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Progressive Formation of DNA Lesions in Cultured Ehrlich Ascites Tumor Cells Treated with Hydroxyurea[1]

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ABSTRACT

We have previously demonstrated an accumulation of strand breaks in mature DNA of cultured Ehrlich ascites tumor cells treated with methotrexate. We postulated that the strand breaks arose from unrepaired spontaneous DNA lesions. The present study describes a progressive accumulation of strand breaks in mature DNA of Ehrlich ascites cells treated with hydroxyurea (HU). Strand breaks were determined by alkaline elution. Accumulation of strand breaks was dependent on the length of incubation (0–16 h) and on HU concentration (0–10 mM). About 70% of strand breaks were repaired when cells were incubated without HU. About 67% of strand breaks were prevented by 0.4 mM deoxyadenosine, deoxyguanosine, and deoxycytidine, with or without thymidine. Prevention was less effective by deoxyadenosine and deoxyguanosine and ineffective by deoxycytidine. Free radical scavengers did not prevent strand breaks. S-phase cells accumulated about twice the number of strand breaks as non-S-phase cells. Cell survival decreased in proportion to the increase in HU concentration (0–10 mM). The results (*a*) demonstrate that lack of purine, as well as of pyrimidine, nucleotides results in strand breaks in mature DNA, (*b*) suggest that HU cytotoxicity is due to fragmentation of mature DNA, and (*c*) caution against the use of HU in DNA repair studies.

INTRODUCTION

In a previous study (1) we described a progressive accumulation of strand breaks in mature DNA of Ehrlich ascites tumor cells during incubation with methotrexate, an inhibitor of dihydrofolate reductase. We postulated that the strand breaks arose from spontaneous DNA lesions, which were not repaired because of decreased supply of dTTP and of purine nucleotides. However, it was not clear whether DNA strand breaks were solely due to a "thymineless" state (2) or to the lack of purine nucleotides as well. This question is difficult to answer using methotrexate, because addition of thymidine to the medium reduces the generation of dihydrofolate by thymidylate synthetase and thus conserves tetrahydrofolate coenzymes for *de novo* purine synthesis.

In order to test the hypothesis that the lack of deoxyribonucleoside triphosphates other than dTTP will result in strand break accumulation, we examined the effect of HU[2], an inhibitor of ribonucleotide reductase, on mature DNA in exponentially growing Ehrlich ascites tumor cells. HU rapidly inhibits ribonucleotide reductase (3) and profoundly decreases dATP and dGTP pools, while dTTP and dCTP pools increase (4–6). As a result, HU effectively inhibits replicative DNA synthesis and, to a lesser extent, repair DNA synthesis (6–8). For this reason HU is frequently used to suppress replicative DNA synthesis in DNA repair studies.

In the present study, we report that HU causes a progressive accumulation of strand breaks in mature DNA of cultured Ehrlich ascites tumor cells. Some of the strand breaks were alkali-labile DNA lesions, which were converted to strand breaks during the alkaline elution procedure. References in the text to strand breaks refer both to strand breaks and to alkali-labile lesions. Most of the strand breaks were prevented by the addition of dAdo, dGuo, and dCyd to the medium. Inclusion of dThd did not increase strand break prevention.

MATERIALS AND METHODS

Chemicals and Cell Cultivation. HU, deoxyribonucleosides, sodium benzoate, catalase, superoxide dismutase, and aphidicolin were obtained from Sigma Chemical Co., St. Louis, MO. SKF 525-A was a gift from Smith, Kline and French, Philadelphia, PA. [2-^{14}C]Thymidine (52.0 mCi/mmol) and [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) were purchased from New England Nuclear, Boston, MA. The sources of other chemicals, calf serum, and culture medium were as described previously (1). Dialyzed serum was prepared by dialyzing calf serum in 40 volumes of PBS for 3 days with daily changes of PBS. Ehrlich ascites tumor cells were grown in T25 plastic bottles (Falcon) in Eagle's minimal essential medium supplemented with 5% calf serum, 1 g/liter of glucose and 25 mM morpholinopropanesulfonic acid in a CO_2 incubator (9). Cells were in exponential growth phase (10–13-h doubling times) before being used for experiments. Cells synchronized in the S phase of the growth cycle were obtained by incubating exponentially growing cells with aphidicolin (1 μg/ml) for 12 h (2). Cells synchronized in the non-S phases of the growth cycle were obtained by incubating S-phase cells in normal growth medium for 6 h. By that time the rate of incorporation of [^{3}H]thymidine into DNA decreased by 85% and did not decrease further over the following 4 h.

