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Accumulation of DNA strand breaks and methotrexate cytotoxicity

(DNA repair/antimetabolites/thymineless death/purineless death)

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ABSTRACT **There was a progressive formation of strand breaks in mature DNA of Ehrlich ascites tumor cells that were treated with methotrexate. Cells were labeled with [^{14}C]thymidine before incubation with methotrexate, and DNA strand breaks were measured by alkaline and by neutral filter elution methods. DNA single-strand breaks accumulated in a linear fashion as a function of time during the first 10 hr of incubation with 2 μM methotrexate. Thereafter, the accumulation of DNA strand breaks deviated from linearity because of progressive cell death. The extent of DNA strand breakage in cells that had been incubated with methotrexate for 24 hr was as high as in cells that had been irradiated with 300 rads. DNA strand breaks persisted in cells that were incubated, after exposure to methotrexate, in medium containing thymidine, hypoxanthine, and nonessential amino acids, indicating that these strand breaks were poorly repaired. Cell death commenced after 10 hr of incubation with methotrexate and continued during the following 3–4 days. These findings suggest that cell death was due to a lethal accumulation of DNA strand breaks. The formation of DNA strand breaks is probably due to inefficient DNA repair, resulting from the inhibition of syntheses of thymidylate and of purine nucleotides. The accumulation of DNA strand breaks was minimal in growth-arrested cells, which are resistant to methotrexate toxicity.**

Cytotoxicity of the antifolate methotrexate (MTX) is generally thought to be related to the inhibition of DNA synthesis, although the precise mechanism of cell death is not known (1). Multiplying cells are sensitive to MTX, especially cells in the S phase of the cell cycle (2), while resting cells are not (2–4). MTX restricts the synthesis of thymidylate and of purine nucleotides by inhibiting dihydrofolate reductase and, to a lesser extent, thymidylate synthetase (1). Hence, MTX cytotoxicity has been postulated to occur both by "thymineless death" and "purineless death" mechanisms (5). DNA synthesis is inhibited rapidly in cells that are incubated with micromolar concentrations of MTX. In CCRF-CEM human lymphoblastoid cells, DNA synthetic rates decrease by 80% within 30 min and then remain stable for at least 6 hr (6). The newly synthesized DNA accumulates in 80S fragments and does mature to bulk-size DNA upon reversal of the MTX block, at least within the first hr of MTX treatment (6). An abnormal progression of DNA synthesis, with accumulation of low molecular weight DNA fragments, also was demonstrated with the antifolate metoprine (7). In addition, there was suggestive evidence that newly formed DNA may be partially degraded, perhaps as a result of misincorporation of dUMP and the subsequent repair reactions (7). Misincorporation of dUMP also has been demonstrated in cells treated with MTX (8), and it was hypothesized that futile repair processes could result in extensive DNA degradation, by analogy to thymineless death in bacteria (9–15).

The inhibition of new DNA synthesis and partial degradation of newly synthesized DNA provide an explanation of a cytostatic effect of MTX but do not account for a cytocidal effect. In the present study, we examined how mature DNA molecules were affected in Ehrlich ascites tumor cells during incubation with MTX. We found a progressive formation of DNA strand breaks and correlated the occurrence of these lesions with the cytotoxicity of MTX. We also found that accumulation of DNA strand breaks was minimal in growth-arrested cells treated with MTX.

MATERIALS AND METHODS

Materials and Cell Cultivation. [2-^{14}C]Thymidine (52.0 mCi/mmol) was obtained from New England Nuclear; polycarbonate membrane filters of 2-μm pore size, from Nuclepore; sodium dodecyl sulfate, from Bio-Rad; and proteinase K (EM Products), from American Scientific Products (Bedford, MA). MTX was purchased from Sigma. MTX was prepared as a 2 mM stock solution in phosphate-buffered saline (pH 7.0) and stored at 4°C. The sources of culture media and of other chemicals were as described (16). Ehrlich ascites tumor cells were grown in continuous spinner cultures in Eagle's minimal essential medium supplemented with 25 mM morpholinopropanesulfonic acid, 1 mg of glucose per ml, and 5% calf serum as described (16). Cells were in exponential growth phase (1–8 × 10^5 cells per ml) before being used in experiments. Growth-arrested cells were obtained by cultivation for 42 hr in 0.5% serum medium, with a change of medium after 24 hr (17).

