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DNA Lesions in Human Neoplastic Cells and Cytotoxicity of 5-Fluoropyrimidines[1]

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ABSTRACT

We have examined the induction of alkali-labile regions in DNA of human neoplastic cells treated with 5-fluorouracil and 5-fluorodeoxyuridine. 5-Fluorouracil induces DNA lesions by two mechanisms: incorporation of drug into DNA and a second mechanism not involving the incorporation. The second mechanism is seen in cells treated with aphidicolin, a specific inhibitor of DNA polymerase α, to stop the movement of the DNA replication forks. 5-Fluorodeoxyuridine is not incorporated into DNA of these cells; only the second mechanism of induction of alkali-labile DNA is detected. The second mechanism is in all probability due to inefficient DNA repair of normally occurring defects in purine and pyrimidine residues. Furthermore there is a correlation between increasing levels of alkali-labile regions in the DNA and cytotoxicity of the drugs. This may be one explanation for the cytocidal effects of 5-fluoropyrimidines.

INTRODUCTION

Methotrexate treatment causes accumulation of DNA strand breaks in the cells (1). The amount of strand breaks correlates with methotrexate cytotoxicity. It was proposed that cell death is due to lethal accumulation of strand breaks and that the accumulation of strand breaks results from inefficient DNA repair, because of the inhibition of synthesis of thymidylic acid and of purine nucleotides.

In this paper we have examined the effect of 5-fluoropyrimidines (5-FUra[3] and 5-FdUrd) on DNA of malignant cells. We have asked the question of whether a similar mechanism for cell death occurs during treatment with 5-fluoropyrimidines as during treatment with methotrexate. 5-FUra is frequently used in the treatment of various human malignancies as, *e.g.*, breast and colon carcinoma. At least three mechanisms are involved in inducing cytotoxicity: inhibition of the enzyme thymidylate synthetase; incorporation of drug into RNA; and the incorporation of drug into DNA. The balance between the different mechanisms can be changed by metabolic modulation (2) involving, *e.g.*, selective induction of normal cell arrest, metabolic canalization, selective protection, and/or inhibition of repair mechanisms.

We have earlier analyzed the interaction between 5-FUra and DNA of human colon adenocarcinoma (3). 5-FUra is to a degree incorporated into DNA, and it induces formation of alkali-labile high-molecular-weight DNA as well as alkali-labile DNA replication intermediates within the first 60 min of drug treatment. The induction of these lesions in the DNA can be prevented by preincubating the cells with aphidicolin, a specific inhibitor of DNA polymerase α (4), to stop the movement of the replication forks and therefore the incorporation of 5-FUra into DNA.

In this paper we show that, even if there is no incorporation of 5-FUra into DNA due to simultaneous treatment with aphidicolin, there appears 12–24 h later alkali-labile DNA regions. Similar results are obtained with 5-FdUrd and methotrexate. There is a progressive formation of alkali-labile regions in the DNA which correlates with the cytotoxicity of the drugs. This may be one explanation for the cytocidal effects of 5-fluoropyrimidines.

MATERIALS AND METHODS

Cells, Culture Methods, Cytotoxicity Analysis, and Labeling with Tritiated Thymidine. Human colon adenocarcinoma cells (WiDr), obtained from American Type Culture Collection, Bethesda, MD, were grown as earlier described (3). Survival of drug-treated cells was determined by the outgrowth method described by Li and Kaminskas (1). 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: 10^6 cells were seeded in a small culture dish (35 x 10 mm) containing 3 ml of medium with added tritiated thymidine (30 μCi; 22 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.

Incubations with tritiated 5-FUra or tritiated 5-FdUrd were performed according to the protocol of Mayor *et al.* (5). Cells were incubated in serum-free medium with 1 μM tritiated 5-FUra (12 Ci/mmol; Amersham, Inc.) or 1 μM tritiated 5-FdUrd (10 Ci/mmol; New England Nuclear, Inc.) for 12 h.

