HYDROXYUREA: INDUCTION OF BREAKS IN TEMPLATE STRANDS OF REPLICATING DNA

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SUMMARY: Hydroxyurea causes the appearance of breaks in the template strand of DNA in cultured mammalian cells that are replicating their DNA but not in the DNA of non-replicating cells. This effect is transient even in the continued presence of hydroxyurea and is preventable when deoxynucleosides are present in the culture medium.

INTRODUCTION

While investigating the effects of hydroxyurea (HU) on the repair of ultraviolet light induced damage to DNA of mammalian cells, we found that HU itself caused the formation of single-strand breaks. Further examination showed that HU induced the formation of single-strand breaks only in cells that were replicating their DNA. These breaks appeared transiently even in the continued presence of HU and their appearance was suppressed by the simultaneous addition of a deoxynucleoside mixture. The phenomenon appears therefore to be a consequence of the reduction of the deoxynucleoside triphosphate (dNTP) pool, but the molecular mechanism remains speculative.

METHODS AND MATERIALS

Petri dish cultures of mouse L-cells were prelabelled in their DNA by overnight growth in alpha MEM medium (Flow Laboratories, Rockville, Md., U.S.A.) containing 0.1 μCi[^{3}H]-thymidine per ml (20 Ci/m mol) and supplemented with 10% fetal calf serum plus penicillin, streptomycin and tylocin (1). These cells were then synchronized by the 5-fluorodeoxyuridine (FdUrd) technique and were exposed to hydroxyurea at various times as indicated in the legend of Figure 1.

Secondary mouse embryo cells were grown in medium containing 3% fetal calf serum (2,3). After 24 hours of growth [^{14}C]-thymidine was added (0.125 μCi/ml, 61 mCi/m mol) and after a further 116 hours the medium was replaced with fresh medium containing 10% fetal calf serum. Ten hours later the cells enter S-phase synchronously (2,3) which we verified by pulse-labelling experiments.

When desired for analysis the cells were scraped from the petri dishes into phosphate buffered saline and a sample of the suspension was introduced into 0.3 ml of lysing layer which overlaid 4.5 ml of a preformed 5-20% alkaline sucrose gradient (1). The gradient rested on a 0.4 ml cushion of 35% sucrose. Centrifugation was done in a Beckman SW 50.1

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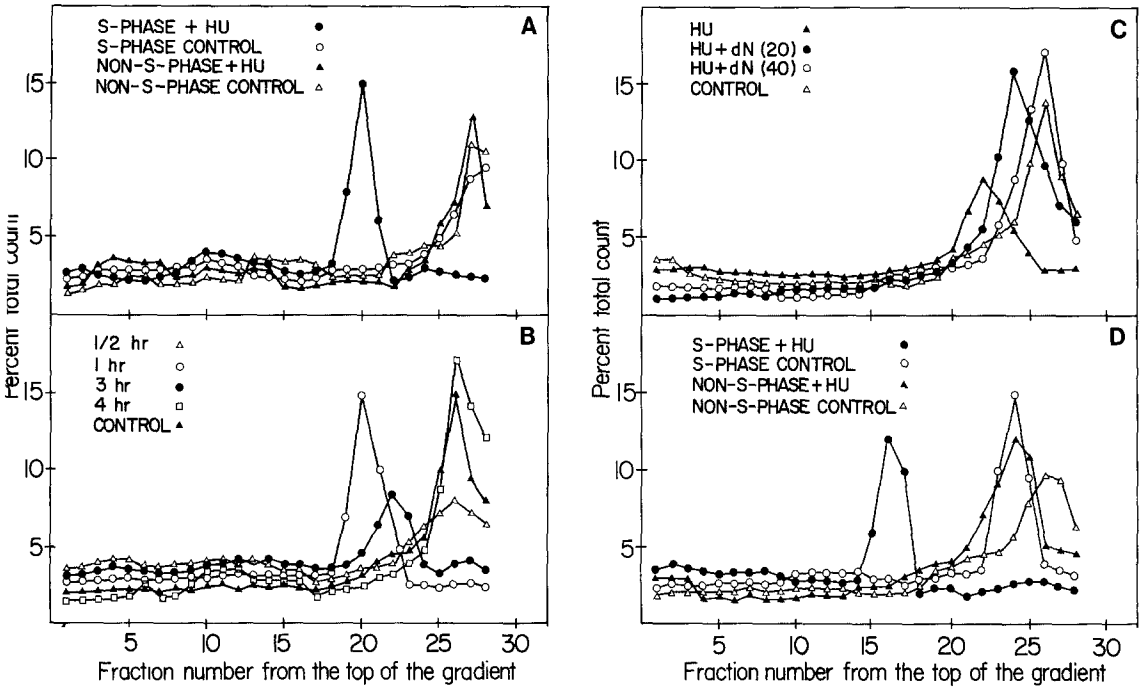

