

Increased Levels of DNA Lesions Induced by Leucovorin-5-Fluoropyrimidine in Human Colon Adenocarcinoma¹

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ABSTRACT

Leucovorin augments the growth inhibitory effect of 5-fluoropyrimidines on neoplastic cells. The effect is paralleled by much higher levels of DNA fragmentation than in cells treated with 5-fluoropyrimidines alone at the same concentration. The lesions are induced by a mechanism independent of incorporation of the drug into DNA, in all probability due to reduced repair of DNA lesions induced independently of the drug treatment. Thymidine added after the treatment with fluoropyrimidines partly rescues the cells and reduces the level of DNA fragmentation.

INTRODUCTION

The 5-fluoropyrimidines are important antineoplastic agents used in the clinic. The effects of 5-fluoropyrimidines against tumor cells have been ascribed traditionally to three major mechanisms: (a) metabolism to FdUMP³, which is an important inhibitor of thymidylate synthase resulting in reduced synthesis of thymidine ("thymineless death"); (b) incorporation of the drug into RNA; and (c) incorporation of the drug into DNA. Drug resistance has been shown to involve amplification (by a factor of 30-100) of the gene for thymidylate synthase (1). So far it has not been possible to determine the relative importance played by each mechanism.

Furthermore the 5-fluoropyrimidines induce DNA lesions without being incorporated into DNA (2). The mechanism responsible for the induction of DNA lesions is not clearly established. A parallel has been made to the induction of DNA lesions by hydroxyurea and methotrexate, proposing that the lesions arise due to reduced levels of nucleotides resulting in inefficient DNA repair of lesions which appear in the DNA independent of the drug treatment (2-4).

The lesions appearing independent of the drug treatment are induced due, e.g., to misincorporation of uridine during DNA synthesis, deamination of cytosine, adenosine, or guanosine, and loss of bases resulting in apurinic/apyrimidinic sites. The number of lesions that appear per day in one human cell has roughly been estimated as 10,000 purines and several hundred pyrimidines (5).

The inhibition of thymidylate synthase involves the formation of a complex in which the enzyme is covalently linked to FdUMP, which in turn is covalently linked to a folate cofactor, 5,10-methylene-tetrahydrofolate (6, 7). Under physiological conditions the complex is stabilized by high concentrations of the folate cofactor (8, 9).

To further examine the ability of the 5-fluoropyrimidines to induce DNA lesions we carried out the experiments reported here, in which the growth inhibition effect of the 5-fluoropyrimidines was augmented by D,L-N⁵-formyltetrahydrofolic acid

(leucovorin). It is known that this combination shows a synergistic effect (10, 11). We report here, using human colon adenocarcinoma cells, that the increased cytotoxicity is paralleled by increased levels of DNA lesions. The lesions arise independently of any incorporation of the drug into DNA.

MATERIALS AND METHODS

Cells, Culture Methods, Growth Inhibition, and Labeling with Tritiated Thymidine. Human colon adenocarcinoma cells (WiDr), obtained from American Type Culture Collection, Bethesda, MD, were grown as described earlier (12). Survival of drug-treated cells was determined by the outgrowth method described by Li and Kaminskas (3). Portions of treated and untreated cells were incubated for 5 days with daily changes of medium. The level of cell survival in treated cultures was measured by determining the difference in the numbers of cell doublings in untreated and treated cell cultures.

To obtain cells with prelabeled DNA, the following incubations were performed: 10⁶ cells were seeded in small culture dishes (35 x 10 mm) containing 3 ml of medium with added [³H]thymidine (30 μCi; 20 Ci/mmol; Amersham, Inc.). After 24 h the medium was replaced with fresh medium without thymidine, and after another 24 h the cells were used for drug treatment.

5-FUra, 5-FdUrd, and leucovorin were obtained from Sigma, Inc. Aphidicolin was obtained from Boehringer/Mannheim.