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 the 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 NaH_2PO_4. For a more detailed description of the cell lysis procedure, see Ref. 6. Finally the solution was made 1% with regard to sodium dodecyl sulfate.

Agarose Gel Electrophoresis and Cs_2SO_4 Gradient Centrifugations. Agarose flat bed gels (0.75%) were made as described earlier (7). 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).

For Cs_2SO_4 gradient centrifugations, the DNA was dissolved in 0.02 M Tris-HCl (pH 8)-0.001 M disodium EDTA, and Cs_2SO_4 was added to a refractive index of 1.375. Centrifugation was carried out at 40,000 rpm at 20°C for 48 h in a Ti 50 rotor using a Beckman ultracentrifuge. After centrifugation, fractions were collected by siphoning from the bottom of the centrifugation tube. The DNA was precipitated with cold trichloroacetic acid, and the radioactivity was measured by scintillation counting.

RESULTS

Treatment with 5-FUra. During a drug treatment for 60 min, there is some incorporation of 5-FUra into DNA (3, 5, 8, 9). This induces alkali-labile regions in the DNA, which appear as strand breaks when the cells are lysed in dilute alkali. The DNA fragments that are released from bulk DNA are separated from the high-molecular-weight DNA by agarose gel electrophoresis. The incorporation of 5-FUra into DNA does not occur in cells treated with aphidicolin, an inhibitor of DNA polymerase α

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[3] The abbreviations used are: 5-FUra, 5-fluorouracil; 5-FdUrd, 5-fluorodeoxyuridine.

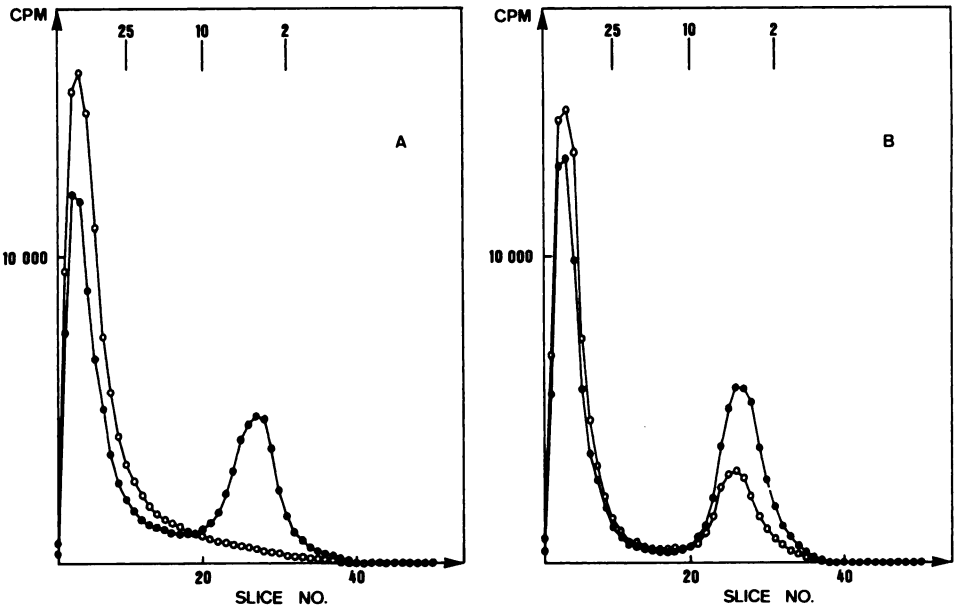

Fig. 1. *A*, treatment with 5-FUra. Human colon adenocarcinoma cells with prelabeled DNA were incubated with 5-FUra (1 mM) for 60 min (●) or aphidicolin (10 μg/ml) for 30 min followed by aphidicolin and 5-FUra for 60 min (○). The cells were lysed in dilute alkali, and the DNA was then separated in 0.75% agarose gels. The *numerals* across the *top* (*25*, *10*, and *2*) denote the size (in kilobases) and location of single-stranded DNA markers. *B*, cells treated with aphidicolin (10 μg/ml) for 30 min and then with 5-FUra (1 mM) in the presence of aphidicolin for 12 h (○) or 24 h (●).

