Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10474–10478, November 1992
Medical Sciences

Variations in patterns of DNA damage induced in human colorectal tumor cells by 5-fluorodeoxyuridine: Implications for mechanisms of resistance and cytotoxicity

(cell death/pulsed-field gel electrophoresis/DNA fragmentation)

CHRISTINE E. CANMAN*, HSIN-YI TANG*, DANIEL P. NORMOLLE†, THEODORE S. LAWRENCE‡, AND JONATHAN MAYBAUM*§

Departments of *Pharmacology and ‡Radiation Oncology, University of Michigan Medical School, Ann Arbor, MI 48109; and †Department of Biostatistics, University of Michigan School of Public Health, Ann Arbor, MI 48109

Communicated by Gertrude B. Elion, July 31, 1992 (received for review December 30, 1991)

ABSTRACT **We have previously shown that treatment of the HT29 human colorectal tumor (HCT) cell line with 100 nM 5-fluorodeoxyuridine (FdUrd) induces DNA fragments ranging from 50 kilobases to 5 megabases. The studies reported here were conducted to characterize the kinetics, concentration dependence, and pharmacologic specificity of this process and to determine if such fragmentation varies among HCT cell lines. HT29 and SW620 cells yielded similar fragment size distributions upon treatment with either FdUrd or CB3717 [a folate analog inhibitor of thymidylate synthase (TS)]. With either of these agents the SW620 line required higher drug concentrations or longer incubation times than HT29 cells to achieve a given level of fragmentation or cytotoxicity, even though the two cell lines are equally sensitive to FdUrd-induced TS inhibition. These data indicate that SW620 resistance is not due to a lesion in the events leading up to TS inhibition but it may be due to a difference in the steps following TS inhibition. Aphidicolin, a DNA polymerase inhibitor, did not cause substantial fragmentation or cytotoxicity in these two cell lines, demonstrating that the fragmentation response to the other two drugs is not a general consequence of DNA synthesis inhibition. A third HCT line, HuTu80, gave rise only to a smaller and more discrete population of DNA fragments, ranging from ≈50 to 200 kilobases, following exposure to FdUrd. Similar patterns were seen in this line upon treatment with CB3717 or aphidicolin, indicating that this fragmentation pattern is not specific to TS inhibition and may be characteristic of a more general response than that seen in the other two cell lines. DNA fragments induced by FdUrd in HuTu80 cells did not degrade into smaller pieces, demonstrating that the process by which they are formed is distinct from apoptosis. We conclude that the responses of HCT cells to FdUrd-induced TS inhibition vary significantly, that these differences may reflect heterogeneity in the mechanism of DNA damage formation, and that, in some cases, FdUrd resistance may be due to alterations in the fragmentation process.**

The fluoropyrimidine fluorouracil (FUra) and its nucleoside 5-fluorodeoxyuridine (FdUrd) are among the most active agents in the treatment of gastrointestinal cancers. FUra and FdUrd have a variety of biochemical effects, including the inhibition of *de novo* thymidylate biosynthesis, incorporation into DNA and RNA, and the formation of altered sugars (1). Although the relative importance of these effects in mediating antitumor action probably varies among individual patients, the overall importance of thymidylate synthase (TS; EC 2.1.1.45) inhibition is underscored by recent improvements in clinical response resulting from the addition of leucovorin to fluoropyrimidine regimens (2). It is therefore likely that the clinical application of these drugs will be well served by a clearer understanding of how TS inhibition leads to cell death.

