[CANCER RESEARCH 50, 1786-1789, March 15, 1990]

Inhibition of DNA Excision Repair by Methotrexate in Chinese Hamster Ovary Cells following Exposure to Ultraviolet Irradiation or Ethylmethanesulfonate[1]

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ABSTRACT

Previous results have suggested that methotrexate (MTX) could interfere with the repair of spontaneous DNA damage. To determine its effects on induced DNA damage, MTX was compared to hydroxyurea and arabinofuranosylcytosine (H/A), a drug combination known to block the DNA polymerase step of excision repair, for its ability to cause the accumulation of single-strand breaks (SSB) following exposure to either UV light or the alkylating agent ethylmethanesulfonate in Chinese hamster ovary cells. SSB were measured by alkaline elution 1, 2, and 6 h after exposure to either 1.8 mg/ml of ethylmethanesulfonate or 10 J/m² of UV in cells pretreated with MTX or H/A. Following exposure to ethylmethanesulfonate, significant accumulation of SSB occurred in cells pretreated with either H/A or MTX. Coadministration of hypoxanthine and thymidine in MTX-treated cells prevented SSB accumulation, indicating that nucleotide depletion by MTX had inhibited repair synthesis. After UV irradiation, SSB accumulation was much less in MTX- than in H/A-treated cells. MTX was found to have no effect on the incision of UV damage. These results indicate that nucleotide depletion by MTX can affect the repair of DNA damage by exogenous agents, and that the extent of inhibition is dependent on the type of damage induced.

INTRODUCTION

As an inhibitor of dihydrofolate reductase (1, 2), thymidylate synthase (3), and aminoimadizolecarboxamide phosphoribosyl transformylase (4), MTX[3] interferes with the *de novo* synthesis of adenylate, guanylate, and thymidylate (5–7). Depletion of these nucleotide pools leads to inhibition of semiconservative DNA synthesis. The particular sensitivity of cells in S-phase to the cytotoxic effects of MTX has led to the general consensus that inhibition of replicative DNA synthesis is the basis for the cytotoxicity of MTX to cycling cells (8).

Li and Kaminskas (9) reported that treatment of logarithmically growing Ehrlich ascites tumor cells with MTX led to a time-dependent accumulation of SSB in preformed DNA. It was proposed that SSB arose because MTX-induced nucleotide depletion led to inhibition of the DNA synthesis step of the repair of spontaneously arising DNA damage. Spontaneous damage, which includes the deamination of cytosine to uracil and depurination as its primary lesions, may lead to as many as 10,000 excision repair events per cell per day (10–12).

The production of strand breaks in preformed DNA upon treatment with MTX has been confirmed in other cell lines, including human colon adenocarcinoma cells (13) and NIH/3T3 cells (14). Apart from these observations, however, further evidence to either support or refute the hypothesis that MTX could function as an inhibitor of excision repair has not been reported. It is relevant to establish whether such a relationship exists, in view of the extensive clinical use of MTX in combination with DNA-damaging agents and the possible implications this might have concerning the cytotoxic and mutagenic potential of such combination therapies. Therefore, the goal of this investigation was to determine if MTX could interfere with the DNA synthesis step of excision repair.

The process of DNA excision repair can be monitored by measuring the level of SSB present at various time points following the induction of DNA damage (15–17). The excision of damaged regions creates SSB, whereas the filling of gaps and their ligation to the parent DNA leads to a reduction in SSB. Thus, following the induction of DNA damage, SSB are observed to accumulate. As repair continues, the rate of gap-filling/ligation will begin to predominate over excision. At that point, the level of SSB reaches a maximum and will thereafter decline until repair is complete. In the presence of inhibitors of DNA repair synthesis such as H/A, an excess accumulation of SSB results due to the buildup of excision gaps along the DNA.