(4), which stops the movement of the replication forks (10).

To examine whether alkali-labile DNA appears independently of the movement of the replication forks, we performed the following experiments. Cells with prelabeled DNA were incubated with aphidicolin (10 μg/ml) for 30 min and then with aphidicolin and 5-FUra (1 mM) for an additional 12 or 24 h.

The cells were lysed in dilute alkali as described earlier. This treatment results in partial fragmentation of the DNA and release from bulk DNA of DNA fragments induced by drug treatment. Cells not treated with drugs do not release DNA fragments. After neutralization of the solution, the DNA fragments appear as single-stranded DNA, whereas the bulk DNA appears as double-stranded DNA. The fragments are separated from bulk DNA by agarose gel electrophoresis.

The analysis shows that, in cells treated with 5-FUra for 60 min in the presence of aphidicolin, there is no DNA fragmentation (Fig. 1*A*). However, in cells incubated simultaneously with aphidicolin and 5-FUra for 12 or 24 h, there is DNA fragmentation, which increases with increasing duration of treatment (Fig. 1*B*).

Hence there exist two types of alkali-labile DNA in 5-FUra-treated cells. One type is due to the incorporation of the drug into DNA, and the other is independent of the incorporation.

Treatment with 5-FdUrd. It is possible that the early induction of alkali-labile regions in DNA due to incorporation of drug does not occur in dells treated with 5-FdUrd. To examine this we incubated cells with prelabeled DNA in medium containing 5-FdUrd (1 mM) for 60 min and then lysed the cells in dilute alkali (Fig. 2*A*). The results showed that, in contrast to the results obtained with 5-FUra, there is no fragmentation of the DNA.

Next we examined cells incubated with 5-FdUrd for 12 or 24 h (Fig. 2*B*) or cells preincubated with aphidicolin and then incubated with aphidicolin and 5-FdUrd for 12 or 24 h (Fig. 2*C*). In both situations one can now detect fragmentation of the DNA. The level of fragmentation is somewhat higher in cells incubated with only 5-FdUrd. In agreement with the data on 5-FUra, there is an increasing level of fragmentation with increasing duration of treatment with 5-FdUrd.

Hence in cells treated with 5-FdUrd, one can only detect the late accumulation of alkali-labile DNA regions.

Treatment with Methotrexate. As a control we performed similar experiments with methotrexate. It has been established earlier that methotrexate induces DNA strand breaks, although the drug is not incorporated into the DNA (1). Therefore we now performed control experiments that were patterned according to the protocol used above.

Cells with prelabeled DNA were incubated with methotrexate (10 μM) for either 60 min, 12 h, or 24 h (Fig. 3*A*). In parallel experiments cells were preincubated with aphidicolin, and then incubated with aphidicolin and methotrexate for either 60 min, 12 h, or 24 h (Fig. 3*B*). The cells were lysed in dilute alkali, and the DNA was then separated in agarose gels. The results showed that initially (treatment with methotrexate for 60 min) there is no fragmentation of the DNA. With increasing duration of the treatment there is increasing fragmentation.

The results are the same as those described in Ref. 1 and parallel the results described above regarding the late appearing DNA strand breaks.

Cytotoxicity Analysis. Cytotoxicity analyses were performed using an outgrowth method (1). Since cells are arrested at the G_1-S block during the treatment with aphidicolin, we have only examined cells not treated with aphidicolin. The results show that cytotoxicity increases with increasing duration of the drug treatment (Fig. 4).

