Resistance to Fluorodeoxyuridine-induced DNA Damage and Cytotoxicity Correlates with an Elevation of Deoxyuridine Triphosphatase Activity and Failure to Accumulate Deoxyuridine Triphosphate

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ABSTRACT

Deoxyuridine triphosphate (dUTP) misincorporation and uracil misrepair have long been implicated in fluoropyrimidine-induced DNA damage; however, the enzymatic activities responsible for these lesions have not been previously identified as critical determinants of overall sensitivity to the antitumor effects of these agents. The purpose of this study was to determine whether differences in uracil misincorporation/misrepair could account for the difference in sensitivity to fluorodeoxyuridine (FdUrd)-induced cytotoxicity and DNA damage in 2 human colorectal tumor cell lines having identical sensitivities to FdUrd-induced thymidylate synthase inhibition. Compared to HT29 cells, SW620 cells were resistant to both cytotoxicity and induction of DNA double-strand breaks, as assessed by pulse field gel electrophoresis. Alkaline elution experiments demonstrated that this resistance coincided with delayed induction of DNA single-strand breaks on parental DNA and, to a lesser extent, on nascent DNA. Following treatment with FdUrd for 24 h, HT29 cells accumulated 904 ± 273 pmol deoxyuridine triphosphate (dUTP)106 cells, whereas SW620 cells accumulated 20 ± 7 pmol dUTP. Consistent with this difference in extent of dUTP accumulation was the observation that deoxyuridine triphosphatase levels in SW620 cellular extracts were 4.4-fold higher than in HT29 extracts. The ability to accumulate dUTP, intracellular deoxyuridine triphosphatase activity, and extent of DNA damage appear to be important determinants for predicting the response to FdUrd treatment in these cell lines.

INTRODUCTION

The fluoropyrimidines FUr4 and FdUrd have antineoplastic activity against a broad spectrum of solid tumors and are among the most active agents in the treatment of gastrointestinal cancers. A major mechanism of action of both agents is the inhibition of thymidylate synthase by their common metabolite, FdUMP, in the presence of a reduced folate cofactor (1). TS inhibition results in depletion of dTTP pools, thereby interfering with DNA synthesis and cell division. However, it is not obvious why transient DNA synthesis inhibition should cause cells to permanently lose their ability to proliferate.

Even though significant benefit is derived from fluoropyrimidine therapy, a large percentage of tumors exhibit intrinsic or acquired resistance to these drugs. Several mechanisms of fluoropyrimidine resistance have been identified, most of which lead to reduced inhibition of TS. These include decreased anabolism of fluoropyrimidines to FdUMP, lowered affinity of target enzyme for FdUMP, or expanded intracellular TS levels (2). We recently have reported that in 3 HCT cell lines (HT29, HuTu80, and SW620), sensitivity to high FdUrd concentrations does not depend on the degree of TS inhibition, but rather on their responses to TS inhibition, involving the induction of apparently nonrandom DNA DSB (3). Furthermore, we have shown that the process of DNA DSB formation varies both qualitatively and quantitatively among these cell lines.

There are apparently 2 different mechanisms by which TS inhibition leads to the formation of DNA DSB among the 3 cell lines. The first mechanism entails the formation of 50- to 200-kilobase DNA fragments and substantial cell killing within one doubling time in response to a variety of insults that perturb cell cycle progression, including (but not limited to) TS inhibition. This response has several features in common with apoptosis; however, oligonucleosomal DNA fragments are not observed following drug treatment. The second mechanism, which involves the generation of a broader distribution of DNA fragments ranging from 50 kilobases to 5 megabases, appears to be a specific consequence of TS inhibition. Furthermore, within this category of response, sensitivity to cytotoxicity depends on the rate and extent of DNA DSB formation. Detailed examination of 2 of the cell lines belonging to this category (HT29 and SW620) revealed that, although they are equally sensitive to TS inhibition by FdUrd, the expression of DNA DSB and cytotoxicity requires much longer exposure times or higher drug concentrations in SW620 cells than in HT29 cells (3). This suggests that SW620 resistance is due to differences in the process whereby TS inhibition is translated into DNA damage.