Treatment with MTX and Cytotoxicity Analyses. Cells were incubated in parallel cultures at about 3 × 10^5 cells per ml in 0.5% serum medium with or without 2 μM MTX for time periods of up to 24 hr. The concentration of serum in the medium was reduced from 5% to 0.5% to reduce the concentrations of thymidine, 5-methyl tetrahydrofolate, and purine ribonucleosides that counteract the effects of MTX. Cells that were incubated in 0.5% serum medium without MTX underwent 1.31 ± 0.19 doublings in 24 hr, while cells in 5% serum medium underwent 1.69 ± 0.08 doublings, in agreement with previously reported data (17).

Survival of cells that were capable of multiplication after MTX exposure was determined by an outgrowth method. Portions of both MTX-treated and untreated cultures were resuspended at about 2 × 10^5 cells per ml in recovery medium (complete growth medium supplemented with 2 μM thymidine, 20 μM hypoxanthine, and nonessential amino acids of Eagle's minimal essential medium) and were incubated for 5–7 days with daily changes of the recovery medium. Concentrations of viable cells were determined daily by quadruplicate counts in a hemacytometer in the presence of 0.07% trypan blue. More than 97% of cells excluded trypan blue in both untreated and MTX-treated cultures, after cells were resuspended in fresh medium. The percentages of surviving cells in MTX-treated cultures were calculated from the differences in the numbers of cell doublings in the untreated

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Abbreviation: MTX, methotrexate.

cultures and in the MTX-treated cultures when cells in both sets of cultures were multiplying exponentially, as originally described for the outgrowth method (18). The time course of cell death was determined by using cells that were labeled with [^{14}C]thymidine (as described below) prior to exposure to MTX. There was no toxicity due to [^{14}C]thymidine incorporation in these cells as well as in others (19). At times as specified during the incubation with MTX and during the subsequent incubation in recovery medium, duplicate samples of MTX-treated and of untreated cultures were withdrawn, and the percentages of radioactivity released into the medium were determined from the radioactivities in the samples before and after centrifugation.

Measurements of DNA Single-Strand and Double-Strand Breaks. Cells were grown for 24 hr in the presence of [^{14}C]thymidine (0.02 μCi/ml, 0.38 μM; 1 Ci = 37 GBq) before treatment with MTX. At the end of the labeling period, more than 90% of the label was incorporated into the cells. At times as specified, samples of 0.5–2 $\times$ 10^6 cells were removed from the cultures, washed once with 30 ml of ice-cold phosphate-buffered saline, resuspended in 20 ml of ice-cold phosphate-buffered saline, and subjected to alkaline or neutral filter elution analyses at room temperature by previously described methods (19, 20). Relative elution values, which are proportional to the frequency of DNA single-strand breaks (21), were calculated by the formula $\log R_0 - \log R$, where R_0 and R represent fractions of DNA retained on the filter in the samples of the untreated and of MTX-treated cells, respectively, at 12 hr of elution (22). Irradiation of cells grown in 5% serum medium was carried out in Dr. John B. Little's laboratory at Harvard School of Public Health (Boston, MA) with a Gammarad Irradiator, model GR9 at a dose rate of 50 rads (0.5 grays)/sec. Irradiated cells as well as the corresponding control cells were kept at 4°C until analyzed by alkaline elution.