Figure 1. Characteristics of hydroxyurea-induced single strand breaks in DNA. (A) Prelabelled L-cells were treated with 1 x 10^{-6} M FdUrd for 12 hours followed by 4 x 10^{-5} M thymidine to release them synchronously into S-phase (6). After 30 minutes HU was added (final concentration 1 x 10^{-2} M) and 1 hour later the cells were removed for analysis. Non-S-phase cells received no thymidine. (B) The protocol was similar to that in (A), but cells were taken for analysis at 30 minutes, 1, 3 and 4 hours after adding HU. (C) The protocol was similar to that in (A), but a mixture of the 4 deoxynucleosides was added along with the HU, final concentration of 20 µg/ml each, closed circles; 40 µg/ml each, open circles. (D) Prelabelled secondary mouse embryo cells were synchronized as described above. Cells in mid S-phase, or prior to commencement of S-phase, were treated for 1 hour with 1 x 10^{-2} M HU and then the cells were taken for analysis.

rotor at 15,000 rpm, 20°C, for 4 hours. Fractions were collected and analysed as described previously (1). The cells were allowed to lyse for 25 ± 5 minutes before starting the centrifuge.

RESULTS

It will be seen in Fig. 1 A that the DNA from HU treated non-S-phase cells, from untreated non-S-phase cells and from untreated S-phase cells all sedimented to the bottom of the centrifuge tube. In contrast DNA from HU treated S-phase cells sedimented more slowly indicating a lower molecular weight and suggesting that the DNA had suffered single strand breaks.

At the lower concentration of 1×10^{-3} M HU no breaks were observed. Similar experiments with HeLa cells gave identical results. We have also examined the effects of cytosine arabinoside, another inhibitor of DNA synthesis (4) and found that it too induced the formation of single-strand breaks in the template strand of DNA of S-phase L-cells, but not in DNA of non-S-phase L-cells. Thus, the effect is not confined to a single cell type nor a single inhibitor of DNA synthesis.

When the duration of exposure of S-phase L-cells to HU was varied the results shown in Fig. 1 B were obtained. An exposure time of more than 30 minutes was required before single-strand breaks appeared in the template strand. The number of breaks reached a maximum by about 1 hour and then declined so that by 4 hours they were no longer apparent.

Fig. 1 C shows that when a mixture of the 4 deoxynucleosides was added along with HU to S-phase L-cells, the extent of single-strand breakage was reduced. At a final concentration of 20 µg/ml for each of the deoxynucleosides this reduction was plainly apparent while at 40 µg/ml single-strand breaks were virtually absent.