In these studies, MTX was compared to H/A for the ability to alter the normal time course of repair of DNA damage induced by the alkylating agent EMS or UV irradiation. The types of damage induced by these agents are repaired, respectively, by the base excision ("short patch") and nucleotide excision ("long patch") repair modes of the excision repair process. Although both of these repair pathways involve excision of damage followed by DNA synthesis and ligation, they differ in several respects, including enzymology, regulation, patch size produced upon removal of damage, and the kinetics of the repair process (for review, see Refs. 10, 12, and 17).

In this report we show that MTX was as effective as H/A in causing SSB accumulation after EMS-induced DNA damage. In MTX-treated cells, SSB accumulation could be prevented by coadministration of 10 μM thymidine and 100 μM hypoxanthine, indicating that nucleotide depletion by MTX was responsible. The results obtained were quantitatively different when UV irradiation was used as the source of DNA damage. Under these conditions, SSB accumulation in MTX-treated cells, although significantly greater than controls exposed to UV only, was markedly less than in cells pretreated with H/A. We conclude that MTX is as effective as H/A as an inhibitor of short patch but not long patch repair.

MATERIALS AND METHODS

Materials. Tissue culture reagents were obtained from Gibco (Grand Island, NY). Tissue culture flasks were obtained from Corning (Corning, NY) and tissue culture dishes from Costar (Cambridge, MA). Unless otherwise indicated, all other reagents were obtained from Sigma (St. Louis, MO).

Cell Culture and Drug Exposure Conditions. The AA8 Chinese hamster ovary cell line used in this work, originally characterized by Thompson *et al.* (18), was grown in monolayer in Dulbecco's modified Eagle's medium supplemented with dialyzed calf serum (5%, v/v), dialyzed fetal calf serum (5%, v/v), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The doubling time of the cells under these conditions was approximately 18 h. In all experiments, cells were allowed to recover

Received 5/4/89; revised 11/27/89.

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[1] From a dissertation presented by A. H. B. to the Department of Pharmacology, the George Washington University Graduate School of Arts and Sciences, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[3] The abbreviations used are: MTX, methotrexate; SSB, single-strand breaks; H/A, hydroxyurea plus arabinofuranosylcytosine; EMS, ethylmethanesulfonate; 3AB, 3-aminobenzamide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

at least 8 h after plating before drug treatment was commenced.

For UV repair experiments, 600,000 cells were plated into 10-cm dishes. Eight h later, fresh MTX stock was prepared and added to the cells. Sixteen h later (immediately prior to irradiation), the medium was transferred to sterile tubes and kept at 37°C. This medium was used to refeed the cells immediately after exposing the cells to 10 J/m^2 of 254-nm UV light delivered from a germicidal lamp. For EMS repair experiments, 600,000 cells were plated into 25-cm^2 flasks. Eight h later freshly prepared MTX was added, and the cells were incubated an additional 16 h. Exposure to EMS was carried out by first transferring 5 of the 10 ml of medium present in each treatment flask to sterile tubes. This was kept at 37°C and used to refeed the cells after treatment with EMS. EMS was then added to the cells, and 1 h later the cells were refed with the medium removed earlier. For both UV and EMS repair experiments, repair was terminated by replacing the medium with ice-cold PBS and keeping the flasks or dishes on ice until harvesting the cells for alkaline elution analysis.