DNA damage has been implicated frequently in the cytotoxic mechanism of TS inhibitors, and at least three proposals have been made to explain how this damage occurs. One hypothesis is that the excessive dUTP/dTTP ratio caused by TS inhibition results in saturation of the uracil-*N*-glycosylase repair system, leading to DNA strand breaks (3). A second hypothesis is based on studies in the FM3A murine mammary carcinoma cell line, in which TS inhibitors caused the formation of nonrandomly distributed DNA double-strand breaks, resulting in a discrete fragment size distribution ranging from 50 to 200 kilobases (kb) (4). Evidence was presented indicating that a double-strand-specific endonuclease activity may be induced by nucleotide pool imbalances in FM3A cells. It was suggested that the relatively open conformation of chromosomal domains undergoing replication may make these regions particularly sensitive to endonuclease cleavage, thus accounting for the nonrandom breakage pattern. A third hypothesis is that fluoropyrimidine treatment initiates a cellular suicide response (apoptosis) characterized by the appearance of a Ca^{2+}/Mg^{2+}-dependent endonuclease that gives rise to DNA fragments (oligonucleosomal ladders) (5) much smaller than those detected in FM3A cells.

The diversity of these responses suggests that the mechanism for fluoropyrimidine-induced DNA damage and cytotoxicity may vary among cell types or among individual cell lines. We have addressed this possibility by examining the distribution, extent, and pharmacological specificity of DNA double-strand breakage induced in three human colorectal tumor (HCT) cell lines, using pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Cell Culture and Drug Treatments. HT29, HuTu80, and SW620 cells were obtained from the American Type Culture Collection and were grown as monolayers in McCoy's 5A medium (GIBCO) with 10% fetal bovine serum (GIBCO), at 37°C. HL60 cells (from W. R. Mancini, University of Michigan) were cultured in suspension under the same conditions.

FdUrd (Sigma) was dissolved in double-distilled water and stored at 4°C for ≤2 months. CB3717 (from A. L. Jackman, Royal Cancer Hospital, Surrey, UK) was dissolved in 10 mM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FdUrd, 5-fluorodeoxyuridine; HCT, human colorectal tumor; Mb, megabase(s); PFGE, pulsed-field gel electrophoresis; TS, thymidylate synthase.

§To whom reprint requests should be addressed at: 4302A Upjohn Center for Clinical Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0504.

$NaHCO_3$ and stored at −20°C. Dialyzed fetal bovine serum was used for experiments with FdUrd or CB3717. Etoposide and aphidicolin (Sigma) were dissolved in dimethyl sulfoxide and stored at −20°C. Media were changed every 24 hr during drug treatments. Cells were trypsinized and plated at a density of 20,000 cells per cm^2, then allowed to recover for at least two doubling times before addition of drug or radioactivity (one doubling time = 22–24 hr for HT29/SW620 cells, 16–18 hr for HuTu80 cells).

For clonogenicity assays cells were plated in six-well dishes. In experiments using FdUrd or CB3717, colonies were grown in medium supplemented with 10 μM thymidine, to provide an immediate and uniform cessation of the thymidylate-deprived state. The plating efficiency of control cells was typically 0.4–0.5 for HuTu80 and 0.6–0.8 for HT29 and SW620. Cells were labeled for quantitating DNA fragmentation by incubation with [2-^{14}C]thymidine [0.15 μCi/ml; 56 mCi/mmol (1 Ci = 37 GBq); Moravek Biochemicals, Brea, CA] for one doubling time, followed by a 6- to 8-hr chase period prior to drug treatment.

Electrophoretic Analysis and Quantification of DNA Fragments. Cell blocks (10^7 cells per ml) were prepared and stored according to standard procedures (6) using low melting point agarose (BRL, 0.7% final concentration). Qualitative PFGE analyses were performed using a CHEF DR-II apparatus (Bio-Rad). Blocks containing 2–3 × 10^5 cells were loaded onto a 0.7% agarose gel and run at 1.9 V/cm with a reorientation angle of 120°. The switching interval was ramped linearly from 30 to 120 sec over 30 hr and then from 2 to 42 min for 51 hr (7). The buffer, 0.5× TBE (45 mM Tris borate, pH 8.0/1 mM EDTA), was recirculated at 10°C. Quantitative PFGE analyses were conducted by using a Hex-a-field apparatus (BRL) with a 45-min constant switching interval for 44 hr at 36 V, in 0.5× TBE at 14°C. Size standards were *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes (Bio-Rad). Lanes were cut into 3-mm slices, melted in 0.1 M HCl, and analyzed by scintillation counting. Data are expressed as the fraction of cpm which migrated into the lane from its corresponding well ($F_{released}$). Control $F_{released}$ values were typically 0.04–0.07.