A leading hypothesis regarding the mechanism for the formation of DNA damage is based on observations that TS inhibition produces an abnormally high intracellular dUTP/dTTP ratio in mammalian cells, and that this ratio correlates with incorporation of dUTP into DNA, and with cytotoxicity (4-6). Under normal circumstances, dUTP levels are virtually undetectable because of the enzyme dUTPase, which efficiently hydrolyzes dUTP to dUMP, thereby making uracil misincorporation an unlikely event (7, 8). When uracil does become incorporated into DNA, it usually is then rapidly removed by an excision repair mechanism initiated by uracil-DNA-glycosylase (9). It was hypothesized that during repair of such sites in the presence of unbalanced dUTP/dTTP ratios, more uracil may be incorporated, thus generating areas of continuous DNA excision/repair. Eventually, DNA strand breaks may accumulate as a result of failure of the repair process (10). The misincorporation of FUr4 into DNA has also been observed in cells treated with fluoropyrimidines and may inflict DNA damage by a similar mechanism (5, 11-14). Because uracil-DNA glycosylase and dUTPase activities have been reported to vary significantly among various cell types (11), the sensitivity of a particular cell line to FdUrd-induced DNA damage and cytotoxicity caused by the misincorporation of uracil or FUr4 may therefore be related to the intrinsic activities of these enzymes.
Based on these data, we hypothesized that SW620 resistance to both FdUrd-induced DNA damage and cytotoxicity may be related to an ability to efficiently prevent (Fd)UTP levels from accumulating (higher dUTPase activity) as compared to HT29 cells. To investigate whether the resistance of the SW620 cell line to the formation of DNA DSB is due to attenuated (Fd)UTP accumulation, we measured dUTP, FdUTP, and dTTP pool sizes and dUTPase activities in HT29 and SW620 cellular extracts. In addition, we further characterized the relationship between DSB formation and cytotoxicity, and measured the time course of DNA SSB induction in both parental and nascent DNA by alkaline elution to investigate whether changes in deoxy- nucleotide pool sizes in response to TS inhibition correlate with the level of DNA damage presumably caused by misrepair of incorporated (F)Ura residues in DNA.

MATERIALS AND METHODS

Cell Culture and Drug Treatments. HT29 and SW620 cells were obtained from the American Type Culture Collection and grown as monolayers in McCoy's 5A medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO), at 37°C. FdUrd (Sigma, St. Louis, MO) was dissolved in double distilled water and stored at 4°C for no longer than 2 months. Aphidicolin (Sigma) was dissolved in dimethyl sulfoxide and stored at -20°C. All FdUrd treatments were conducted with media supplemented with diazylated fetal bovine serum. Media were changed every 24 h during drug exposure. Cells were trypsinized and plated at a density of 20,000 cells/cm², then allowed to recover for at least 2 days prior to addition of drug or radiolabel (for experiments involving quantification of DNA damage). For clonogenicity assays, treated cells were diluted and plated in 6-well dishes, in medium containing 10 μM thymidine to provide immediate and uniform cessation of the thymidylate-deprived state. The plating efficiency of control cells was typically 0.6 to 0.8 for both cell lines.

Quantification of DNA Fragmentation by PFGE. Cells were prelabelled with [2-14C]thymidine (0.03 μCi/ml; specific activity, 56 mCi/mmol; Moravek, Brea, CA) for 48 h, followed by a 4- to 8-h chase period prior to drug treatment. Cell blocks (107 cells/ml) were prepared and stored according to standard procedures (15) using low melting point agarose (0.7% final concentration; BRL, Grand Island NY). PFGE analysis was performed using a CHEF DR-II apparatus (Biorad, Richmond, CA). Blocks containing 2-3 x 107 cells were loaded onto a 0.7% agarose gel and electrophoresed at 1 V/cm for 15 to 20 h. At each end of elution, was quantitated by scintillation counting. As external

...external standards for damage on parental DNA, prelabeled control cells were exposed on ice to 3 Gy of radiation prior to processing for alkaline elution. Elution curves from irradiated DNA were consistently found to be convex rather than linear when fraction remaining was plotted against fraction number. Ordinarily, a linear relationship is obtained when fraction remaining of irradiated DNA is plotted on a double log scale versus an internal standard (16). In preliminary experiments (data not shown), we found that the addition of internal standards affected elution rates of our experimental cells, and we were therefore forced to exclude them from these experiments.