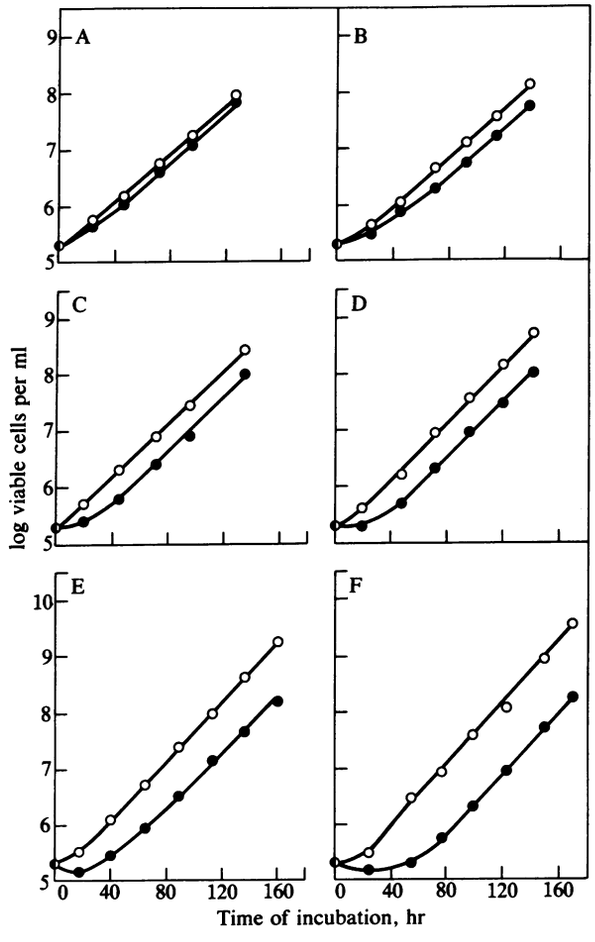

FIG. 1. Effect of MTX on outgrowth of Ehrlich ascites tumor cells. Exponentially growing cells were incubated in 0.5% serum medium with (●) or without (○) MTX for 3 (*A*), 6 (*B*), 8 (*C*), 10 (*D*), 16 (*E*), and 24 hr (*F*) and then in recovery medium. Changes in the numbers of cells are plotted from the time of incubation in recovery medium.

RESULTS

MTX Cytotoxicity. Within certain limits, cell killing by MTX is dependent both on the length of exposure to MTX and on the concentration of MTX (23). In this study we incubated exponentially growing Ehrlich ascites tumor cells with 2 μM MTX in 0.5% serum medium for periods of 3–24 hr and determined the percentages of surviving cells by an outgrowth method. During MTX exposure in 0.5% serum medium (and also in 5% serum medium), the number of cells decreased by 23.5 ± 6.4% after 16 hr and by 50.4 ± 8.7% after 24 hr. Cultures that were treated with MTX for increasing periods of time exhibited increasing periods of delay before resuming exponential growth (Fig. 1). These delays were due to the progressive decrease in the number of cells that survived and multiplied (18). The data from such outgrowth experiments showed that the percentages of surviving cells decreased exponentially as a function of the time of exposure to MTX (Fig. 2). Most of the nonsurviving cells disintegrated not during the time of exposure to MTX but during the subsequent incubation in the recovery medium, as indicated by the release of radioactivity from [^{14}C]thymidine-labeled cells into the medium. There was no release of radioactivity during the first 8 hr of incubation with MTX. The percentages of the incorporated radioactivity released were 2 ± 0.5%, 13%, and 47 ± 2.8% (values and SD are from two to four experiments) after 10, 16, and 24 hr of incubation with MTX, re-

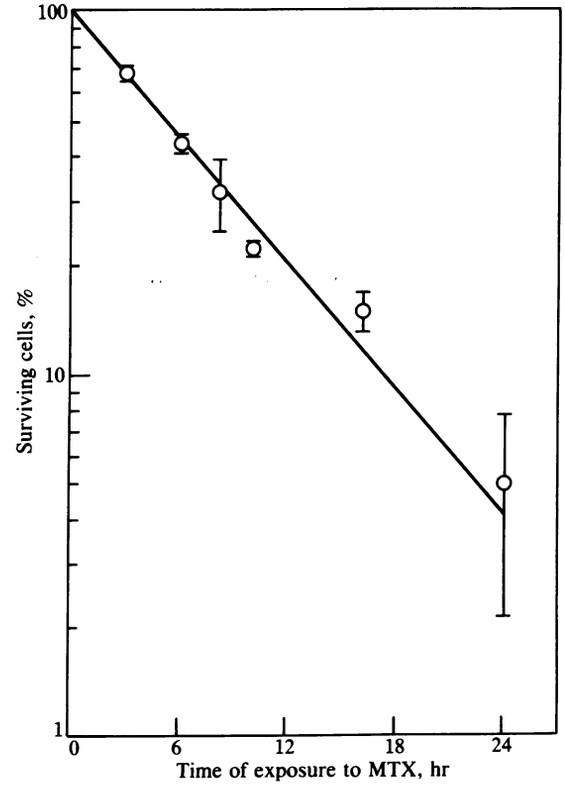

FIG. 2. Survival of cells as a function of time of incubation with MTX as determined by the outgrowth method. Each point is the mean of three to five experiments. Bars indicate the SD.

spectively. Cells that were exposed to MTX for 24 hr released 77.5 ± 1.1%, 85.9 ± 0.6%, and 91.6 ± 0.6% of the initial radioactivity after 1, 2, and 3 days of cultivation in recovery medium, respectively.