For detection of oligonucleosomal fragments, 2 × 10^6 cells were harvested, washed in Hanks' buffer, resuspended in TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA) containing 0.4% Triton X-100, and allowed to lyse for 15 min on ice. After a 15-min centrifugation at 13,000 × *g*, the supernatant was extracted with 1:1 (vol/vol) phenol/chloroform. DNA in the supernatant was precipitated in ethanol, dissolved in 40 μl of TE buffer, and treated with RNAse at 40 μg/ml at 37°C for 2 hr. Fifteen microliters of this solution was then analyzed by conventional gel electrophoresis (8).

Measurement of TS Activity in Intact Cells. Cells were seeded onto 24-well plates at 5 × 10^4 (HT29/SW620) or 2.5 × 10^4 (HuTu80) cells per cm^2, then allowed to recover for 48 hr. After 2-hr exposure to FdUrd, 10 nM [5-^{3}H]dUrd (20 Ci/mmol; Moravek Biochemicals) was added. At 20- or 30-min intervals, a plate containing replicates of each cell line was placed on ice and 2 ml of an ice-cold suspension of 5% activated charcoal in 0.2 M trichloroacetic acid was added to each well. One milliliter of this solution was centrifuged at 13,000 × *g* for 6 min, and the radioactivity in 500 μl of the supernatant was assayed by scintillation counting (9). To quantitate the concentration dependence of TS inhibition, data were modeled by a 4-parameter logistic function (10). Parameters of the function were estimated by a weighted nonlinear regression (weight = 1/response) using PROC NLIN in SAS.

RESULTS

Concentration Dependence of Fragmentation and Cytotoxicity Induced by FdUrd. In HT29 cells fragmentation was usually not detectable at FdUrd concentrations below 10 nM (Fig. 1). At each concentration from 10 to 1000 nM a similar, broad distribution of fragment sizes was observed, ranging from ≈5 Mb to 50–100 kb. Unlike radiation-treated cells, none of the FdUrd-treated samples contained a substantial mass of DNA between the longest *Schizosaccharomyces pombe* marker and the well, suggesting that the double-strand breaks in the drug-treated cells may not be randomly distributed (11). In the SW620 line high concentrations of FdUrd induced fragment size distributions similar to those found in HT29 cells, although the mass of DNA fragments at each FdUrd concentration was less in SW620 than in HT29. Fragment size distributions in FdUrd-treated HuTu80 cells had a narrower range (≈50–200 kb) and a much smaller average size than the other two lines. This pattern closely resembles the one obtained in FdUrd-treated FM3A cells (4).

In HT29 and SW620 cells the fraction of DNA released into the gel ($F_{released}$) had a sigmoidal dose–response relationship (Fig. 2). These data confirm that significantly higher drug concentrations are required to produce a given degree of fragmentation in SW620 cells than in HT29 cells, with half-maximal fragmentation occurring at 300 and 30 nM, respectively. The relative resistance of SW620 cells to FdUrd-induced fragmentation is accompanied by resistance to FdUrd-induced cytotoxicity (Fig. 2). For example, the FdUrd concentration needed to reduce surviving fraction to 0.1 is almost 6-fold higher in SW620 cells than in HT29 cells (200 nM vs. 35 nM). In HuTu80 cells $F_{released}$ and surviving fraction both begin to differ from control values over the range 10–100 nM and then change more steeply at concentrations above 100 nM. We conclude that, although there may be differences among these cell lines in the quantitative relationship between $F_{released}$ and cytotoxicity, within each cell line the concentration dependences of these two parameters are similar.