Cell Lysis. The incubation medium was drained off from the culture dish, and the cells were rinsed twice with cold phosphate-buffered saline. Cell lysis was performed in the dark at 0°C by addition of 2.25 ml of 0.03 M NaOH. After 30 min the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl/0.02 M NaH₂PO₄. For a more detailed description of the procedure see Refs. 2 and 13. Finally the solution was made 1% with regard to sodium dodecyl sulfate.

Agarose Gel Electrophoresis. Agarose flat bed gels (0.75%) were made as described earlier (12). The electrophoretic separation of the DNA was performed using an LKB Multiphor electrophoretic system. After the separation was terminated the gels were sliced into 1-mm-thick slices. The slices were incubated in scintillation fluid containing 3% Soluene 100 (Packard) and finally the radioactivity was measured in a scintillation counter (Packard).

RESULTS

5-Fluoropyrimidines induced DNA lesions via two different mechanisms, one involving the incorporation of drug into DNA and the other not involving the incorporation (2). 5-FUra shows both types of mechanisms whereas 5-FdUrd shows only the second type. Operationally one can distinguish between the two mechanisms by incubating the cells with or without aphidicolin, a specific inhibitor of DNA polymerase α (14). Polymerase α is responsible for the movement of the DNA replication fork and there is no incorporation of 5-fluoropyrimidines into DNA when the enzyme is inhibited, thus allowing the detection only of the second mechanism.

DNA-damaging agents can give rise to many different types of modifications in DNA, the damage often resulting in alkali-labile regions (15). The regions arise either through enzymatic strand scission as part of a repair process or through direct chemical alterations in the DNA molecule, e.g., incorporation of drug into DNA.

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³ The abbreviations used are: FdUMP, 5-fluorodeoxy-uridine monophosphate; 5-FUra, 5-fluorouracil; 5-FdUrd, 5-fluorodeoxyuridine.

We detect the DNA lesions by lysing the cells in dilute alkali (2, 13). The DNA is denatured during the alkaline treatment and if lesions exist in the DNA, fragments are released from bulk DNA. When the solution is then neutralized the high molecular weight DNA is renatured whereas the released DNA fragments remain as single-stranded DNA. The fragments can then be separated from bulk DNA by agarose gel electrophoresis.