Alkaline Elution. The set-up of the alkaline elution apparatus was essentially that as described in detail by Kohn *et al.* (19). Three days prior to the experiment two 150-cm^2 flasks containing 50 ml of growth medium and 0.06 μCi/ml of [^{3}H]thymidine or 0.015 μCi/ml of [^{14}C] thymidine were seeded, respectively, with 300,000 and 400,000 cells. Nine h before drug treatment, the [^{14}C]thymidine-labeled cells were harvested by trypsinization, and 600,000 cells were transferred into 25-cm^2 flasks (one flask per treatment group). After this point, all handling of the cells was done under a safelight. After the desired drug treatment (described above), the cells were scraped and made into single-cell suspensions by repeatedly pipetting the cells. The same was done with the [^{3}H]thymidine-labeled cells, which were then irradiated with 300 rads. Both the [^{14}C]thymidine- and [^{3}H]thymidine-labeled cells were quantitated for cells/ml and cpm/ml. For each treatment flask, approximately 20,000 cpm (and less than 500,000 cells) were transferred to a test tube on ice. Each tube then received about 15,000 cpm (and less than 500,000 cells) of the [^{3}H]thymidine, 300-rad-irradiated cells. The cells were then deposited onto 2-μm membranes (Nucleopore, Pleasonton, CA) held in 25-mm Swinnex filter holders (Millipore, Bedford, MA) and lysed with 5 ml of lysis buffer (2% SDS:0.025 M EDTA:0.1 M glycine, pH 10.0). Two ml of 0.5 mg/ml of proteinase K (Boehringer, Mannheim, West Germany) dissolved in lysis buffer were added. After 20 min, 30 ml of elution buffer (0.1 M tetrapropylammonium hydroxide:0.02 M EDTA:0.1% SDS, pH 12.15) were added. Fractions were collected every 1.5 h for 13.5 h. For each sample, cpm were determined for the lysis solution, each fraction, and the filter. Total cpm were determined, and the cpm of ^{14}C and ^{3}H remaining on the filter after each fraction were calculated. Rad-equivalent breaks were calculated as described by Kohn *et al.* (19).

Cell Survival Assays. On Day 1 of the experiment, cells in exponential growth were seeded at appropriate densities into 6-cm culture dishes. Nine h later, cells were exposed as described above. After the desired exposure time, the drug-containing medium was aspirated off the plates and replaced with 5 ml of fresh medium supplemented with 10 μM thymidine and 100 μM hypoxanthine. After a 10-day incubation period to allow survivors to proliferate into visible colonies, the plates were fixed and stained with crystal violet (2.5 g/liter in 90% methanol/10% formalin), and the surviving fraction was calculated. Synergism (S) was calculated by dividing the product of the fractional survivals (FS) produced by treatment with EMS and MTX alone by the FS produced by treatment with both agents.

$$S = (FS_{MTX})\ (FS_{EMS})/FS_{MTX+EMS}$$

RESULTS

The cytotoxicity of a 16-h exposure to various concentrations of MTX alone or in combination with EMS is shown in Fig. 1. The survival curve for MTX illustrates the characteristic self-limiting cytotoxicity of this agent. When administered together, MTX and EMS acted synergistically in causing cell death. Cell kills between 3- and 10-fold greater over additivity were obtained for the combination at MTX concentrations of 1 μM or greater (see "Materials and Methods" for calculation).

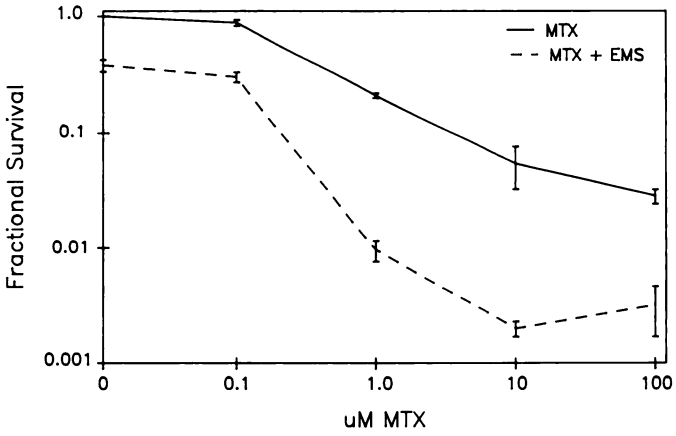

Fig. 1. Cytotoxicity of MTX alone and in combination with EMS as measured by colony formation ability. Cells were pretreated with various concentrations of MTX for 16 h and then one group exposed to 1.8 mg/ml of EMS during the final hour of MTX exposure. All cells were then refed with medium supplemented with 10 μM thymidine and 100 μM hypoxanthine and returned to the incubator to allow survivors to proliferate into colonies. *Bars*, SE.

