA methylation (6–15, 26). The potent demethylating activity of subcytotoxic concentrations of rMETase provides a tool to investigate these processes and potentially correct their defects in cancer cells.

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