Quantification of dUTP, FdUTP, and dTTP Pools by HPLC. Intracellular deoxyribonucleotides were initially quantified by sequential boronate and strong anion exchange HPLC procedures as described (17). Because FdUTP coeluted with dUTP during anion exchange HPLC, the combined (Fd)UTP and dTTP fractions were collected and enzymatically converted to nucleotides. The resulting FdUrd, deoxyuridine, and thymidine were then quantified by reverse phase HPLC.

Duplicate samples containing 3-4 x 107 cells were extracted in 1 ml 0.4 perchloric acid and centrifuged at 4°C. The supernatant containing deoxy- nucleotide triphosphates was then neutralized with 10 μl KOH and stored at -20°C until analysis. Boronate chromatography (Affi-gel 601; Biorad) was used to remove the majority of ribonucleotides from acid soluble extracts prior to HPLC analysis. Approximately 3 pmol of [methyl-3H]dUTP (96 Ci/mmol; Amersham, Arlington Heights, IL) were added to extracts in order to calculate recovery. Ammonium bicarbonate and MgCl2 were then added to adjust extracts to boronate chromatography elution buffer (0.05 M NH4HCO3, pH 8.9, 0.015 M MgCl2) and applied to 1 ml boronate columns. Columns were eluted with equilibration buffer to isolate deoxyribonucleotides and then washed with 0.1 M sodium borate, pH 8.9, to remove remaining ribonucleotides and to regenerate the column.

Following boronate chromatography, samples (1.5-1.7 ml) were adjusted to pH 2.5-3.5, loaded onto a 5-μm strong anion exchange column (4.6 x 150 mm; Astec, Whippany, NJ), and eluted with a linear gradient of HPLC grade ammonium phosphate buffer (0.15 M to 0.60 M, pH 4.1) for 24 min at a rate of 2 ml/min using a Rainin dual pump HPLC system equipped with a Waters 990 diode-array UV absorbance detector. Under these conditions, (Fd)UTP and dTTP were separated from all other deoxyribonucleotides and contaminating ribonucleotides. The recovery of [3H]dUTP collected in the combined (Fd)UTP and dTTP peaks ranged from 60 to 95%. These fractions were desalted by adsorbing the deoxyribonucleotides onto activated charcoal, washing the charcoal 3 times with deionized H2O, and then eluting the deoxyribonucleotides with 1 ml of 62% ethanol containing 2% ammonium hydroxide. Two nmol of ATP were added to each sample prior to the addition of charcoal to calculate recovery. The elution buffer was then evaporated and the remaining deoxyribonucleotides were redissolved in 200 μl of solution containing 50 mM ammonium bicarbonate, pH 8.5, 10 mM magnesium chloride and 1 mM sodium bicarbonate, pH 8.9, 0.015 M MgCl2 and 1 unit of calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemical). Following a 1-h incubation at 37°C, the samples were again evaporated to dryness and redissolved in reverse phase HPLC running buffer (0.05 M ammonium phosphate, pH 4.0, 7% methanol). Samples were injected onto a Rainin C18 (80-225-C5) reverse phase HPLC column and eluted with 0.05 M NH4H2PO4, 7% MeOH at 1 ml/min. Deoxyuridine, FdUrd, and thymidine were identified by their retention times and absorbance spectra and were quantitated using an external standard curve. Cellular concentrations of deoxyribonucleotides were calculated by normalizing for recovery in the boronate/anion exchange and desalting/ dephosphorylation procedures.