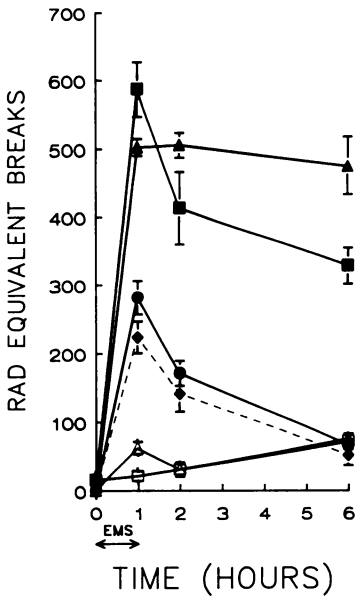

Fig. 2. Time course of SSB formation and removal during and after EMS exposure in control, MTX-, or H/A-treated cells. Cells were pretreated 16 h with 10 μM MTX or 1 h with 2 mM hydroxyurea plus 50 μM arabinofuranosylcytosine (H/A) before EMS exposure. Immediately prior to addition of EMS, 5 of the 10 ml of medium in each flask were transferred to sterile tubes. EMS was then added to the remaining 5 ml at a dose of 1.8 mg/ml. One h later the cells were refed with the same medium removed earlier. Repair was stopped at the times indicated by replacing the medium with ice-cold PBS. DNA was then analyzed by alkaline elution, and strand breaks were calculated as described in "Methods." ●, EMS; △, H/A; ▲, H/A + EMS; □, MTX; ■, MTX + EMS; ◆, MTX + hypoxanthine + thymidine + EMS. *Bars*, SE.

The effects of H/A and MTX on the repair of EMS-induced damage are shown in Fig. 2. In these studies, a dose of 10 μM MTX was used, based on the results of preliminary experiments in which 10 μM MTX was found to produce the greatest net accumulation of SSB after EMS exposure. In cells treated with EMS alone, SSB were formed as long as EMS was present. This is consistent with previous reports and reflects the rapid rate of incision at sites of EMS-induced damage (17). In cells pretreated with either MTX or H/A, twice as many breaks accumulated during the period of EMS exposure as compared with control cells. Over the next 5 h, essentially no breaks disappeared in the H/A-treated cells, whereas in MTX-treated cells, a detectable level of DNA synthesis/ligation was observed. As shown also in Fig. 2, the ability of MTX to cause accumulation of SSB was completely prevented by the presence of 10

μM thymidine and 100 μM hypoxanthine. This observation indicates that SSB accumulation was a consequence of MTX-induced nucleotide depletion.

To investigate the possibility that MTX treatment may somehow interfere with ligation, its effect on the resealing of SSB induced by ionizing radiation was investigated (Fig. 3). The repair of X-ray-induced damage displays both a fast and slow component (20, 21). The slow component, which involves excision repair of X-ray-induced base damage, takes several hours. In contrast, the fast component, which represents the repair of X-ray-induced SSB, involves the action of exonucleases to clean up the ends prior to ligation and is completed within minutes. The fast component is largely unaffected by inhibitors of DNA repair synthesis (22, 23). Consistent with these findings, MTX, like H/A, displayed only a slight inhibitory effect on strand break repair. These results suggest that the large degree of SSB accumulation after EMS damage was not due to an effect on ligation, but occured due to inhibition of DNA synthesis associated with repair.

Experiments similar to those done with EMS were also performed in cells exposed to 10 J/m² of UV irradiation (Fig. 4). When this form of damage was used, MTX was much less efficacious than H/A in causing SSB accumulation. In comparison to cells exposed to only UV, pretreatment with H/A led to the formation of 8.5-fold more SSB, whereas in cells pretreated with MTX, an increase of only 2.7-fold more breaks was found.