DNA Strand Breaks in MTX-Treated Cells. There was a progressive accumulation of DNA single-strand breaks during the 24 hr of incubation with 2 μM MTX in 0.5% serum medium. Fig. 3 *A–E* shows typical alkaline elution patterns of DNA from cells that were incubated with or without MTX for 3, 6, 8, 16, and 24 hr. For purposes of comparison, the elution pattern of cells irradiated with 300 rads is also included in Fig. 3*E*. The increase in the incidence of DNA single-strand breaks as a function of time of incubation with MTX was assessed by calculating the relative elution values, which increased in a linear fashion during the first 10 hr of incubation with MTX (Fig. 4). Subsequently, the increase deviated from linearity, presumably because cells with a lethal number of strand breaks started dying and disintegrating; hence, the radioactivity in their DNA was washed through before the intact cells were lysed. The dashed line in Fig. 4 represents a hypothetical rate of accumulation of DNA strand breaks in absence of cell death. In addition to the accumulation of DNA single-strand breaks, neutral filter elution revealed the presence of some DNA double-strand breaks in cells exposed to MTX for 24 hr (Fig. 3*F*). Presumably these breaks arose as a result of the proximity of single-strand breaks on the two complementary strands of DNA.

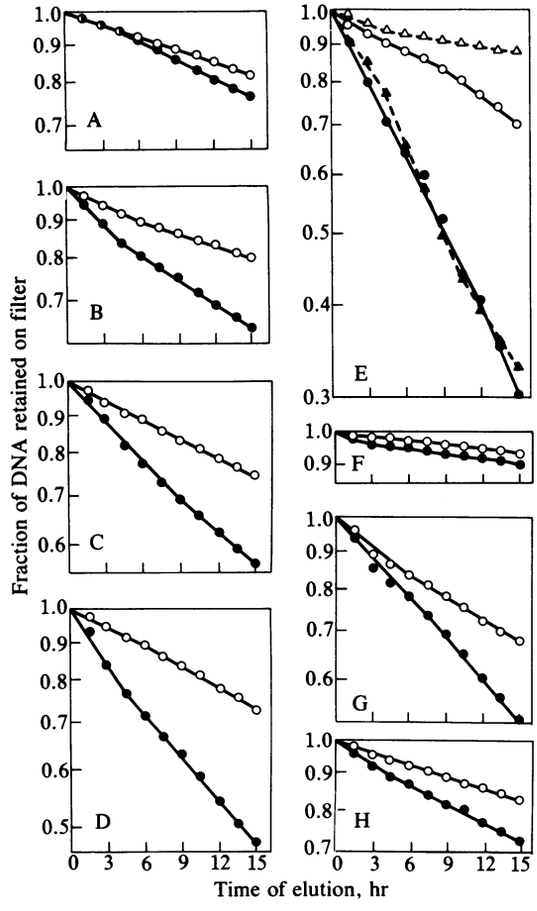

FIG. 3. Filter elution patterns in MTX-treated cells. Cells were incubated with (●) or without (○) MTX for 3 (*A*), 6 (*B*), 8 (*C*), 16 (*D*), and 24 (*E* and *F*) hr. Cells that had been incubated with MTX for 24 hr were incubated in recovery medium for 24 (*G*) and 48 (*H*) hr. In *E*, exponentially growing cells were irradiated with 300 rad (▲). △, Cells not irradiated. *A–E* and *G* and *H* are alkaline elution patterns. *F* shows neutral filter elution patterns, which were obtained from portions of the same cultures that were analyzed by alkaline elution in *E*.