Cs_2SO_4 Gradient Centrifugations. We have earlier shown that tritiated 5-FUra is incorporated into the DNA of our cells (3). Using the same labeling protocol we now also examined whether tritiated 5-FdUrd is incorporated into DNA. The results show that we cannot detect incorporation of tritiated 5-FdUrd into DNA (Fig. 5).

DISCUSSION

In this paper we have analyzed the induction of alkali-labile regions in the DNA of cells treated with 5-fluoropyrimidines (5-FUra or 5-FdUrd). The results show that 5-FUra induces lesions in DNA by two mechanisms, whereas 5-FdUrd induces lesions by one mechanism. Furthermore with increasing levels of DNA lesions, there is increasing cell death.

5-FUra differs by showing two mechanisms of induction of alkali-labile DNA. The first mechanism is the incorporation of

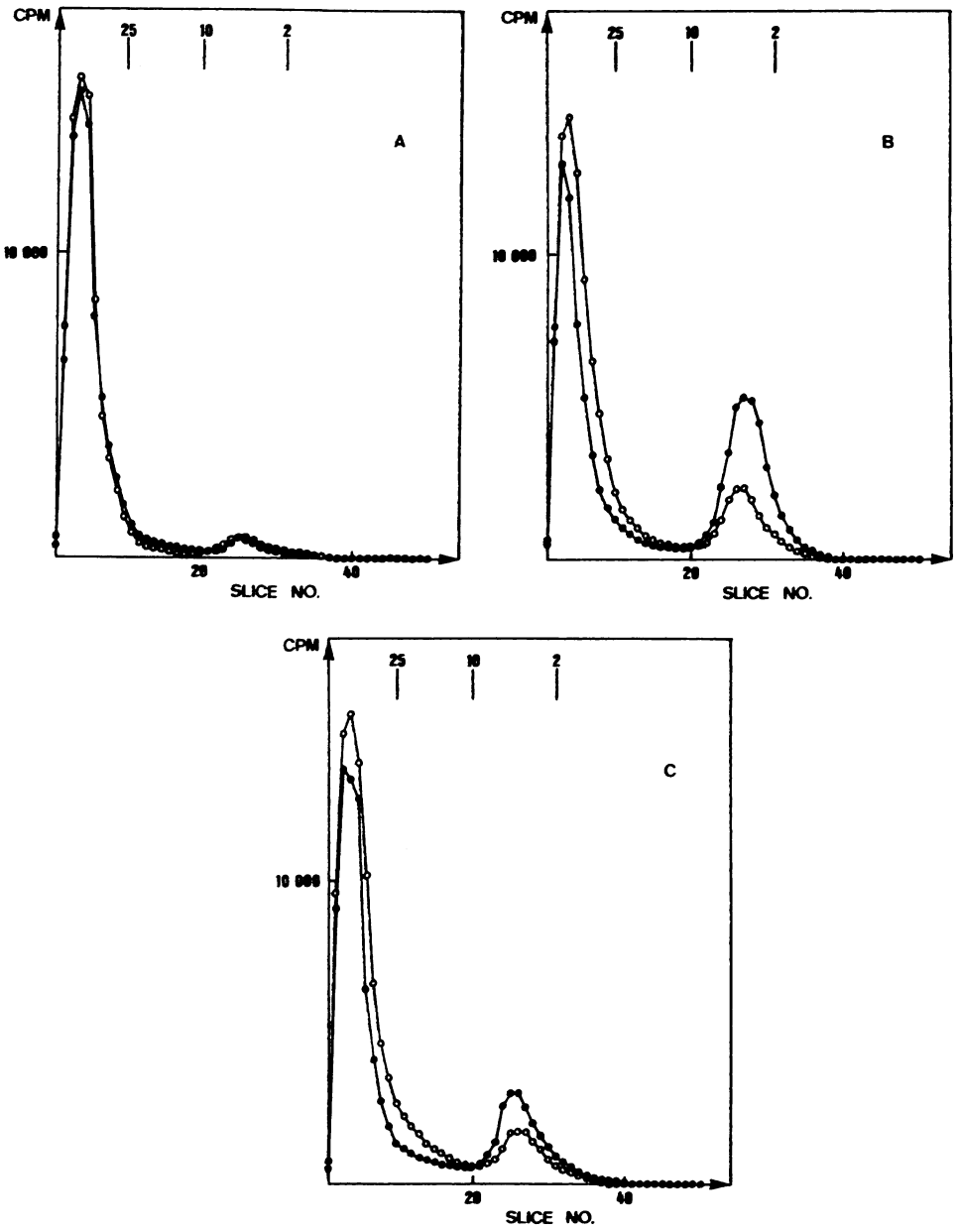

Fig. 2. *A*, treatment with 5-FdUrd. Human colon adenocarcinoma cells with prelabeled DNA were incubated with 5-FdUrd (1 mM) for 60 min (●) or aphidicolin (10 μg/ml) for 30 min followed by aphidicolin and 5-FdUrd for 60 min (○). The cells were lysed in dilute alkali, and the DNA was then separated in 0.75% agarose gels. The *numerals* across the *top* (*25*, *10*, and *2*) denote the size (in kilobases) and location of single-stranded DNA markers. *B*, cells treated with 5-FdUrd (1 mM) for 12 h (○) or 24 h (●). *C*, cells treated with aphidicolin (10 μg/ml) for 30 min and then with 5-FdUrd (1 mM) in the presence of aphidicolin for 12 h (○) or 24 h (●).

5-FUra into DNA. The second mechanism occurs independently of DNA synthesis. This mechanism is the same as that occurring in cells treated with 5-FdUrd and can operate in cells treated with aphidicolin. Aphidicolin is a specific inhibitor of DNA polymerase α (4) and therefore stops the movement of the replication forks. Consequently the second mechanism involves the appearance of strand breaks in mature DNA molecules.