Treatment with HU and Measurement of DNA Single-Strand Breaks. Cells were seeded at 2×10^5/T25 bottle, allowed to grow overnight, and then incubated for 24 h in growth medium containing [^{14}C]thymidine (10 nCi/ml). Cells were then incubated for 4 h in medium containing 5% dialyzed serum before the addition of HU. After periods of incubation as specified, cells were detached by a 3-min incubation in 0.5 ml of PBS containing 0.4 mM EDTA and 0.04% trypsin. Cells were then diluted with 20 ml of ice-cold PBS, collected by centrifugation, and processed for alkaline elution as described previously (1). Alkaline elution was performed by the method of Kohn *et al.* (10). Relative elution values, which are proportional to the frequency of DNA single-strand breaks (11), were calculated by the formula

$$\log R_o - \log R,$$

where R_o and R represent fractions of DNA retained on the filter in the samples of the untreated and of the HU-treated cells, respectively, after 9 h of elution. Percentages of strand breaks repaired were calculated by the method described by Bradley and Kohn (12).

Cytotoxicity Analyses. Survival of cells after HU treatment was determined by colony formation in soft agar. Two hundred cells in 1.5 ml of growth medium supplemented with 10% calf serum and 0.33% Noble agar (Difco Laboratories, Detroit, MI) were plated in triplicate in 100-mm plastic dishes containing a solidified base layer of 0.5% Noble agar in the same medium. Colonies were counted 12 to 15 days after plating.

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[1] Presented in part at the 17th Meeting of the Federation of European Biochemical Societies, August 1986, West Berlin (31).

[2] The abbreviations used are: HU, hydroxyurea; PBS, phosphate buffered saline, pH 7.3; dAdo, deoxyadenosine; dGuo, deoxyguanosine; dCyd, deoxycytidine; dThd, thymidine.

RESULTS

In these studies 0.5–10 mM HU was used; this range of HU concentrations is frequently used in studies of DNA repair, of HU cytotoxicity, and of other effects of HU. When exponentially growing [^{14}C]thymidine-labeled Ehrlich ascites tumor cells were incubated with HU, there was a progressive accumulation of strand breaks in mature DNA. Fig. 1*A* shows typical alkaline elution patterns of cells that were incubated in parallel cultures with 10 mM HU for up to 16 h. Relative elution values, which are a measure of the frequency of single-strand breaks, were calculated from the above elution curves and are shown in Fig. 2*A*. The rate of strand break accumulation appears to be very rapid during the first h of incubation and is constant thereafter. Other experiments showed that strand breaks continued to accumulate in a linear fashion for at least 24 h. For purposes of comparison, irradiation of these cells with 300 rads results in about the same number of strand breaks (1) as a 4-h incubation with 10 mM HU. There was no loss of cell viability, as measured by trypan blue exclusion, during the 16-h incubations with HU. There was a gradual release of incorporated [^{14}C]-thymidine from the cells into the medium, reaching 8 ± 5% (SD) above that of untreated cells after 16 h of incubation.

Accumulation of DNA strand breaks was also dependent on the concentration of HU. Fig. 1*B* shows typical alkaline elution patterns of cells that were incubated in parallel cultures for 16 h with concentrations of HU between 0.5 and 10 mM. Fig. 2*B* shows the relative elution values calculated from the above alkaline elution patterns. The extent of strand break accumulation increased with the concentration of HU but was not precisely proportional. As the HU concentration was increased from 0 to 2 mM, the rate of increase in the frequency of strand breaks was almost twice that when HU was increased from 2 to 10 mM.

The kinetics of repair of the HU-induced strand breaks is shown in Fig. 3. Cells were incubated with 10 mM HU for 4 h, washed, and then incubated in normal growth medium. There was a rapid phase of repair during the first 4 h, during which time about 55% of strand breaks were rejoined. Thereafter, the rate of repair decreased by about 90%. After 16 h of repair incubation, 73% of strand breaks were rejoined.