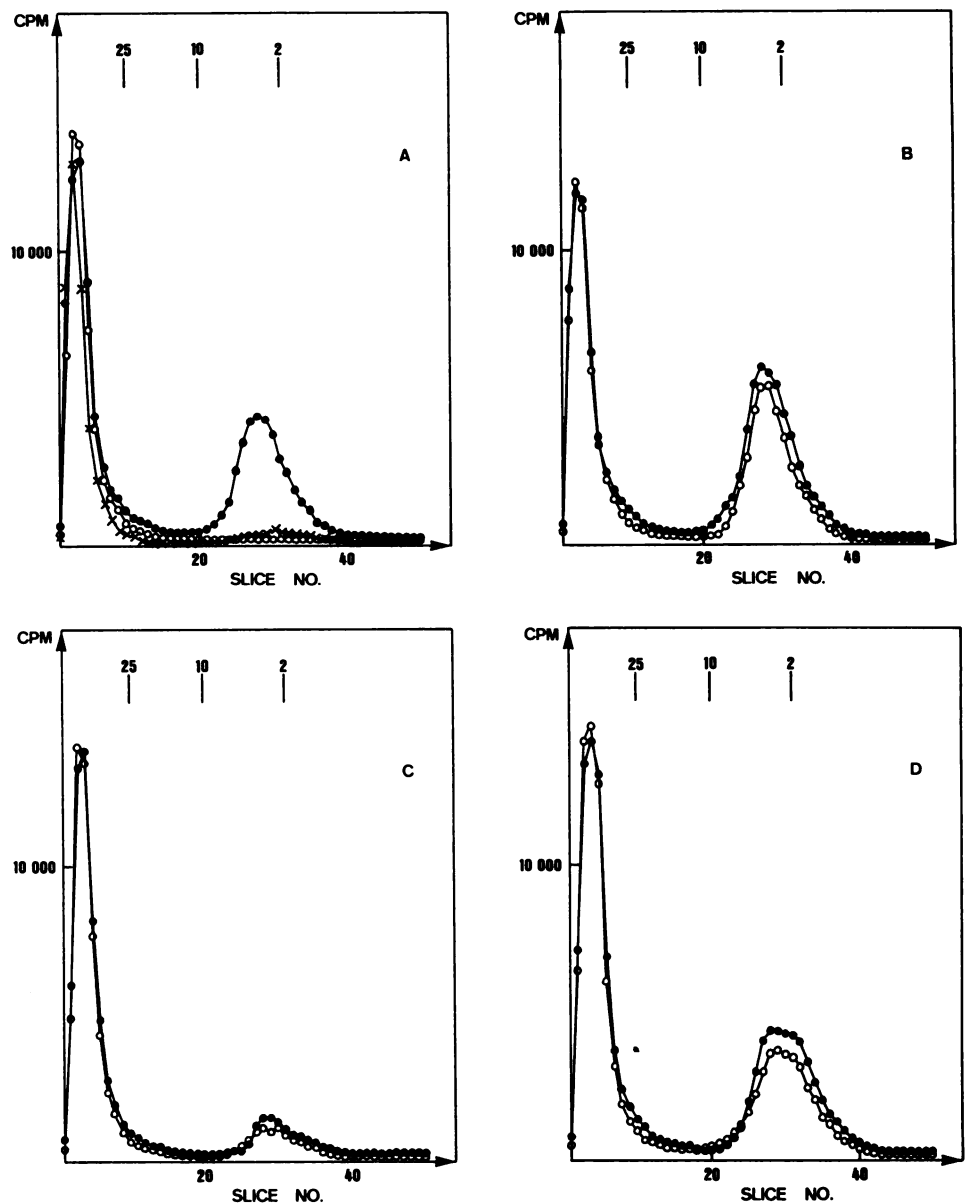
Fig. 1 shows gel separations of prelabeled DNA obtained from human colon adenocarcinoma cells treated with 5-FUra (or 5-FdUrd) in the absence or presence of aphidicolin. The high molecular weight DNA is located at slices 3–6 whereas the DNA fragments, released from bulk DNA during the cell lysis in dilute alkali, are located at slices 20–30. Aphidicolin prevents the appearance of DNA fragmentation in cells treated with 5-FUra for short times (60 min) whereas prolonged incubation (24 h) with 5-FUra or incubation with 5-FdUrd is not affected by aphidicolin. Hence the mechanism involving the incorporation of drug into DNA dominates during short incubations whereas during prolonged incubations the second mechanism dominates. The data are essentially the same as described in (2).

Combined Treatment with Leucovorin and 5-FUra, 5-FdUrd. Treating cells with leucovorin is known to increase the growth inhibitory effect of 5-fluoropyrimidines. It has recently been reported that there is a synergistic effect with high cytotoxicity (10, 11). Our own outgrowth experiments show the same results (see below, "Growth Inhibition"). To examine the DNA we incubated cells with prelabeled DNA in medium containing leucovorin (10 μ M) for 4 h and added 5-FUra (or 5-FdUrd) during the last 60 min. The cells were then immediately lysed or lysed after incubation for 24 h in the absence or presence of leucovorin.

Fig. 2, *A* and *B* shows that in the cells lysed immediately after the treatment with 5-fluoropyrimidines there is a high level of DNA fragmentation, much higher than in cells treated with 5-fluoropyrimidines alone. In cells incubated with leucovorin for the next 24 h all DNA is fragmented. Furthermore by varying the concentration of leucovorin from 0.1–10 μ M one increases the level of DNA fragmentation with the higher concentrations (Fig. 2*C*).

Hence DNA fragmentation is dramatically increased when leucovorin is added.