To rule out the possibility that MTX treatment affected the incision of UV dimers, the influence of MTX on SSB accumulation in H/A-pretreated cells was determined. If MTX affected the incision of UV dimers, the rate of SSB accumulation in cells pretreated with MTX and H/A would be slower than in cells pretreated with H/A alone. As shown in Fig. 4, this was not the case. These data suggest that the inability of MTX to cause comparable accumulation of SSB after UV damage could not be attributed to an effect of MTX on the incision of UV dimers.

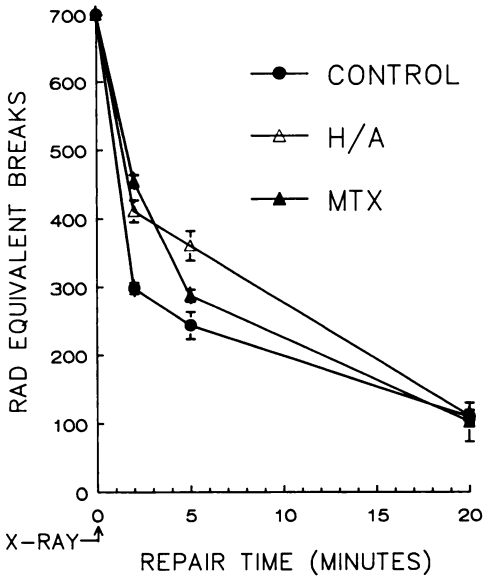

Fig. 3. Time course of single-strand break removal in control cells *versus* cells pretreated with MTX or H/A. Cells were pretreated with MTX or H/A as described in Fig. 2. Immediately prior to irradiation, the medium was transferred from each flask to sterile tubes, and ice-cold PBS was added to the cells. These were kept on ice until all groups had received 700 rads. The cells were then refed with the same medium (kept at 37°C) removed earlier. At the indicated times, break resealing was stopped by again refeeding with cold PBS. DNA was then analyzed by alkaline elution, and strand breaks were calculated as described in "Methods." *Bars*, SE.

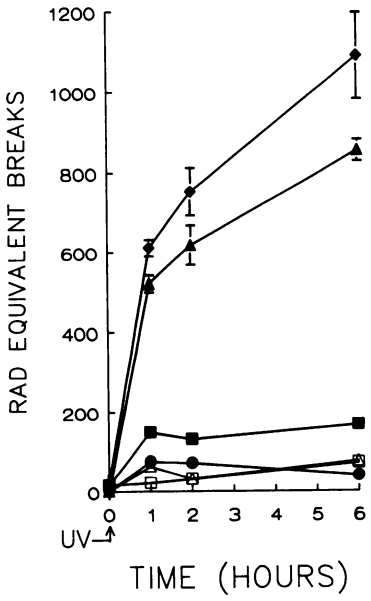

Fig. 4. Time course of SSB formation and removal during and after UV exposure in control, MTX-, or H/A-treated cells. Cells were pretreated with MTX or H/A as described in Fig. 2. Immediately prior to UV irradiation, the medium was transferred from each dish to sterile tubes. The cells were promptly irradiated with 10 J/m² UV and then refed with the same medium. Repair was stopped at the times indicated by replacing the medium with ice-cold PBS. DNA was then analyzed by alkaline elution, and strand breaks were calculated as described in "Methods." ●, UV; △, H/A; ▲, H/A + UV; □, MTX; ■, MTX + UV; ◆, MTX + H/A + UV. *Bars*, SE.

DISCUSSION

The goal of the present study was to determine if treatment of cells with MTX can inhibit DNA excision repair. Several groups have documented that treatment of cells with MTX leads to the formation of SSB in preformed DNA (9, 13, 14). Despite the extensive clinical use of MTX in combination with DNA-damaging agents, direct evidence to indicate whether or not MTX inhibits repair of such damage has not been reported. To address this question, we compared the ability of MTX with that of H/A to alter the normal time course of repair of EMS- and UV-induced DNA damage.