Time Dependence of Fragmentation and Cytotoxicity Induced by FdUrd. In both the HT29 and SW620 lines the first fragments detected had sizes in the low megabase range (Fig. 3). At later times this distribution extended down to ≈50 kb, although little DNA was detected below this size. In HuTu80 cells fragments were usually first detected at 24 hr. Although the intensity of this staining increased with longer drug exposures, the size distribution did not change appreciably. We therefore conclude that the fragments seen in HuTu80 cells do not represent breakdown products of larger intermediates, such as those found in the other two lines. Fig. 3 also demonstrates that, as was the case for the concentration dependence of fragmentation (described above), the time dependence of fragmentation in HT29 and SW620 cells is

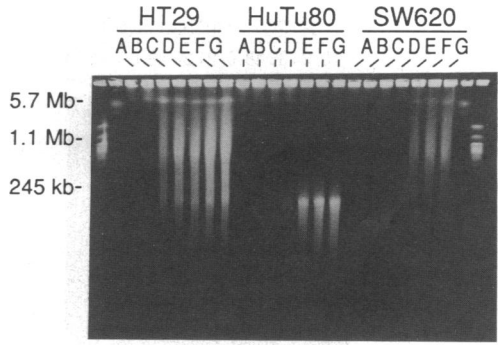

FIG. 1. Cell line and concentration dependence of DNA fragmentation patterns induced by FdUrd. Cells were treated with FdUrd for 48 hr, then analyzed by PFGE. Drug concentrations (nM) are A, 0; B, 3; C, 10; D, 30; E, 100; F, 300; and G, 1000. *S. cerevisiae* chromosome markers were run in the outermost lanes, adjacent to *Schizosaccharomyces pombe* markers. Mb, megabases.

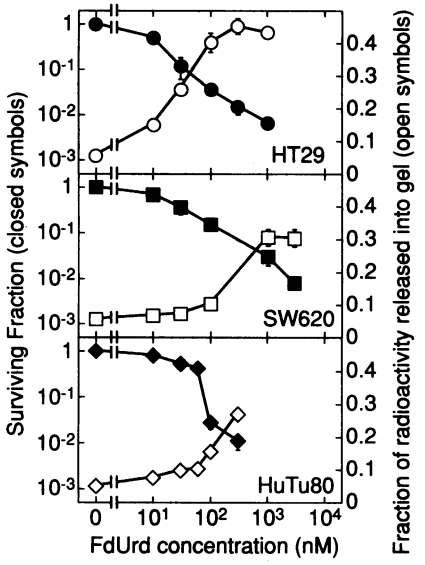

FIG. 2. Quantitative analysis of FdUrd-induced DNA fragmentation and cytotoxicity in HCT cells. Cells were treated with FdUrd for 48 hr and either processed for PFGE followed by quantification of DNA fragmentation or assayed for clonogenicity. For quantitative PFGE analysis, cells were prelabeled with [2-^{14}C]thymidine prior to drug treatment. Data are expressed as fraction of DNA released into the gel ($F_{released}$) for DNA fragmentation (open symbols) and surviving fraction (closed symbols) for cytotoxicity. Values are the mean ± SEM of at least three independent experiments. $F_{released}$ and cytotoxicity were determined in separate experiments.

different as well, with SW620 cells requiring longer drug exposures to elicit fragmentation. The time course for loss of clonogenicity is similarly offset in these two lines (Fig. 4).

In none of the samples studied by PFGE did we detect a significant mass of DNA fragments smaller than 15 kb. However, it has been shown that DNA fragments up to 30 kb in length can diffuse out of agarose plugs during PFGE sample preparation (12), and it was possible that small fragments (such as those found in cells undergoing apoptosis) might have been present in our samples but not detected by PFGE analysis. Conventional electrophoresis revealed that, although oligonucleosomal ladders were found in our positive controls (HL60 cells exposed to etoposide; Fig. 5) (13), they were not detectable in HT29 or HuTu80 cells treated for 48 hr with 100 nM FdUrd.