Fig. 1. Fragmentation of DNA from 5-fluoropyrimidine-treated cells. Cells with pre-labeled DNA were treated with 5-FUra (0.1 mM) for 60 min (*A*) or 24 h (*B*). In parallel cells were also incubated with aphidicolin (10 μ g/ml) for 30 min prior to the addition of 5-FUra. The subsequent incubation with 5-FUra was then performed in the presence of aphidicolin (O). Cells treated with 5-FUra (●) or aphidicolin (x) alone. The cells were lysed in dilute alkali and the DNA was then separated in 0.75% agarose gels. Numbers at the top, (25, 10, and 2), size (in kilobases) and location of single-stranded DNA markers. Experiments were also performed using 5-FdUrd (0.1 mM) instead of 5-FUra. Incubations were for 60 min (*C*) and for 24 h (*D*).



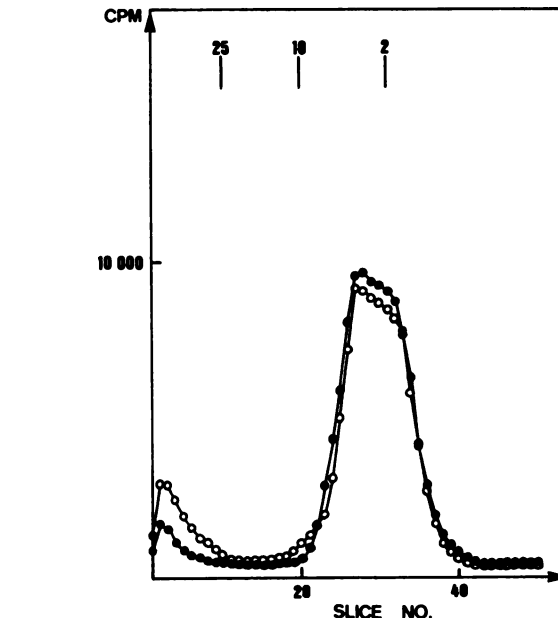
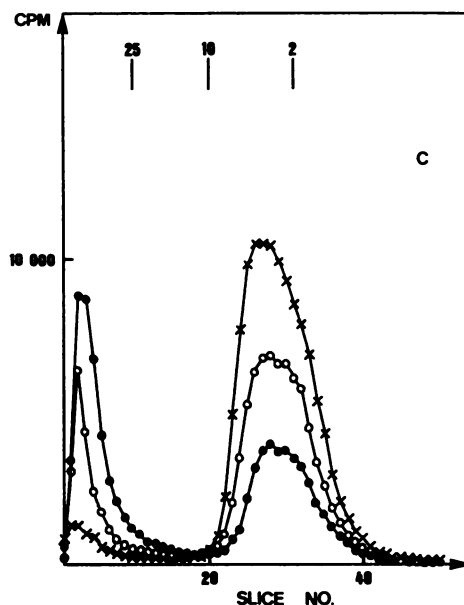
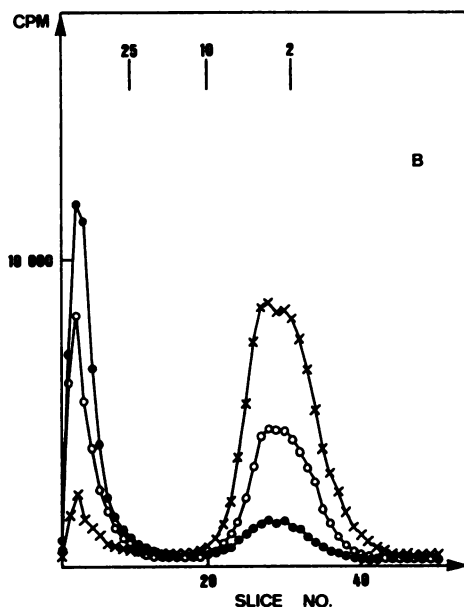
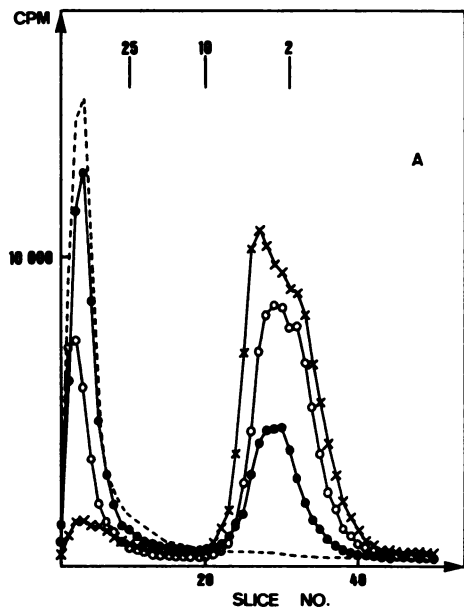