Accumulation of strand breaks was markedly decreased by the addition of deoxyribonucleosides to the medium (Fig. 4). In four experiments in which cells were incubated with 10 mM

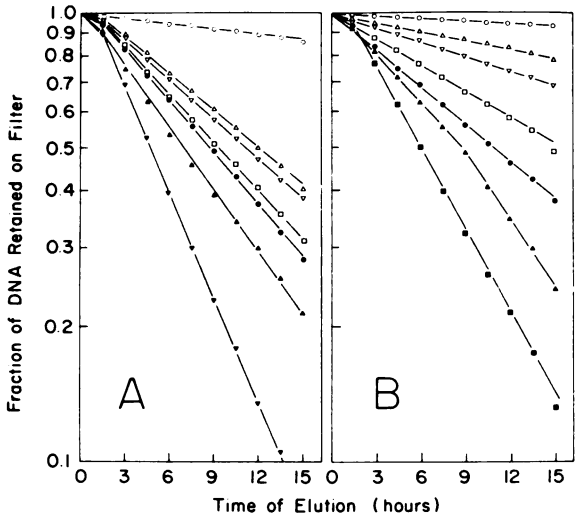

Fig. 1. Alkaline elution patterns of cells treated with HU. In *A*, parallel cultures of exponentially growing cells were incubated for 0 (○), 1 (△), 2 (▽), 4 (□), 6 (●), 8 (▲), and 16 (▼) h with 10 mM HU. In *B*, parallel cultures of exponentially growing cells were incubated for 16 h with 0 (○), 0.5 (△), 1 (▽), 2 (□), 4 (●), 6 (▲), and 10 (■) mM HU.

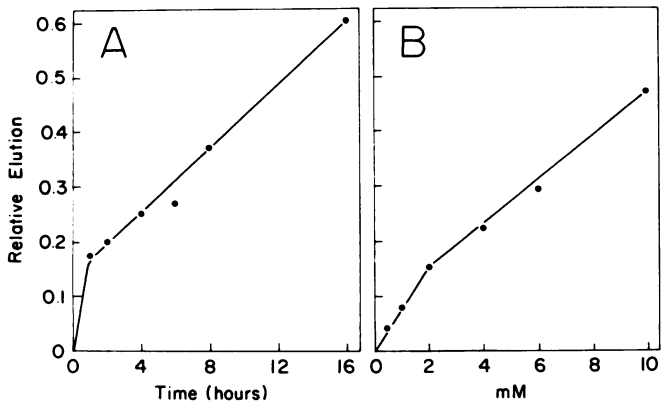

Fig. 2. Relative elution values of cells treated with HU. *A*, increases in relative elution as a function of time of incubation with HU (data from Fig. 1*A*). *B*, increases in relative elution as a function of HU concentration (data from Fig. 1*B*).

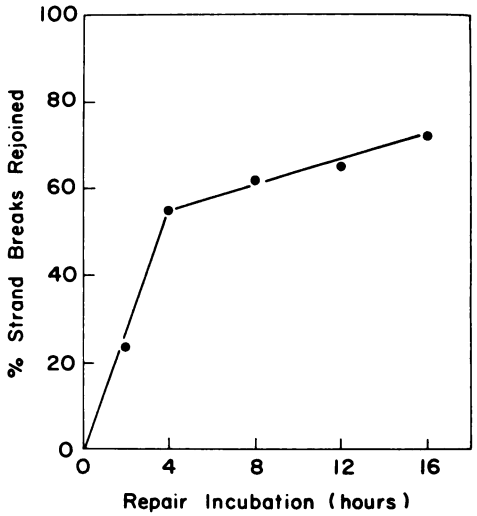

Fig. 3. Repair of HU-induced DNA strand breaks. Cells were incubated for 4 h with 10 mM HU, washed, and then incubated in normal growth medium for time periods as shown in parallel cultures. Alkaline elution patterns were determined and percentages of strand breaks rejoined were calculated by the method of Bradley and Kohn (12).

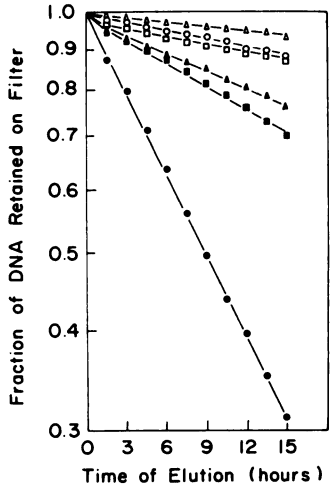

Fig. 4. Effect of deoxyribonucleosides on the accumulation of DNA strand breaks in HU-treated cells. Alkaline elution patterns of cells incubated in parallel cultures with (●, ■, ▲) or without (○, □, △) 10 mM HU for 4 h in normal growth medium (○, ●,); in medium supplemented with 0.4 mM dThd, dAdo, dGuo, and dCyd (□, ■); or in medium supplemented with 0.4 mM dAdo, dGuo, and dCyd (△, ▲).