Regarding the metabolism of 5-fluoropyrimidines, there exist differences in different cell types as well as different metabolism of 5-FUra and 5-FdUrd in the same cell (11). In our experimental material, a human colon adenocarcinoma cell line, one can detect incorporation of tritiated 5-FUra into DNA using Cs_2SO_4 gradients, but not the incorporation of tritiated 5-FdUrd. Our results are in agreement with the finding that 5-FUra and 5-FdUrd show different metabolic pathways. This may be different in different cell lines, *e.g.*, the human breast carcinoma cell line examined by Major *et al.* (5) and the human lymphoblasts examined by Ingraham *et al.* (9), where both tritiated 5-FUra and tritiated 5-FdUrd appear in the DNA.

Li and Kaminskas (1) have proposed that the second mechanism occurs due to inefficient DNA repair, resulting from deranged deoxyribonucleotide pools. It is known that human cells lose about 10,000 purine residues and several hundred pyrimidine residues from their DNA every day irrespective of whether the cells have been treated with drugs or not, *e.g.*, by deamination of cytosine to uracil, depurination, or depyrimidation (12). Therefore decreased availability of deoxyribonucleotide triphosphates should result in inefficient DNA repair and consequently increased levels of alkali-labile DNA. It is well established that 5-fluoropyrimidines change/deplete nucleotide pools (13).

The major growth-inhibitory effect of 5-FUra is believed to be associated with 5-fluorodeoxyuridine monophosphate, which in the presence of $N^{5,10}$-methylene-tetrahydrofolate binds covalently to the enzyme thymidylate synthetase (13–15). By blocking this enzyme, which catalyzes the final reaction in the *de novo* synthesis of thymidylic acid, eventually DNA synthesis