Fig. 3. Treatment with aphidicolin. Cells with pre-labeled DNA were treated with leucovorin ($10 \mu\text{M}$) for 4 h with aphidicolin ($10 \mu\text{g/ml}$) added during the last 90 min. and 5-FUra (0.1 mM) (\odot) or 5-FdUrd (0.1 mM) (\circ) added during the last 60 min. The cells were then incubated for 24 h in the presence of aphidicolin and leucovorin before cell lysis. The DNA was then separated in 0.75% agarose gels. Numbers (25, 10, and 2) at the top, size (in kilobases) and location of single-stranded DNA markers.

Nonprevention by Aphidicolin of Leucovorin Enhanced DNA Fragmentation. Next we examined whether aphidicolin prevents the leucovorin enhanced DNA fragmentation. Cells with pre-labeled DNA were treated with leucovorin for 4 h with aphidicolin added during the last 90 min and 5-FUra (or 5-FdUrd) added during the last 60 min. The cells were then lysed after incubation for 24 h in the presence of leucovorin and aphidicolin.

Fig. 3 shows that aphidicolin does not prevent the DNA fragmentation. The levels of DNA fragmentation are the same level as in cells not treated with aphidicolin. This indicates that the second mechanism to induce DNA lesions dominate.

Prevention by Thymidine of Leucovorin Enhanced DNA Fragmentation. Purines can be used to protect cells from 5-fluoropyrimidine cytotoxicity (16). In agreement with this thymidine added after drug treatment substantially rescues the cells from cytotoxicity of leucovorin-5-fluoropyrimidines (see below, "Growth inhibition" and Ref. 11). We therefore tested if this effect is paralleled by a reduced level of DNA lesions. Cells with pre-labeled DNA were treated with leucovorin for 4 h with 5-FUra (or 5-FdUrd) added during the last 60 min. The cells were then incubated for 24 h in the presence of thymidine ($0.1 \mu\text{M}$) and leucovorin.

Fig. 4 shows that the addition of thymidine partly, but not completely, prevents DNA fragmentation.

Growth Inhibition. Outgrowth experiments were performed as described by Li and Kaminskas (3). The cells were treated with leucovorin for 4 h and 5-FUra/5-FdUrd added during the

Fig. 2. Treatment with leucovorin and 5-fluoropyrimidines. A, cells with pre-labeled DNA were treated with leucovorin ($10 \mu\text{M}$) for 4 h with 5-FUra (0.1 mM) added during the last 60 min (\odot) and then incubated for 24 h in the absence (\circ) or presence of leucovorin (\times). (---), cells treated with leucovorin alone for 24 h. The cells were lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. Numbers at the top (25, 10, and 2), size (in kilobases) and location of single-stranded DNA markers. B, same protocol as in A but 5-FdUrd was used instead of 5-FUra. C, incubations with increasing doses of leucovorin, $0.1 \mu\text{M}$ (\odot), $1 \mu\text{M}$ (\circ), or $10 \mu\text{M}$ (\times). The incubation after the treatment with 5-FUra was always done in the presence of leucovorin. Otherwise the protocol was the same as in A.

