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LEUCOVORINE PREVENTS METHOTREXATE FROM INDUCING DNA LESIONS

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SUMMARY: Methotrexate induces DNA strand fragmentation. We show here that the induction of DNA lesions can be overcome by treatment with leucovorine, a folic acid analogue.

During treatment of cells with anti-neoplastic agents many different types of lesions in DNA appear, such as the formation of monoadducts, crosslinks and the incorporation of drug into DNA (1). Methotrexate is an inhibitor of the enzyme dihydrofolate reductase, which is responsible for maintaining the intracellular pool of folate in a reduced state (2,3).

Methotrexate does not in itself attack DNA. Nevertheless there exist DNA lesions in treated cells (4,5). It has been proposed that this is due to reduced and/or inhibited DNA repair of normally occuring DNA lesions, i.e. DNA lesions occuring in the DNA irrespective of the treatment with drug (4). It has been estimated that untreated cells normally lose about 10,000 purine residues and several hundreds of pyrimidine residues from its DNA every day (6).

Leucovorine, D,L-N^5-formyl-tetrahydrofolic acid, is used in clinical practice to overcome the toxic effect of methotrexate. We have now examined the effect of leucovorine on the ability of methotrexate to induce DNA lesions.

MATERIALS AND METHODS

A human colon adenocarcinoma cell line (WiDr), obtained from American Type Culture Collection, Bethesda, MD., was grown as monolayers (). For experiments the cells were seeded in small culture dishes (35x10 mm) containing 3 ml medium 24 h before the addition of 50 µCi

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tritiated thymidine (22 Ci/mmole, Amersham, U.K.) and the incubation performed for 24 h. The cells were used in experiments after cultivation in fresh medium for another 24 h.

Cell lysis was performed in the dark at 0°C by the addition of 2.25 ml of 0.03 M NaOH (pH 12.1). After 30 min the solution was neutralized by the addition of 0.09 ml 0.067 M HCl, 0.02 M NaH_2PO_4 and then made 0.5 % with regard to SDS (7,8,9,10).

The labelled DNA was separated in 0.75 % agarose gels (10). The gels were sliced in 1-mm-thick slices and the radioactivity was then measured in a Packard scintillation counter.

RESULTS

Although DNA damaging agents can give rise to many different types of modifications in DNA, the damage often results in alkali-labile regions (1). The regions either arise through enzymatic strand scission as part of a repair process or through direct chemical alterations of the DNA molecule.

We use a procedure to lyse cells in dilute alkali to partly denature the DNA. During the cell lysis the DNA starts to uncoil in the alkaline milieu. The uncoiling, which is initiated at

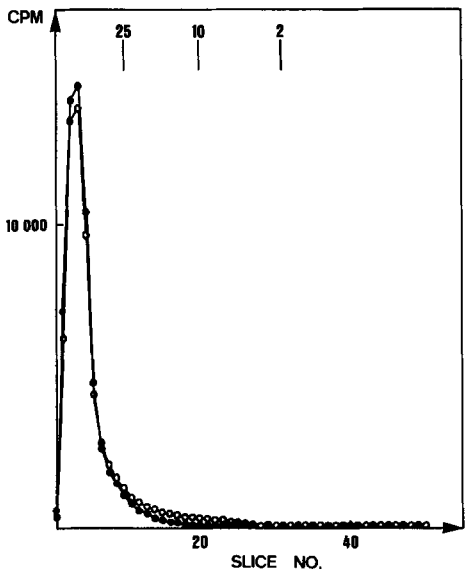

Fig. 1. Treatment with leucovorine. Human colon adenocarcinoma cells with pre-labelled DNA were incubated with leucovorine (10 µM) for 90 min (-●-). Control cells not treated with leucovorine (-o-). The cells were lysed in dilute alkali and the DNA then separated in 0.75 % agarose gels. The numerals across the top (25, 10 and 2) denote the size (in kb) and location of single-stranded DNA markers.

single-stranded gaps (and/or alkali-labile regions) results in denaturation of 20 kb regions of the DNA. When the solution is neutralized the DNA strands larger than 20 kb renature and form double-stranded DNA. The fragments smaller than 20 kb remains free in solution as single-stranded DNA (10). We have earlier shown that when cells with pre-labelled DNA are treated with methotrexate or 5-fluoropyrimidines, one can detect the release of single-stranded DNA fragments (5,7). The fragments are separated from the large double-stranded DNA by agarose gel electrophoresis.

First we examined whether leucovorine in itself induces alkali-labile regions in the DNA. Fig. 1 shows that leucovorine (10 μM) does not induce alkali-labile regions in the DNA of cells with pre-labelled DNA.

Next we examined cells treated with methotrexate. Cells were incubated with leucovorine (10 μM) for 30 min and then with methotrexate (1 μM) and leucovorine for 60 min. The cells were either immediately lysed in dilute alkali or lysed in alkali after incubation of the cells in leucovorine and methotrexate for 24 h or 48 h. In parallel we incubated cells with only methotrexate.

Fig. 2 shows that DNA fragmentation increases with increasing time of methotrexate treatment. This is in agreement with our earlier data (5) and the work of (4). In contrast in cells treated with leucovorine and methotrexate there is greatly reduced DNA fragmentation. This is most clearly seen in cells incubated for 48 h. Hence the presence of leucovorine prevents methotrexate from inducing DNA fragmentation.