TS Inhibition. To determine if the relative resistance of SW620 cells to FdUrd-induced fragmentation and cytotoxicity is attributable to incomplete TS inhibition, we measured

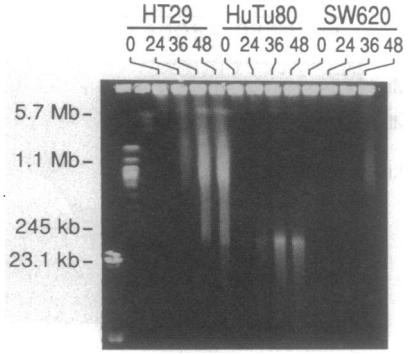

FIG. 3. Time dependence of FdUrd-induced DNA fragmentation. Cells were treated with 100 nM FdUrd for the indicated number of hours, then analyzed by PFGE. Markers in the first three lanes are, left to right, phage λ DNA digested with *Hin*dIII, *S. cerevisiae* chromosomes, and *Schizosaccharomyces pombe* chromosomes.

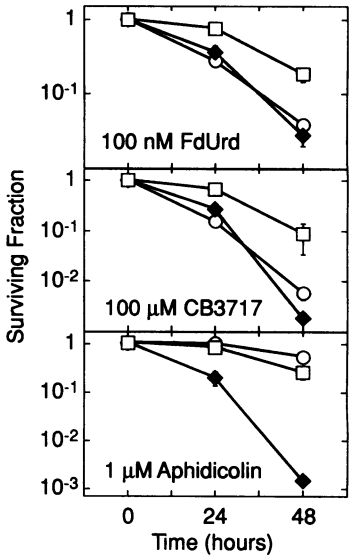

FIG. 4. Time dependence of drug-induced cytotoxicity. HT29 (○), HuTu80 (◆), and SW620 (□) cells were treated with 100 nM FdUrd, 100 μM CB3717, or 1 μM aphidicolin for the indicated times and assayed for clonogenicity. Data are the mean ± SEM of at least three experiments.

the effect of FdUrd treatment on apparent TS activity in intact cells by using the *in situ* tritium release assay. TS activity was not detectable in either HT29 or SW620 cells at FdUrd concentrations ≥ 10 nM (Fig. 6). The curves for HT29 and SW620 were not significantly different from each other ($F_{4,33}$ = 0.864, P = 0.49), but the HuTu80 line was significantly different from the other two lines ($P < 0.0001$). This result is consistent with previous data indicating that HuTu80 cells contain severalfold higher levels of TS than do HT29 cells (14). The estimated values for EC_{50} (nM) ± 95% confidence interval were: HuTu80, 4.15 ± 1.26; HT29, 2.18 ± 0.67; SW620, 1.91 ± 0.53. Therefore, differences in TS inhibition do not account for the resistance of SW620 cells to drug-mediated fragmentation or cytotoxicity.

Fragmentation and Cytotoxicity Induced by CB3717 and Aphidicolin. To test whether DNA incorporation of FdUTP is required to produce fragmentation, we exposed cells to the folate analog CB3717, which inhibits TS but which cannot become incorporated into DNA (15). In preliminary experi-

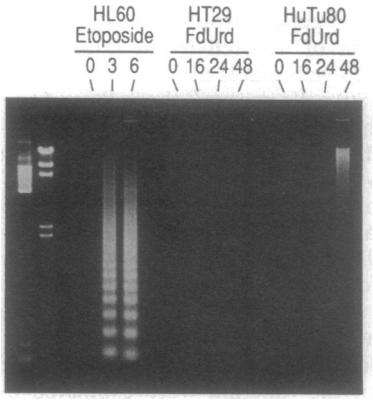

FIG. 5. Induction of oligonucleosomal fragments by FdUrd. HT29 or HuTu80 cells were treated with 100 nM FdUrd for the indicated number of hours, then analyzed by conventional agarose gel electrophoresis. As positive controls, HL60 cells were treated with etoposide at 10 μg/ml for 3 or 6 hr (13). Markers in the leftmost lanes are 123-bp ladder and λ DNA digested with *Hin*dIII. This gel is representative of four independent experiments.