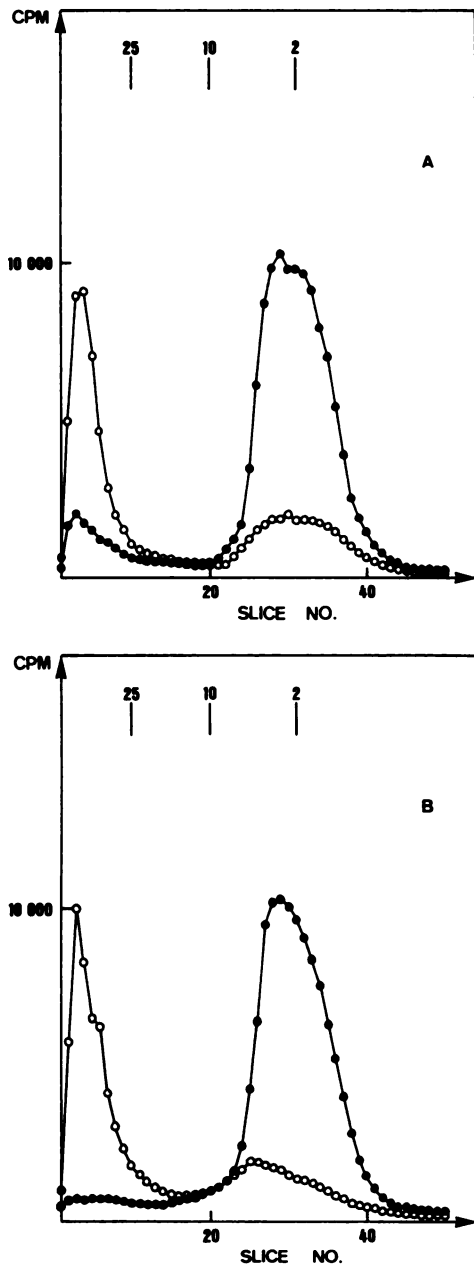


Fig. 4. Treatment with thymidine. *A*, cells with prelabeled DNA were treated with leucovorin ($10 \mu\text{M}$) for 4 h with 5-FUra (0.1 mM) added during the last 60 min and then incubated for 24 h in the presence of leucovorin (\bullet) or leucovorin and thymidine ($0.1 \mu\text{M}$) (\circ). The cells were lysed in dilute alkali and the DNA was then separated in 0.75% agarose gels. Numbers (25, 10, and 2) at the top, size (in kilobases) and location of single-stranded DNA markers. *B*, incubations with 5-FdUrd instead of 5-FUra but otherwise the same protocol as in *A*.

last 60 min. The cells were then incubated for 24 h in the presence of leucovorin or thymidine and leucovorin.

Fig. 5 shows that there is a pronounced growth inhibition of cells treated with leucovorin-5-fluoropyrimidines. The addition of thymidine partly rescues the cells. The results are the same as described in Refs. 10 and 11.

Inhibition of DNA Synthesis. The rate of DNA synthesis was measured by incubating 10^5 cells with [^3H]thymidine for 5 min after cell treatment with leucovorin for 4 h with 5-FUra/5-FdUrd added during the last 60 min. The trichloroacetic acid-precipitable portion was then measured. The results show that for both 5-FUra and 5-FdUrd the DNA synthesis is reduced to 5–10% of the control value.