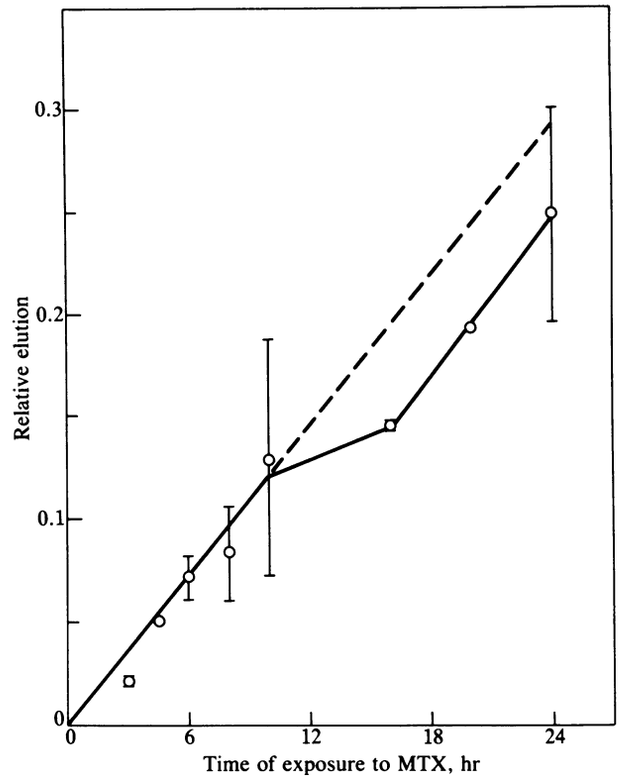

FIG. 4. Relative elution values in cells treated with MTX for variable time periods. Relative elution values after 4.5 and 20 hr of exposure to MTX are from single experiments; all other values are mean values from three to six experiments. Bars indicate the SD.

Most of the strand breaks accumulated in mature rather than in nascent DNA molecules. Although no chase period was provided in the above experiments between the [^{14}C]-thymidine labeling period and the incubation with MTX, virtually identical elution patterns were obtained with cells that underwent 2 doublings between the end of the labeling period and treatment with MTX. Kohn *et al.* (19) also had found that in rapidly growing cultures a chase period was unnecessary, because the rapid consumption of [^{14}C]thymidine provided an automatic chase.

DNA strand breaks persisted during cultivation in recovery medium (Fig. 3 *G* and *H*). Their incidence decreased, probably as a result of continuing cell death. These residual strand breaks may not be repairable and most likely are in cells that are destined to die. It is of interest that DNA strand breaks accumulate during cultivation of cells in 0.5% serum medium without MTX (Fig. 3 *A–E*). The differences in elution patterns of cells cultivated for 24 hr in 5% and in 0.5% serum media are evident in Fig. 3*E*. However, MTX treatment resulted in the same number of strand breaks in cells that were exposed to MTX for 24 hr in 5% and in 0.5% serum media in parallel cultures, since the relative elution values were virtually identical.

Growth-Arrested Cells. Ehrlich ascites tumor cells can be growth-arrested by cultivation in 0.5% serum medium for 2 days; they survive for several days and resume exponential growth upon resuspension in normal medium (17). Like other growth-arrested cells (2–4), they are resistant to MTX cytotoxicity (not illustrated). Fig. 5 shows an alkaline elution pattern of growth-arrested cells that were incubated for 24 hr with 2 μM MTX. The elution pattern of the growth-arrested cells was markedly different from that of growing cells incu-

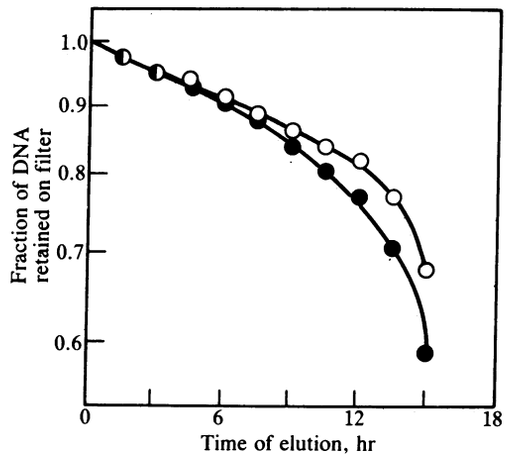

FIG. 5. Alkaline elution patterns in growth-arrested cells that were incubated with (●) or without (○) MTX for 24 hr.

bated for 24 hr with MTX and is comparable to the elution pattern of growing cells that were incubated with MTX for 3 hr. During the period of incubation with MTX the numbers of viable cells decreased by about 25%; a similar decrease occurred in a parallel culture incubated without MTX as a result of serum deprivation (17).

DISCUSSION

In growing tumor cells treated with MTX, strand breaks appeared in mature DNA molecules and accumulated in a time-dependent fashion (Fig. 4). What mechanisms can best account for strand-break formation? In growing cells that enter the S phase of the cell cycle, deoxyribonucleoside triphosphates are needed for both DNA replication and DNA repair. Thus, strand breaks most likely occurred in mature DNA molecules as a result of defective repair caused by the restriction of supply of dTTP and of purine nucleotides. The need for constant repair in mature DNA molecules arises from a relatively frequent occurrence of some types of DNA lesions (24). Of special pertinence to MTX-treated cells are the following: (*i*) deamination of cytosine to uracil, (*ii*) misincorporation of dUMP instead of dTMP, (*iii*) deamination of adenine, (*iv*) possibly misincorporation of dIMP instead of dGMP, (*v*) depurination, and (*vi*) depyrimidation. Some of these lesions occur at high frequency. For example, it has been estimated that each actively growing human cell loses approximately 10,000 purine residues and several hundred pyrimidine residues from its DNA during each generation (24). Hence, it should not be surprising that inefficient DNA repair due to decreased availability of deoxyribonucleoside triphosphates would result in DNA strand breaks. DNA strand breaks appear to be poorly repaired in MTX-treated cells, even when cells are incubated in recovery medium (Fig. 3 *G* and *H*). It is of interest that DNA strand breaks in bacterial (11–15) and in mammalian cells (25) undergoing thymineless death appear not to be repaired when cells are supplemented with thymine or thymidine.