Measurement of dUTPase Activity in Cell Extracts. dUTPase activity in cell extracts was assayed by measuring the conversion of [3H]dUTP to [3H]dUMP according to published procedures (18). Approximately 2-4 x 107 exponentially growing cells were scrapped into ice-cold PBS containing 15 mM EDTA, washed, and resuspended to 5 x 107 cells/ml in a buffer containing 0.01 M Tris HCl, pH 7.5, 2 mM β-mercaptoethanol, 4 mM MgCl2, 10% glycerol, and 0.2 mM phenethylsulfonyl fluoride. The cell suspension was subjected to 3 freeze/thaw cycles, adjusted to 0.15 M KCl, and then sonicated. The resulting extract was centrifuged at 13,000 x g for 20 min at 4°C. Protein contained in the supernatant was quantitated using the Bio-Rad protein assay [based on the Bradford (19) dye binding procedure] using bovine serum albumin as a standard. Extracts were stored at -70°C until further analysis.

The dUTPase reaction mixture contained 0.05 M Tris HCl, pH 7.5, 4 mM MgCl2, 2 mM β-mercaptoethanol, 10 mg/ml bovine serum albumin, 25 mM NaF, 0.1 mM dUTP, 0.05 μCi/ml [5-3H]dUTP (21 Ci/mmol; Amersham), and extract containing 2-3 μg of protein, in a final volume of 20 μl. Reactions were carried out at 37°C for a period of 0 to 20 min and stopped by placing...
developed in one dimension using 0.5 M LiCl in 2 N acetic acid. Following separation, dUTP, deoxyuridine diphosphate, and dUMP were cut out based on the migration of known standards, and the amount of radioactivity determined by scintillation counting. In several experiments, nonspecific phosphatase activity was determined under the same conditions by measuring the conversion of [methyl-3H]dUTP (30 Ci/mmol; Amersham) to dTMP by cell extracts. Less than 2% of dTTP was degraded under these conditions.

RESULTS

Time Dependence of FdUrd-induced Cytotoxicity and DNA DSB. Because 100 nm FdUrd is equally cytostatic and produces complete TS inhibition in both cell lines [20-fold higher than the concentration needed to reduce TS activity below detectable levels (3)], this concentration was chosen for studies presented here. The 50% inhibitory concentration for FdUrd-induced growth inhibition is 3 and 4 nm, respectively, for HT29 and SW620 cells (20). Initial characterization of the time dependence of FdUrd-induced DNA fragmentation and cytotoxicity in HT29 and SW620 cells indicated that these events are delayed in the latter cell line (3). A more detailed analysis (Fig. 1) confirms that SW620 cells require a longer exposure to FdUrd to induce an equivalent degree of cytotoxicity as compared to HT29 cells. SW620 cells do not begin to lose clonogenicity until 24 to 32 h of exposure as compared to 10 to 16 h in HT29 cells. This difference appeared to widen slightly as incubation times increased (e.g., time to reach 0.2 surviving fraction was 28 h for HT29 cells, and 48 h for SW620 cells). The addition of 100 μM leucovorin failed to increase cytotoxicity in either cell line. Both cell lines have approximately the same doubling time (22 to 24 h); therefore, it is unlikely that differences in the length of the cell cycle account for delay in cytotoxicity in SW620 cells, assuming the length of S-phase is also similar in each cell line.

The delay in SW620 loss in clonogenicity is paralleled by a delay in the induction of DNA DSB as assayed by PFGE (Fig. 2). SW620 cells required approximately 40 h of treatment before DNA damage became detectable, whereas in HT29 cells, DNA DSB were detectable following 16 h of treatment and accumulated over time as indicated by the increase in F Release. By 48 h of treatment, F Release was 3-fold higher in HT29 cells as compared to SW620 cells, consistent with previous results demonstrating that the fragmentation response is greatly attenuated in the resistant cell line following this exposure to FdUrd (3). Cells treated for 48 h still excluded trypan blue, indicating that DNA damage was not a result of cell lysis.