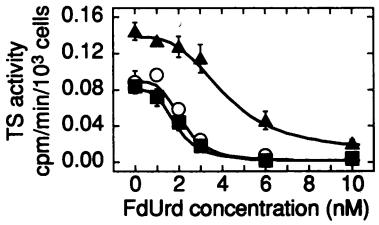

FIG. 6. Inhibition of TS activity by FdUrd. HT29 (○), HuTu80 (▲), and SW620 (■) cells were exposed to FdUrd for 2 hr and then assayed for TS activity by the *in situ* tritium release assay. Data from three or four experiments were pooled and analyzed. The points plotted are the mean (where $n = 2$) or mean ± SEM (where $n \geq 3$) for replicates at each drug concentration. The curves indicate the values expected from fitting the individual data from each cell line to a four-parameter logistic function.

ments (not shown) we found that the EC_{50} for growth inhibition by CB3717 was similar in all three cell lines, ≈10 μM. We therefore chose 100 μM (i.e., $EC_{50} \times 10$) for examining the effects of this drug. In each cell line responses to CB3717 were almost identical to those of FdUrd (Fig. 4, compare Figs. 3 and 7). We conclude that FdUTP incorporation into DNA is not obligatory for formation of megabase fragments in these cell lines.

To determine if the fragmentation patterns seen here are a specific response to TS inhibition, as opposed to a response to DNA synthesis inhibition in general, we measured fragmentation and cytotoxicity caused by aphidicolin, a DNA polymerase inhibitor. As for CB3717, the drug concentration used (1 μM) was 10-fold higher than the EC_{50} for growth inhibition. Exposure to 1 μM aphidicolin for 24 or 48 hr caused little accumulation of fragments in the low megabase range (Fig. 8), as had been seen after exposure to FdUrd or CB3717, in HT29 and SW620 cells. In HuTu80 cells aphidicolin produced DNA fragment patterns similar to those induced by the TS inhibitors. Cytotoxicity of aphidicolin treatments in these cell lines mirrored the presence or absence of fragmentation (Fig. 4): surviving fractions of HT29 and SW620 cells were reduced only slightly, whereas clonogenicity in HuTu80 cells declined to about 0.001.

DISCUSSION

We have found that, even within a small panel of HCT cells, there is diversity in both the extent and the distribution of DNA double-strand breaks formed in response to FdUrd treatment. Two types of fragment size distributions were seen in these cell lines, "broad" (≈0.05–5 Mb) and "narrow"

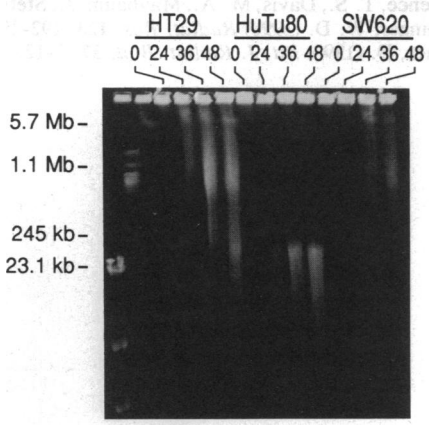

FIG. 7. Time dependence of DNA fragmentation induced by CB3717. Cells were treated with 100 μM CB3717 for the indicated number of hours, then analyzed by PFGE. Markers are as in Fig. 3.

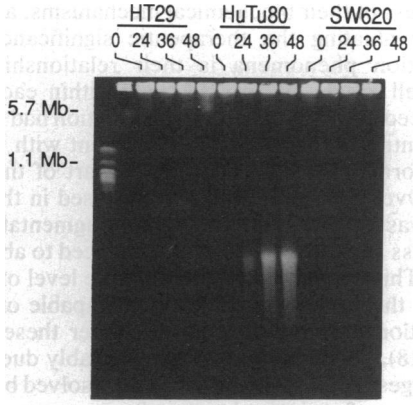

FIG. 8. Time dependence of DNA fragmentation induced by aphidicolin. Cells were treated with 1 μM aphidicolin for the indicated number of hours, then analyzed by PFGE. Markers are as in Fig. 1.

(≈50–200 kb). In preliminary experiments with four other human tumor cell lines (not shown) we observed that fragmentation patterns generated in response to cytotoxic FdUrd treatments resembled one or the other of the patterns described here, suggesting that there may be two major modes of response to FdUrd-induced TS inhibition, of which these patterns are characteristic.