HU for 4 h in parallel cultures, 67% (mean value; range, 50–82%) of strand breaks were prevented by 0.4 mM concentrations of all four deoxyribonucleosides and 67% (mean value; range, 46–88%) were prevented by 0.4 mM dAdo, dGuo, and dCyd. Thus, the inclusion of dThd was not essential. Addition of 0.4 mM dAdo and dGuo or of either one was 25–50% as effective as the combination of dAdo, dGuo, and dCyd. Addition of 0.4 mM dCyd alone was ineffective. Deoxyribonucleosides at 0.8 mM were not more effective than at 0.4 mM.

Because deoxyribonucleosides did not completely prevent strand break accumulation, the possibility arose that some of the strand breaks resulted from a direct action of HU metabolites on DNA. One mechanism is the degradation of DNA by carbamoyloxyurea (13); however, the formation of this oxidation product of HU requires longer incubations than were used in the above experiments. Another mechanism is the genotoxic action of free radicals derived from HU, as reported in human lymphoblastoid cells incubated with liver microsomes and NADPH (14) and in cytochrome P-450-containing hepatoma cells (15). The HU-induced DNA damage was counteracted by catalase, superoxide dismutase, several free radical scavengers and, in the case of hepatoma cells, SKF 525-A, an inhibitor of cytochrome P-450. Accordingly, we incubated Ehrlich ascites cells with HU in the presence of catalase (25–200 μg/ml), superoxide dismutase (50 μg/ml), glutathione (2 mM), sodium benzoate (50 mM), ethanol (100 mM), or SKF 525-A (0.1 mM). None diminished the extent of strand breakage induced by HU.

HU kills cells in the S phase of the cell cycle (16–18) although an additional mode of killing that is not S phase specific has been reported (18). If unrepaired strand breaks are responsible for cell killing, the question arises whether strand breaks form only during the S phase or in other phases as well. In the following experiments we compared the accumulation of strand breaks in cells synchronized in S phase, in cells that were incubated for 6 h in normal medium after synchronization in S phase (non-S-phase cells), and in unsynchronized, actively growing cells in parallel cultures. We found that more strand breaks accumulated in S phase cells than in non-S-phase cells. After 4-h incubations with 10 mM HU, S-phase cells had accumulated 110 ± 28% (mean ± SD of 3 experiments) more strand breaks than non-S-phase cells and 45 ± 6% more strand breaks than unsynchronized cells. Fig. 5 shows the results of one such experiment.

We also examined the correlation between the frequency of DNA strand breaks and the survival of cells, as measured by

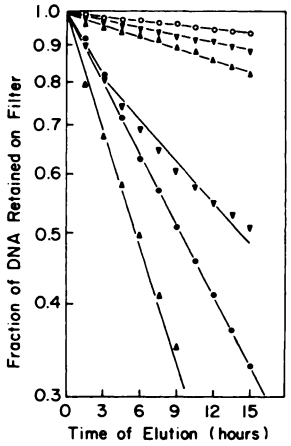

Fig. 5. Alkaline elution patterns of cells synchronized in S phase (△, ▲) and of non-S-phase cells (▽, ▼) and of exponentially growing cells (○, ●) that were incubated without (△, ▽, ○) or with (▲, ▼, ●) 10 mM HU for 4 h in parallel cultures.

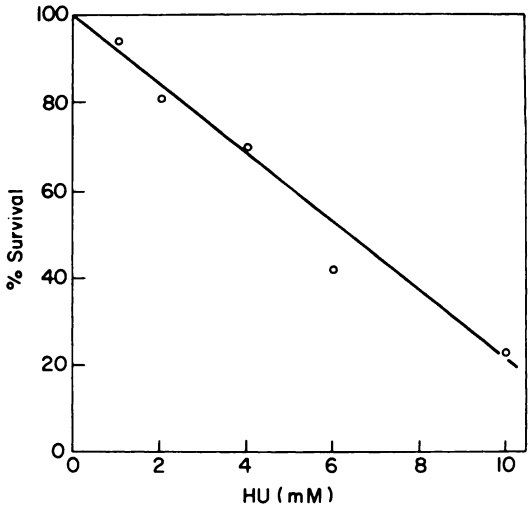

Fig. 6. Survival of exponentially growing cells after treatment with HU. Cells were incubated in parallel cultures with concentrations of HU as shown for 4 h and survival was determined by a colony formation method.

colony-forming ability in soft agar. As shown in Fig. 6, when cells were incubated with 0–10 mM HU for 4 h, the percentages of surviving cells decreased in proportion to the increase in HU concentration.