Alkaline Elution of Parental DNA. The delay in DNA DSB formation detected by PFGE (Fig. 2) in SW620 cells may reflect a slower rate of DNA SSB formation in parental DNA as compared to HT29 cells. In Fig. 3, A and B show the elution patterns of 14C-labeled parental DNA following FdUrd treatment for various exposure times. At the time points used in this experiment, we observed a substantial degree of damage in HT29 cells and only a small amount of damage in SW620 cells. To compare the kinetics of SSB formation in both cell lines, the fraction of 14C-labeled DNA remaining on the filter corresponding to fraction 3 from several experiments was combined (Fig. 4). (We obtained similar results comparing other elution fractions.) The formation of SSB in parental DNA was markedly delayed in the SW620 cell line. DNA damage was not detected until 32 h of treatment, whereas in HT29 cells damage was evident by 10 h of treatment. DNA SSB appeared to accumulate in both cell lines with similar kinetics relative to the appearance of DSB (Fig. 2), with single-strand breaks preceding the formation of double-strand breaks. Therefore, the level of damage occurring on the parental strand measured as either DNA SSB or DSB reflected the sensitivity of each cell line to the cytotoxic action of FdUrd.

In addition, Fig. 3A demonstrates that the shapes of the elution curves of DNA obtained from HT29 cells treated with FdUrd differed significantly from the curves generated from cells treated with 3 Gy of radiation, suggesting that FdUrd treatment may be inducing nonrandom DNA damage. This conclusion is further supported by the rela-
slightly faster than the control rate. At 16 and 24 h, HT29 DNA eluted faster than SW620 DNA, suggesting increased degradation of newly synthesized DNA. A compilation of alkaline elution data for nascent DNA is shown in Fig. 6 using a similar analysis as in Fig. 4.

The increase in elution rate of DNA following treatment with FdUrd could be due to the accumulation of replication intermediates as a result of DNA synthesis inhibition, in addition to increased damage due to the misincorporation and removal of (F)Ura. For comparison, cells were also exposed to aphidicolin, at a concentration that was equally growth inhibitory in the 2 cell lines, but was not cytotoxic over a 24-h exposure period. One μM aphidicolin increased the elution rate of nascent DNA to a similar extent as FdUrd treatment (compare Fig. 5, A and B). Therefore, the increase in elution rates of nascent DNA following exposure to FdUrd may represent a combination of effects consisting of (to a large extent) the inhibition of replication fork movement in addition to increased degradation of newly synthesized DNA as a result of (F)Ura misincorporation.

Quantification of dUTP,FdUTP, and dTTP Pools by HPLC. One explanation for the low level of DNA damage detected in SW620 cells could be that these cells accumulate less (F)dUrd than HT29 cells in response to TS inhibition. To test this possibility, dUTP, FdUTP, and dTTP pools were measured in both cell lines following treatment with FdUrd for 0, 6, or 24 h (Table 1). Control SW620 cells contained approximately 2-fold higher levels of dTTP than did HT29 cells. Following 6 h of FdUrd treatment, the levels of dTTP were depressed in both cell lines to approximately the same values. Following 24 h of treatment, dTTP levels partially recovered in SW620 cells and approached control levels in HT29 cells, possibly due to the combination of DNA synthesis inhibition, expansion of TS levels, and the recovery of enzyme activity (21-23). Although the levels of dTTP rose in HT29 cells between the 6- and 24-h time points, dTTP levels increased even more, such that the ratios of dUTP/dTTP were 3.8 and 8.6, respectively, at these times. SW620 cells accumulated little dUTP following 24 h of drug treatment. The presence of FdUrd was more difficult to assess. In some samples, we observed a peak near the lower limit of sensitivity of our system that may have been FdUrd derived from FdUTP. However, because this peak was not present in all replicates of any sample, we can only conclude that FdUtp accumulation did not exceed 10 pmol/10⁷ cells.

Measurements of dUTPase Activity in Cellular Extracts. The failure to accumulate high levels of (F)UTP in SW620 cells suggests that this cell line may have higher dUTPase activity than HT29 cells. dUTPase activity in cellular extracts obtained from exponentially growing HT29 and SW620 cells was measured and found to be 9.7 ± 1.1 and 45.5 ± 7.6 pmol dUMP formed/min/μg protein (n = 4), respectively. Therefore, SW620 cells contained 4.4-fold higher enzyme activity than HT29 cells.