It is not clear whether these fragmentation patterns represent two different manifestations of a single process or two fundamentally different processes. Fragment size distributions like those induced in HuTu80 cells were previously found in FM3A cells exposed to a number of cytotoxic conditions (16). It was noted that the 50- to 200-kb size range of these fragments corresponds to the estimated range of sizes of individual replicons in mammalian cells, and it was proposed that this damage results from preferential scission of stalled replicons by an induced endonuclease (4). Separate studies showed that 50- to 200-kb fragments were also induced in drug-treated rat thymocytes undergoing apoptosis, as a result of endonuclease action (17), and that these fragments appeared to be precursors in the formation of oligonucleosomal ladders. Fragmentation in HuTu80 cells is clearly distinct from apoptotic damage, in that it does not lead to generation of oligonucleosomes. However, like apoptosis, HuTu80 fragmentation is caused by a diverse set of insults, including not only treatment with DNA synthesis inhibitors (as detailed here) but also treatment with Colcemid and growth for several days without media changes (data not shown). Therefore, it is possible that this fragmentation may be part of a general response to stress or cell-cycle perturbation in HuTu80 cells. It remains to be determined if this response shares any features with apoptosis, such as endonuclease induction, and if the discrete fragment size distribution bears any relationship to replicon size or organization.

In comparison with that in HuTu80 cells, fragmentation in HT29 and SW620 cells is different in terms of both fragment size distribution and pharmacological specificity. Neither aphidicolin or Colcemid (not shown) induced the broad fragment size distributions caused by CB3717 or FdUrd in HT29 and SW620 cells, indicating that this fragmentation response is not a general consequence of inhibiting DNA synthesis or cell cycle progression and that it may be a more specific consequence of TS inhibition. One explanation which would fit this profile is the "futile repair" hypothesis proposed by Goulian *et al.* (3), which proposes that fluoropyrimidine-induced DNA damage stems from intracellular accumulation of dUTP, leading to dUTP misincorporation and, eventually, to saturation of the uracil-*N*-glycosylase repair system.

Regardless of their biochemical mechanisms, an important issue in evaluating the therapeutic significance of these fragmentation phenomena is their relationship to drug-induced cell killing. We found that, within each cell line, drug-induced cytotoxicity and fragmentation had similar time and concentration dependence, consistent with the hypothesis that formation of this damage is part of the cytotoxic process. Over the range of conditions used in these experiments it was generally observed that fragmentation did not occur unless surviving fraction was reduced to about 0.1–0.2 or lower. This is approximately the same level of cell killing caused by the lowest radiation doses capable of producing fragmentation detectable by PFGE under these conditions (≈5 Gy) (18). These thresholds are probably due to the fact that the largest DNA pieces that can be resolved by PFGE are a few percent of an intact human chromosome. As a result, double-strand DNA breaks induced by either drugs or radiation can be detected only when treatment conditions are used that produce (on the average) many lesions per cell and, correspondingly, a substantial degree of cell killing.

More precise analysis of the relationship between fragmentation and cytotoxicity is difficult because $F_{released}$ is not necessarily a linear correlate of the number of DNA double-strand breaks present in drug-treated cells. Even in γ-irradiated cells, in which gross DNA breakage is presumably random, the function relating $F_{released}$ and radiation dose is sigmoidal rather than linear (19). In the present case the relationship between double-strand break frequency and $F_{released}$ is further complicated by differences in the distribution of break sites among the three cell lines. Therefore, although there may be a consistent relationship between $F_{released}$ and break frequency within a particular cell line, this relationship may vary between cell lines, and it is probably not informative to compare $F_{released}$ values between cell lines having markedly different fragment size distributions.