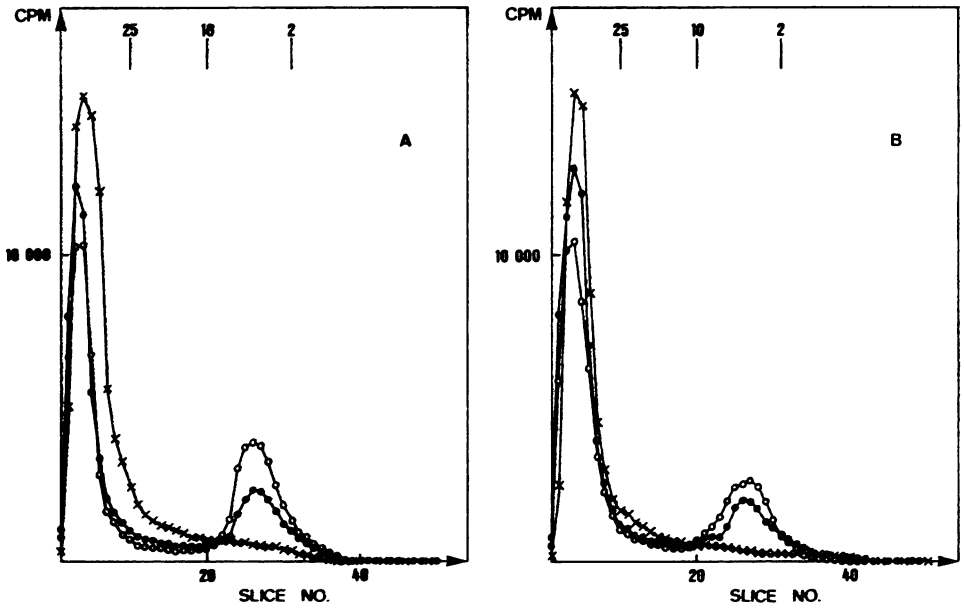

Fig. 3. *A*, treatment with methotrexate. Human colon adenocarcinoma cells with prelabeled DNA were incubated with methotrexate (10 μM) for 60 min (×), 12 h (●), or 24 h (○). The cells were lysed in dilute alkali, and the DNA was then separated in 0.75% agarose gels. The *numerals* across the *top* (*25*, *10*, and *2*) denote the size (in kilobases) and location of single-stranded DNA markers. *B*, cells treated with aphidicolin (10 μg/ml) for 30 min and then with methotrexate (10 μg/ml) in the presence of aphidicolin for 60 min (×), 12 h (●), or 24 h (○).