Alkaline Elution of Nascent DNA. If the majority of (F)Ura misincorporation occurs during replicative DNA synthesis, then the greatest amount of damage caused by misrepair of these lesions would be expected to accumulate on nascent DNA rather than parental DNA. To assess DNA damage occurring in nascent DNA, we analyzed elution rates of DNA labeled during the last 4 h of FdUrd exposure. Fig. 5A shows alkaline elution patterns of nascent DNA following exposure to FdUrd for various times. Following 6 h of drug treatment, nascent DNA eluted at about the same rate for both cell lines, which was slightly faster than the control rate. At 16 and 24 h, HT29 DNA eluted faster than SW620 DNA, suggesting increased degradation of newly synthesized DNA. A compilation of alkaline elution data for nascent DNA is shown in Fig. 6 using a similar analysis as in Fig. 4.

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Quantification of dUTP, FdUTP, and dTTP Pools by HPLC. One explanation for the low level of DNA damage detected in SW620 cells could be that these cells accumulate less (F)dUrd than HT29 cells in response to TS inhibition. To test this possibility, dUTP, FdUTP, and dTTP pools were measured in both cell lines following treatment with FdUrd for 0, 6, or 24 h (Table 1). Control SW620 cells contained approximately 2-fold higher levels of dTTP than did HT29 cells. Following 6 h of FdUrd treatment, the levels of dTTP were depressed in both cell lines to approximately the same values. Following 24 h of treatment, dTTP levels partially recovered in SW620 cells and approached control levels in HT29 cells, possibly due to the combination of DNA synthesis inhibition, expansion of TS levels, and the recovery of enzyme activity (21-23). Although the levels of dTTP rose in HT29 cells between the 6- and 24-h time points, dTTP levels increased even more, such that the ratios of dUTP/dTTP were 3.8 and 8.6, respectively, at these times. SW620 cells accumulated little dUTP following 24 h of drug treatment. The presence of FdUrd was more difficult to assess. In some samples, we observed a peak near the lower limit of sensitivity of our system that may have been FdUrd derived from FdUTP. However, because this peak was not present in all replicates of any sample, we can only conclude that FdUtp accumulation did not exceed 10 pmol/10⁷ cells.

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DISCUSSION

Although (F)dUTP misincorporation and (F)Ura misrepair have long been implicated in fluoropyrimidine-induced DNA damage, the enzymatic activities responsible for these lesions have not been previously identified as critical determinants of overall sensitivity to the enzymatic activities responsible for these lesions have not been previously identified as critical determinants of overall sensitivity to the antitumor effects of these agents. Even though HT29 and SW620 cells are equally sensitive to both growth inhibition and TS inhibition by various concentrations of FdUrd, SW620 cells differed significantly in both the rate and extent of DNA DSB formation (i.e., in their response to TS inhibition). This resistance correlated with delayed induction of DNA SSB, attenuated dUTP accumulation, and a relative elevation of dUTPase activity in the SW620 cell line. Based on these data, we propose that, at least in some cell lines, sensitivity to fluoropyrimidine-induced cytotoxicity may be related to the cellular capacity for (F)Ura misrepair accumulation resulting from differences in dUTPase activity.

Resistance to fluoropyrimidine treatment can occur through a variety of mechanisms. Cells selected for resistance to FdUrd have been found to contain a reduction in thymidine kinase activity, enhanced thymidylate synthase levels, or decreased enzyme affinity for FdUMP (2). Resistance has been correlated with low levels of reduced folate thymidylate synthase levels, or decreased enzyme affinity for FdUMP. However, these investigations were limited by the use of the DNA polymerase assay for dUTP pool measurements, a technique incapable of distinguishing dUTP from dFdUMP, since dUTP can be incorporated efficiently by DNA polymerase (29). The increase in dUTP pools following an initial depression has been attributed to recovery of TS activity, expansion of enzyme levels, and buildup of DNA precursors resulting from general perturbation of DNA replication (21). Surprisingly, even though dUTP pools partially recovered (in the absence of enlarged dUTP pools) in SW620 cells following 24 h of drug exposure, alkaline elution experiments suggested that DNA synthesis continued to be disrupted as indicated by the further increase in elution rate of nascent DNA, compared to control. Furthermore, the recovery of dUTP levels did not protect either cell line from the eventual loss of clonogenicity.