DISCUSSION

Methotrexate induces accumulation of DNA strand breaks in methotrexate treated cells (4,5). Furthermore it has been shown that the amount of strand-breaks correlate well with methotrexate cytotoxicity (4). In agreement with this it has been shown, using thymidylate

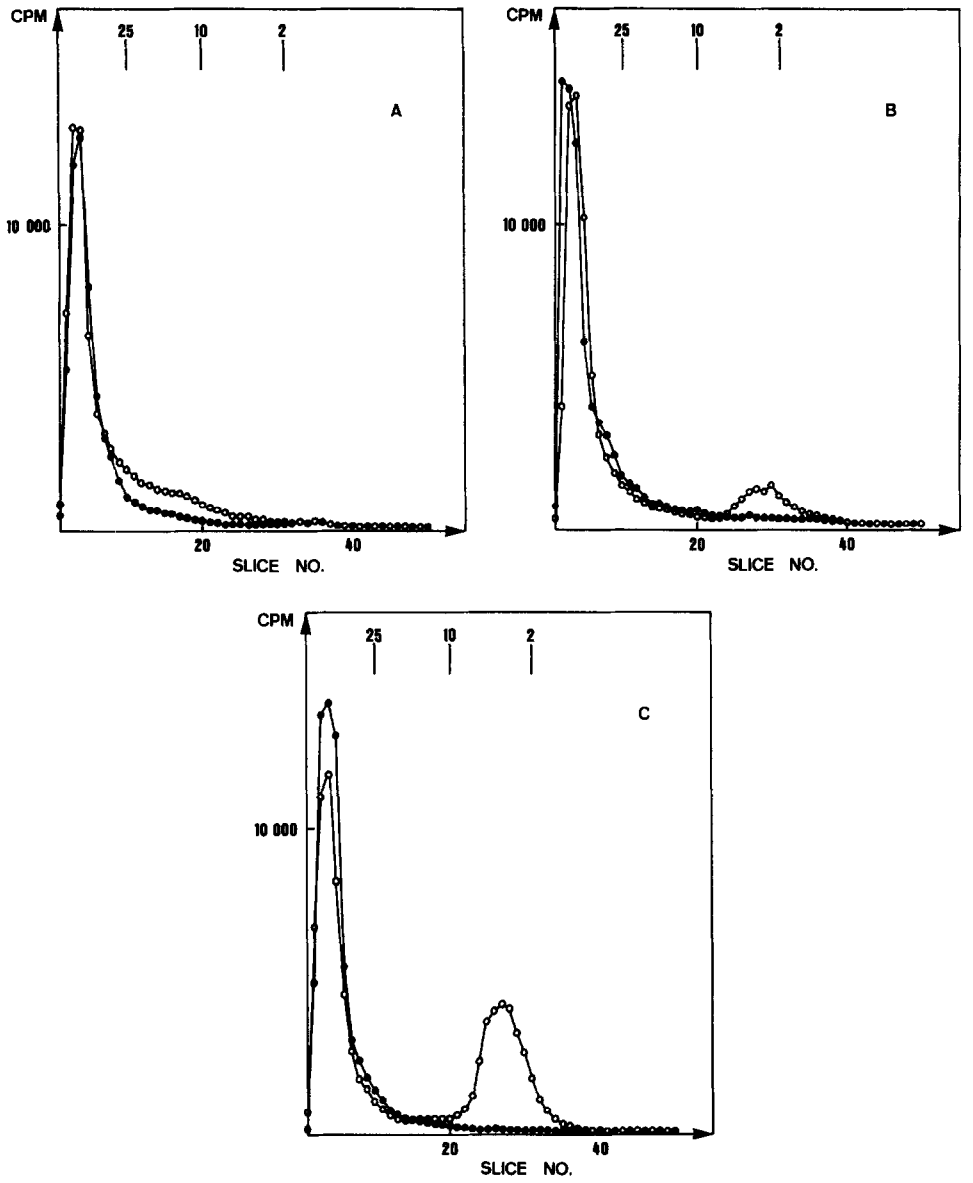

Fig. 2. (a) Treatment with leucovorine and methotrexate. Cells with pre-labelled DNA were either treated with methotrexate (1 μM) for 60 min (-o-) or leucovorine (10 μM) for 30 min followed by leucovorine and methotrexate for 60 min (-•-). The cells were lysed in dilute alkali and the DNA then separated in 0.75 % agarose gels. The numerals across the top (25, 10 and 2) denote the size (in kb) and location of single-stranded DNA markers.

(b) Cells with pre-labelled DNA were either treated with methotrexate for 48 h (-o-) or leucovorine for 30 min followed by leucovorine and methotrexate for 24 h (-•-).

(c) Cells with pre-labelled DNA were either treated with methotrexate for 24 h (-o-) or leucovorine for 30 min followed by leucovorine and methotrexate for 48 h (-•-).

synthetase-negative mutants, that induction of a thymine-less state by removal of thymidine from culture media results in chromosome breaks (11).

In this paper we show that leucovorine prevents the appearance of DNA strand breaks. The main effect of the methotrexate block of dihydrofolate reductase is to deplete the cells of tetrahydrofolates, which is necessary for the synthesis of purines and pyrimidines. The addition of leucovorine provides a source of tetrahydrofolate cofactor substrate, which circumvents the methotrexate block and therefore allow nucleotide biosynthesis to occur (2).

The results provide support for the proposal that methotrexate induces DNA lesions by interfering with nucleotide biosynthesis.

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