Although HT29 and SW620 cells give rise to fragment populations with similar size distributions, SW620 cells require either longer incubation times or higher concentrations of FdUrd to achieve a given level of fragmentation or cytotoxicity than do HT29 cells. Because these two cell lines are equally sensitive to inhibition of TS activity, FdUrd resistance in SW620 cells is not likely due to factors leading up to ternary complex formation, such as decreased FdUrd uptake, deficiency of reduced folate cofactor, or TS overproduction. If the uracil misincorporation/misrepair mechanism discussed above is responsible for DNA damage in these two cell lines, it is possible that SW620 resistance might be traced to a difference in dUTP accumulation, uracil-*N*-glycosylase activity, or some other component of the uracil excision-repair system. Such a finding would represent the characterization of a heretofore undocumented mode of resistance to fluoropyrimidines and would offer a potential target for overcoming such resistance.

In summary, we have demonstrated that significant diversity exists in the processes by which FdUrd-treatment induces DNA double-strand breaks among HCT cell lines. The data presented here do not exclude participation of some previously hypothesized mechanisms for FdUrd-induced damage, such as uracil misincorporation/misrepair or induction of endonuclease activity. Rather, they indicate that the dominant mechanism for fragmentation may vary from one cell line to another and that both must be considered in the analysis of the mechanisms of cytotoxicity stemming from TS inhibition.

This work was supported by National Institutes of Health Grant P01-CA42761, a grant from the Biomedical Research Council of the University of Michigan Medical School, a Lawrence Upjohn Fellowship (to C.E.C.), and a Predoctoral Fellowship from the University of Michigan Medical School Cancer Research Committee (to C.E.C.).

1. Grem, J. L. (1990) in *Cancer Chemotherapy: Principles and Practice*, eds. Chabner, B. A. & Collins, J. M. (Lippincott, Philadelphia), pp. 180–224.
2. Grem, J. L., Hoth, D. F., Hamilton, J. M., King, S. A. & Leyland-Jones, B. (1987) *Cancer Treat. Rep.* **71**, 1249–1264.
3. Goulian, M., Bleile, B. M., Dickey, L. M., Grafstrom, R. H., Ingraham, H. A., Neynaber, S. A., Peterson, M. S. & Tseng, B. Y. (1986) *Adv. Exp. Med. Biol.* **195**, 89–95.
4. Ayusawa, D., Arai, H., Wataya, Y. & Seno, T. (1988) *Mutat. Res.* **200**, 221–230.
5. Kyprianou, N. & Isaacs, J. T. (1989) *Biochem. Biophys. Res. Commun.* **165**, 73–81.
6. Finney, M. (1989) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Wiley, New York), Vol. 1, pp. 2.5.11–15.
7. VanDevanter, D. R. (1992) *Nucleic Acids Res.* **20**, 1148.
8. Sellins, K. S. & Cohen, J. J. (1987) *J. Immunol.* **139**, 3199–3206.
9. Yalowich, J. C. & Kalman, T. I. (1985) *Biochem. Pharmacol.* **34**, 2319–2324.
10. DeLean, A., Munson, P. J. & Rodbard, D. (1978) *Am. J. Physiol.* **235**, E97–E102.
11. Dusenbury, C. E., Davis, M. A., Lawrence, T. S. & Maybaum, J. (1991) *Mol. Pharmacol.* **39**, 285–289.
12. Fritz, R. B. & Musich, P. R. (1990) *BioTechniques* **9**, 542–550.
13. Kaufmann, S. H. (1989) *Cancer Res.* **49**, 5870–5878.
14. Washtien, W. L. (1984) *Mol. Pharmacol.* **25**, 171–177.
15. Jackson, R. C., Jackman, A. L. & Calvert, A. H. (1983) *Biochem. Pharmacol.* **32**, 3783–3790.
16. Wataya, Y., Hazawa, T., Watanabe, K., Hirota, Y. & Yoshioka-Hiramoto, A. (1988) *Nucleic Acids Symp. Ser.* 53–55.
17. Walker, P. R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J. F. & Sikorska, M. (1991) *Cancer Res.* **51**, 1078–1085.
18. Lawrence, T. S., Davis, M. A., Maybaum, J., Stetson, P. L. & Ensminger, W. D. (1990) *Radiat. Res.* **123**, 192–198.
19. Blöcher, D. (1990) *Int. J. Radiat. Biol.* **57**, 7–12.