In this study, as in previous ones, the extent of damage inflicted upon mature DNA measured as either SSB or DSB reflected the sensitivity of cells to FdUrd-induced cytoxicity. Depending on the nature of the enzymatic activity involved, DNA DSB can be the result of a single event, in which both strands are broken at once, or by the accumulation of a large number of SSB such that they eventually lie in proximity on opposite strands, effectively becoming a double strand break. In HT29 cells, we found that the appearance of SSB preceded that of DSB, consistent with the (F)Ura misincorporation/misrepair mechanism, which is expected to yield a large number of SSB prior to the formation of DSB. The relative time course of appearance of single- and double-strand breaks in SW620 cells was more difficult to assess, due to the low magnitude of these effects. Nevertheless, SSB accumulated at a faster rate in HT29 cells as compared to SW620 cells, supporting a role for (F)Ura misincorporation in this process.

It still remains unclear how DNA SSB, presumably caused by (F)Ura misrepair, are converted into DSB. The nonlinearity of alkaline elution curves of parental DNA from cells treated with FdUrd suggests that these breaks are nonrandomly distributed when compared to the curves obtained from irradiated cells. As noted in “Results,” the level of DNA SSB caused by 3 Gy of radiation does not account for the extent of DNA damage detected by PFGE. Furthermore, we have already demonstrated through PFGE that FdUrd-induced double-stranded fragments are much smaller than those generated by doses of radiation that are equitoxic (approximately 8 Gy) (30). A dose of 40 to 80 Gy is necessary to generate a similar size distribution of fragments similar to those caused by FdUrd treatment. Li and Kaminiska (31) postulated that cell death caused by deoxyribonucleotide pool perturbations is due to a lethal accumulation of DNA SSB on mature DNA resulting from defective repair of spontaneous lesions that occur naturally over time. Therefore, DSB could be the result of coincident misrepair of template parental DNA opposite damaged nascent DNA. Another possibility is that the DNA template opposite regions of constant repair is more susceptible to single-strand endonuclease action. The nonrandom generation of DNA SSB and DSB could be
explained by recent observations suggesting that DNA excision/repair may occur preferentially in regions of the genome that code for actively transcribed genes (32, 33). In addition, these may be concerted DNA DSB produced by some unidentified activity, such as a topoisomerase or an endonuclease.

In contrast to DNA damage measured on the parental strand, alkaline elution data comparing HT29 and SW620 nascent DNA damage were less conclusive. The increase in elution rates of nascent DNA obtained from FdUrd-treated cells appeared to be largely influenced by general DNA synthesis inhibition since aphidicolin produces the same effect. It is also important to note that aphidicolin treatment for 24 h was noncytotoxic but completely growth inhibitory to both cell lines, even though this treatment significantly increased the elution rates of nascent DNA. Furthermore, the time dependence of the increase in nascent DNA elutability in SW620 cells did not correlate with FdUrd-induced cytotoxicity. Therefore, damage inflicted on newly synthesized DNA may represent the cytostatic (e.g., inhibition of replication fork movement) rather than the cytotoxic (induction of SSB on mature DNA) consequence to TS inhibition as originally proposed by Li and Kaminskas (31). This hypothesis is consistent with the observation that SW620 resistance to FdUrd-induced cytotoxicity correlated with attenuation of DNA SSB induction on parental DNA.

Although the data presented here are not extensive enough to evaluate the general significance of variations in dUTPase activities as a factor in fluoropyrimidine sensitivity, Caradonna and Cheng (11) measured dUTPase activity in cellular extracts obtained from a variety of sources and found values from 2 to 14 pmol dUMP formed per μg protein. HT29 dUTPase activity falls within this range, whereas SW620 cells contain approximately 3-fold higher enzyme activity than the cell line displaying the highest activity in that previous report. Therefore, the naturally occurring variations of this activity appear to be sufficiently heterogeneous so that they may indeed be an important determinant of response to treatment with TS inhibitors. In support of this hypothesis, Beck et al. (34) reported an inverse relationship between intracellular dUTPase levels and methotrexate cytotoxicity.

REFERENCES

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