DISCUSSION

The results of the present study extend the evidence that agents which reduce the supply of deoxyribonucleoside triphosphates cause a progressive accumulation of strand breaks in mature DNA molecules. We first noted this effect in methotrexate-treated tumor cells (1). Our results were recently confirmed by Lönn and Lönn (19), who also extended them to the action of 5-fluoropyrimidines. The present results with HU are important in that HU restricts the supply of both purine and pyrimidine deoxyribonucleotides and its action is unrelated to a proposed mechanism of "thymineless death" in cells treated with antifolates (20–22). The latter hypothesis postulates a misincorporation of dUMP into DNA and the subsequent excision-repair reactions as the mechanism of DNA fragmentation. This hypothesis would not explain strand breaks in HU-treated cells. Moreover, a recent study failed to detect any misincorporated uracil in DNA of methotrexate-treated cells (23). We (1) postulated that strand breaks in mature DNA of methotrexate-treated cells arose from unrepaired spontaneous DNA lesions of various types. Lindahl (24) has provided some estimates of the rates at which some of the spontaneous DNA lesions occur. We have no direct evidence for this hypothesis, but it is the best explanation of the accumulation of strand breaks in mature DNA of cells treated with methotrexate, 5-fluorodeoxyuridylate, and HU.

The above described mechanism of strand break accumulation in HU-treated cells is based on the well-documented inhibition of ribonucleotide reductase by HU and on the finding that deoxyribonucleosides greatly diminished strand break accumulation (Fig. 4). The reason that deoxyribonucleosides did not completely prevent strand breaks, even when used at high concentrations, is probably because only small proportions of dAdo and dGuo are phosphorylated to dAMP and dGMP; they are largely degraded and excreted (see Ref. 25 for a more complete discussion). A partial prevention of HU cytotoxicity also requires a high concentration (about 0.4 mM) of deoxyribonucleosides (18).

Alternatively, a portion of strand breaks could have been contributed by genotoxic metabolites derived from HU. Our results do not provide support for this mechanism; neither do they rule it out.

The increase in the frequency of strand breaks with the time of incubation with HU (Figs. 1*A* and 2*A*) presumably represents the rate of net accumulation of unrepaired spontaneous DNA lesions. A comparison of the rates of accumulation of strand breaks in methotrexate-treated cells (Ref. 1, Fig. 4) and in HU-treated cells (Fig. 2*A*) shows that strand breaks accumulate more than twice as rapidly in HU-treated cells than in methotrexate-treated cells. The faster accumulation of strand breaks in HU-treated cells may be due to a more complete inhibition of DNA repair. The increase in the accumulation of strand breaks as a function of HU concentration (Figs. 1*B* and 2*B*) probably reflects the concentration-dependent decrease in DNA repair capacity.

A portion of the lesions that arise during the incubation with HU may not be repairable. About 50–70% of strand breaks are repaired during the first 4 h (Fig. 3). Thereafter, the repair process becomes very slow, raising the possibility that the remaining lesions may persist and may account for HU cytotoxicity.

A number of investigators using HU in studies of DNA repair have commented that HU by itself does not cause DNA strand breaks (6–8). A transient formation of strand breaks (26) and fragmentation of native DNA after 24-h incubations with HU (27) have been reported. We cannot explain the discrepancy between these findings and ours. Possible factors include shorter incubation times in DNA repair experiments, differences in sensitivity to HU among cell types (28) and, most importantly, the sensitivity of methods used to detect DNA lesions. The alkaline elution method is far more sensitive than methods used in previous studies (10). The recent report (29) of numerous chromosomal aberrations of various types in cells that had been incubated for 6 h with HU during S phase is consistent with our results.

HU is generally held to be an S-phase-specific agent (16–18) and its cytotoxicity has been ascribed to the inhibition of DNA replication. Since this inhibition is readily reversed on removal of HU (28), it is not obvious how cell killing would result. Our results (Fig. 5) suggest a different reason for S phase specificity; more strand breaks accumulate in S-phase cells than in non-S-phase cells. This finding may be explained by the greater frequency of spontaneous DNA lesions in replicating DNA molecules (24) and by a greater tolerance of DNA damage during DNA replication (30). The specificity of HU cytotoxicity for cells in S phase was reported to be absolute in some studies (16, 17), while in others it was accompanied by a non-S-phase-specific mode (18). If the accumulation of strand breaks is causally related to cell toxicity (as suggested by the data in Fig. 6), our findings explain both the greater vulnerability of cells in S phase and the cytotoxicity to cells not in S phase.

The results of the present study help to elucidate the mechanism of cytotoxicity of HU and of other antimetabolites that inhibit the generation of precursors for DNA synthesis. The results also provide strong evidence against the use of HU in DNA repair studies and may have implications for gene amplification mechanisms (see Ref. 29).

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