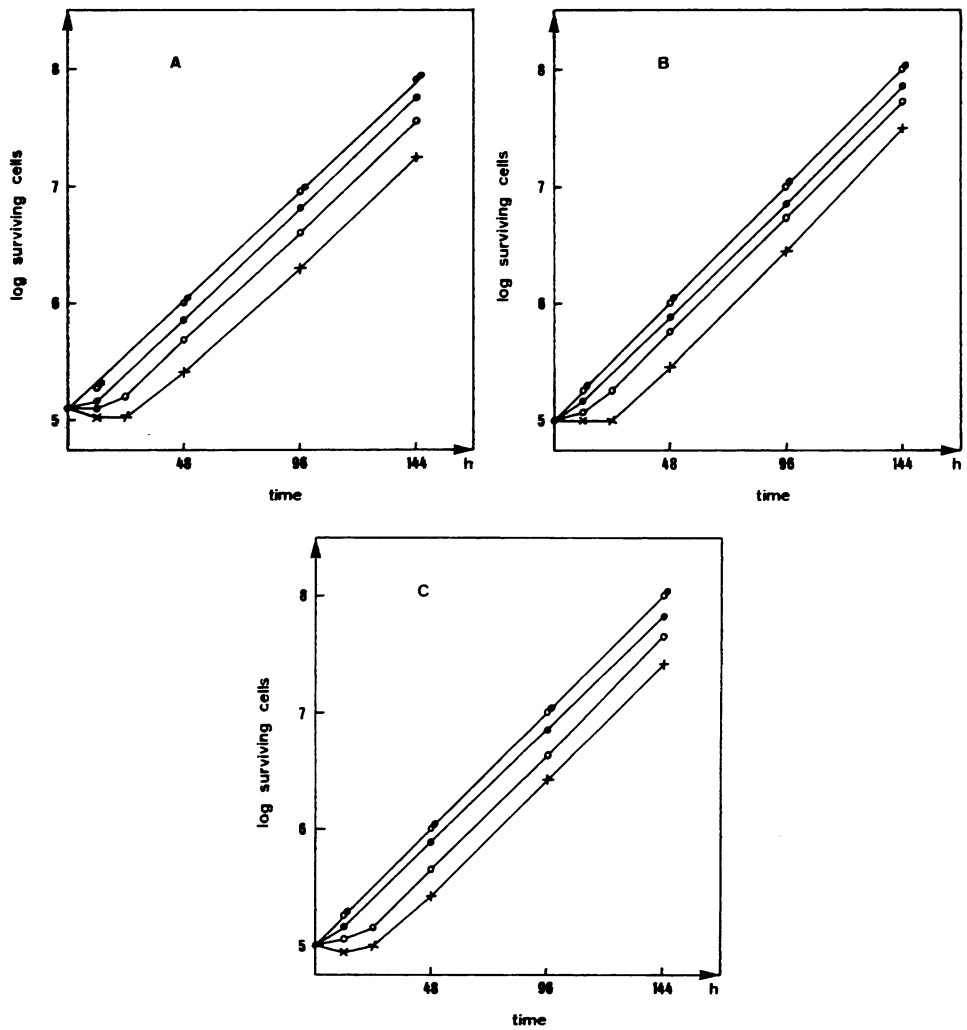

Fig. 4. Effect of 5-FUra (1 mM) (*A*), 5-FdUrd (1 mM) (*B*), and methotrexate (10 μM) (*C*) on outgrowth of human colon adenocarcinoma cells. The treatments with the drugs were 60 min (●), 12 h (○), or 24 h (×). ○●, untreated cells. Changes in the numbers of cells are plotted *versus* the time of incubation in fresh medium.

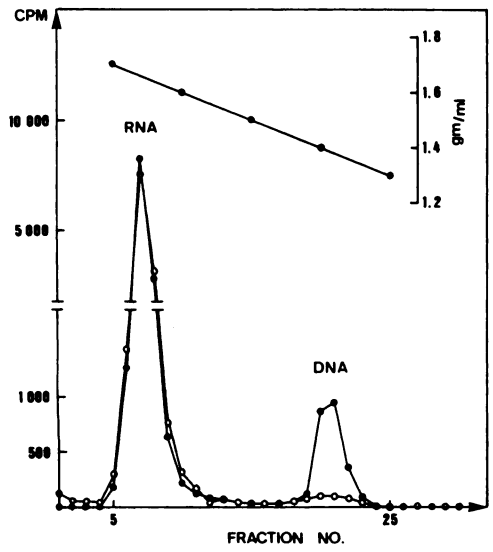

Fig. 5. Banding of DNA labeled with tritiated 5-FUra (●) or tritiated 5-FdUrd (○) in neutral Cs_2SO_4 gradients. The protocol was patterned after Major *et al.* (5). The cells were incubated with labeled drug for 12 h. *Inset scale,* amount of Cs_2SO_4 in g/ml in the different fractions.

is blocked.

One cannot, therefore, assume a priori that the association between 5-fluorodeoxyuridine monophosphate and thymidylate synthetase explains a cytocidal effect of the drugs. It is more likely that it explains a cytostatic effect. Cells can conceivably survive without growth or replication in the absence of DNA synthesis. The cytocidal effect may be due to the accumulation of alkali-labile DNA regions described here, possibly in combination with the incorporation of 5-fluoropyrimidines into RNA.

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