The results of our experiments clearly indicate that inhibition of repair by MTX is comparable to H/A after EMS- but not UV-induced DNA damage. Several explanations could account for these results. If the repair of EMS damage itself consumed more nucleotides than the repair of UV damage, this additional strain on pool size could have caused inhibition of repair synthesis. However, this explanation is unlikely because the total level of DNA synthesis required after UV damage was probably greater than after EMS damage. Comparison of Figs. 2 and 4 reveals that, in H/A-treated cells, the total breaks formed after UV were greater than after EMS, indicating that more gaps (sites of damage repair) were formed in UV- than in EMS-damaged cells. Furthermore, most estimates for the gap size formed upon removal of UV dimers range from 60 to 100 nucleotides, whereas gaps of only 1 to 10 nucleotides are generated at sites where alkylation damage has occurred (12, 24–26). Consequently, the total amount of DNA synthesis required to fill in the repair gaps was probably lower in EMS- than in UV-exposed cells. This would argue that the SSB accumulation specific to EMS damage in MTX-pretreated cells was not due to greater demand on nucleotide levels to repair this form of damage.

The selectivity with which MTX interfered with repair synthesis after EMS damage may reside in intrinsic differences between the "long patch" and "short patch" repair modes used

in response to these two types of damage. The interpretation that short patch repair was more sensitive to nucleotide perturbations induced by MTX is not inconsistent with earlier reports. Li and Kaminskas (9) hypothesized that SSB formation in preformed DNA after MTX treatment resulted from inhibition of repair of spontaneous damage. The most important lesions in this category are depurination and deamination of cytosine to uracil (12). The removal of both apurinic sites and uracil from DNA is followed by short patch repair (12).

The effects of MTX on the accumulation of SSB are similar to results reported in cells treated with 3AB. Pretreatment of cells with 3AB, an inhibitor of poly(ADP) ribosylation, leads to accumulation of SSB after alkylation but not UV damage (27–30). This is the same response we are reporting for MTX, suggesting that both compounds act through a similar mechanism. Several reports have implied that ligation is dependent on activation by poly(ADP) ribosylation (28, 31, 32). Therefore, the accumulation of breaks in 3AB-treated cells after alkylation but not UV damage has been interpreted as evidence that a poly(ADP) ribosylation-dependent ligase (presumably ligase II) participates in the repair of alkylation damage, whereas a different ligase may participate in UV repair (33). The results of our studies could be explained through an inhibition of poly(ADP) ribosylation as a consequence of MTX exposure. However, the observation from two separate laboratories that poly(ADP) ribosylation is increased upon MTX treatment (34, 35) renders this explanation unlikely. 3AB, although a potent inhibitor of poly(ADP) ribosylation, affects several other metabolic processes, including purine synthesis (33, 36). Therefore, accumulation of SSB after 3AB treatment may be due to its effects on nucleotide pools rather than a poly(ADP) ribosylation-mediated inhibition of ligation (33). A perturbation of nucleotide pools by 3AB might explain its differential effects on UV and alkylation repair. This would be consistent with the results reported here with MTX, which suggest that nucleotide depletion can differentially affect UV and alkylation repair.

In conclusion, our results support the hypothesis that nucleotide depletion by MTX affects the ability of cells to carry out the excision repair of DNA damage. This observation may provide an additional explanation for the clinical success obtained in therapies combining MTX with DNA-damaging agents. A current concern with existing treatments is the mutagenic potential of these protocols, which may cause the emergence of second primary tumors. It is possible that inhibition of repair by MTX may also affect the mutagenic potential of DNA-damaging agents. To address this possibility, we are investigating the influence of repair inhibition by MTX on the mutagenic potential of EMS-induced DNA damage.

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