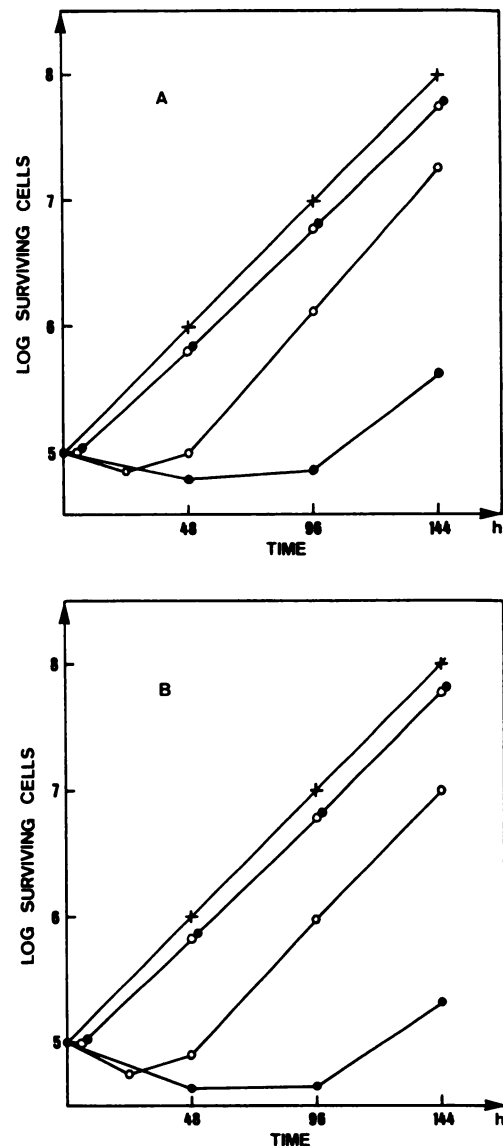


Fig. 5. Outgrowth experiments. *A*, effect of treatment with leucovorin ($10 \mu\text{M}$) for 4 h with 5-FUra (0.1 mM) added during the last 60 min. The cells were then incubated for 24 h in the presence of leucovorin (\bullet) or leucovorin and thymidine ($0.1 \mu\text{M}$) (\circ). This point represent 0 h. Changes in the numbers of cells are plotted versus the time of incubation in fresh medium. \circ , \bullet , cells treated with 5-FUra alone; \times , control cells. *B*, incubations with 5-FdUrd instead of 5-FUra but otherwise the protocol was the same as in *A*.

DISCUSSION

Cytotoxicity of 5-fluoropyrimidines is dramatically increased by a pretreatment with leucovorin, a folic acid analogue (10, 11). In this paper we have examined the effect of this combination on the DNA of human colon adenocarcinoma cells. We show that the level of DNA fragmentation of prelabeled DNA is dramatically increased. Twenty-four h after treatment with 5-FUra (or 5-FdUrd) almost all prelabeled DNA is fragmented when the cells have also been treated with leucovorin ($10 \mu\text{M}$). The higher the dose of leucovorin the more efficient is DNA fragmentation. In agreement when the levels of cellular folate have been determined it has been found that cells with high levels of folates are more sensitive to 5-fluoropyrimidines (17, 18).

Aphidicolin does not prevent DNA fragmentation, indicating that the second mechanism by which the fluoropyrimidines induce DNA lesions prevails during this experimental situation. The second mechanism represents the induction of DNA le-

sions without the incorporation of drug into DNA. The lesions are probably due to inefficient DNA repair of DNA lesions occurring independently of the drug treatment (2-4).

The inefficient DNA repair is due to reduced levels of nucleotides. This proposal is based on the fact that methotrexate and hydroxyurea treatment results in progressive formation of DNA lesions and that all three drugs reduce the synthesis of nucleotides. 5-Fluoropyrimidines reduce or abolish production of thymidylate because of their effect on thymidylate synthase. Presumably the increase in the level of DNA fragmentation represents the rate of net accumulation of unrepaired DNA lesions. With more efficient inhibition of DNA repair there is faster accumulation of DNA fragments.

In earlier publications of the combined action of 5-fluoropyrimidines and leucovorin it has been proposed that the high level of leucovorin should stabilize the complex between 5-FdUMP, thymidylate synthase, and folic acid, resulting in more efficient reduction in the production of nucleotides (10, 11). If this proposal is correct one should expect further reduction in the efficiency of DNA repair resulting in higher levels of DNA fragmentation. The experimental data reported here show higher levels of DNA fragmentation in support of this proposal. Also in agreement with this suggestion we have found that DNA synthesis has rapidly declined. Furthermore control experiments with added thymidine rescued the cells from the toxic effect and caused a decrease in the level of DNA fragmentation. It is believed that the ability of thymidine to rescue cells is due to substitution for the shortage of this nucleoside, restoring the ability of the cells to synthesize and repair DNA.

Both 5-FUra and 5-FdUrd are prodrugs which require intracellular metabolism in order to induce cytotoxicity. There exist many possibilities to modulate cytotoxicity because of the complicated pathways whereby the drugs exert their cytotoxicity. The combination of leucovorin and 5-fluoropyrimidines is an obvious example of these possibilities.

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