DNA strand break formation was minimal in growth-arrested tumor cells (Fig. 5). In part, this could be explained by a lower rate of formation of DNA lesions that require repair. For example, hydrolytic deamination of cytosine occurs at a higher rate in unwound double-stranded and in single-stranded DNA that are present during DNA replication and transcription (24). However, the main reason is the lower requirement for deoxyribonucleoside triphosphates in the absence of DNA replication. Pool sizes of deoxyribonucleoside triphosphates have not been determined in growth-arrested cells that were treated with MTX. However, ribonucleoside triphosphate pools change little in these cells, as compared to the marked depletions of ATP and GTP and the elevation of UTP that occur in MTX-treated growing cells (26). In growth-arrested cells, *de novo* purine synthesis is much less inhibited than in growing cells (26), suggesting a greater availability of tetrahydrofolate coenzymes and, thus, a decreased generation of dihydrofolate during the synthesis of dTMP. Thus, it is likely that pools of dTTP, dATP, and dGTP are not as depleted in growth-arrested cells as they are in growing cells (27). Uptake of MTX and the inhibition of dihydrofolate reductase are the same in growing and in growth-arrested cells (4, 26).

The evidence presented here strongly suggests that cell death was due to a lethal accumulation of DNA strand breaks. Presumably, as the cells enter the S phase of the cell cycle, thymidylate synthesis depletes tetrahydrofolate coenzymes; dTTP, dATP, and dGTP pools become depleted; and DNA repair is inhibited. Hence, DNA strand breaks start accumulating. Since the doubling time of Ehrlich ascites tumor cells is 12–15 hr (16, 17), virtually all of the cells will have passed through the S phase by 10 hr, if DNA synthetic rates were not suppressed. However, the decrease in the rates of DNA synthesis (6) probably extends the S phase; thus, both the accumulation of DNA strand breaks (Fig. 4) and the increase in cytotoxicity (Fig. 2) continued over a 24-hr period. The small fraction of cells that survived a 24-hr exposure to MTX may be cells that never entered the S phase or that repaired the DNA lesions successfully. Why it takes as long as 3–4 days for the lysis of cells to be completed is not clear, since the pathogenesis of cell death, from the time of accumulation of DNA strand breaks to eventual cytolysis, is presently not understood. Cell lysis after thymidine deprivation in thymidylate synthetase-negative mammalian cell strains was far more rapid (complete within 1 day), was far greater, and was accompanied by an extensive formation of double-strand breaks as well as of single-strand breaks (25). Cell lysis was correlated with the extent of double-strand breaks in these cells (25) and in bacterial auxotrophs undergoing thymineless death (14). In MTX-treated Ehrlich ascites cells, double-strand breaks were not prominent (Fig. 3*F*), and the formation of single-strand breaks was not diminished in medium supplemented with thymidine (unpublished data). Thus, DNA strand breaks in MTX-treated cells are not due only to induction of thymineless state.

The results of this study are pertinent not only to the toxicity of MTX and cell death due to folate and vitamin B_{12} deficiencies but also to the toxicity of other antimetabolites that inhibit the supply of deoxyribonucleoside triphosphates for DNA synthesis. It is of interest that cells exposed for 1–4 hr to hydroxyurea and cytosine arabinoside consistently show a small number of DNA strand breaks (22). Ehrlich ascites cells exposed to either of these drugs for 24 hr show very extensive DNA strand breakage (unpublished data). It is also of interest that methotrexate is most commonly used in cancer chemotherapy in combination with other agents (usually alkylating agents). The results of this study suggest that the efficacy of MTX in these forms of therapy may in part be due to the inhibition of repair of alkylated sites on the DNA. This notion is supported by the finding of a marked increase in the formation of strand breaks in cells treated with DNA–protein cross-linking agents in the presence of hydroxyurea and cytosine arabinoside (22).

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