The block in DNA replication in L-cells produced by FdUrd is never complete; small pieces of DNA continue to be formed (5). Also, after release by the addition of thymidine, S-phase is compressed to about 4 hours from a physiological value of 8 hours (6). It seemed possible, therefore, that the single-strand breakage described above was not strictly ascribable to a general effect of HU on S-phase cells and may have been due to a peculiarity of FdUrd treatment. An alternative method of obtaining synchronized cell populations is to grow a secondary culture of mouse embryo cells for 140 hours in medium containing a low serum concentration; ten hours after the serum concentration has been restored to the usual value of 10%, the cells enter S-phase synchronously and the duration of S-phase is not compressed (2,3). When this system of synchronized cells was employed it was seen again (Fig. 1 D) that hydroxyurea induced single-strand breaks in the DNA from S-phase cells but not from non-S-phase cells.

DISCUSSION

What is the explanation of the HU-induced breakage and repair pattern for template-strand DNA in replicating cells? The strand-breaking is not likely to be the result of a chemical reaction between DNA and HU or a product derived from HU for the following reasons. Freshly recrystallized HU gave the same results as unrecrystallized HU. The longest experiments lasted 4 hours and formation of a reactive HU derivative

requires 24 hours (7). DNA in non-replicating cells did not develop single-strand breaks and it is unlikely that this DNA would be significantly less susceptible to chemical attack. The formation of single-strand breaks was inhibited by the addition of deoxynucleosides. Finally, in the continued presence of HU, the single-strand breaks instead of increasing in number or remaining constant gradually disappeared.

It seems likely that HU, by decreasing the availability of deoxynucleoside triphosphates is interfering with the normal completion of DNA chains. HU inhibits the enzyme ribonucleoside diphosphate reductase and the supply of deoxynucleotide precursors for DNA synthesis is thereby reduced (8). Although the ensuing inhibition of DNA synthesis is considerable and appears to be responsible for cell death, it is rarely if ever complete. This has been noted by a number of investigators who have used HU to suppress replicative DNA synthesis while examining repair synthesis (9-11). Coyle and Strauss (12) found that ^{3}H-thymidine continued to be incorporated into small DNA fragments in the presence of HU. The small fragments, in the presence of HU, attained a size of about 2×10^{7} daltons but were never incorporated into the very large sized DNA seen in control cells. When the HU was removed the DNA fragments could be 'chased' into DNA with a size equal to that from control cells. Studies of the effect of HU on replication of polyoma virus DNA by Magnusson et al (13) and of SV40 virus DNA by Laipis and Levin (14) demonstrated an accumulation of DNA fragments which were slowly incorporated into large pieces of DNA. In order to explain why HU caused a gap to remain between DNA fragments and yet allowed the fragments to be synthesized, Laipis and Levin suggested that two DNA polymerases are involved in SV40 DNA replication. The larger polymerase which may be responsible for construction of the DNA sub-chains, has a Km value for deoxynucleoside triphosphates about 5-8 fold lower than that of the smaller polymerase which could be responsible for uniting the sub-chains (15,16). Thus, when the deoxynucleoside triphosphate concentration reached a critically low level some initiation and elongation of DNA chains would occur but joining together of these fragments would be drastically curtailed.

During replication of DNA the template strand must be broken and rejoined in the vicinity of the replicating fork in order to prevent restrictive supercoiling. Taylor et al (17) have provided evidence for this occurrence in replicating mammalian cells. The proposed breaking and rejoining of the template strand conceivably could be brought about by an enzyme such as omega, first described by Wang (18) in E. coli and subsequently shown to occur in mammalian nuclei (19) or, perhaps by the

combined action of a specialized endonuclease and polynucleotide ligase. In either case there is no apparent role for deoxynucleoside triphosphates and yet, the effects observed in this study appear to have their basis in altered deoxynucleoside triphosphate levels. Perhaps under the non-physiological conditions that prevail in the presence of HU, a small number of nucleotides is removed in association with nicking. Repair of these gaps would then occur slowly in the continued presence of HU in accordance with the availability of deoxynucleside triphosphates. This proposal can be tested in a manner analogous to the way that repair of damaged DNA is measured, that is, one would look for the incorporation of labelled bromodeoxyuridine in parental strands of DNA.

Finally, it may be asked why single-strand breaks do not continue to appear in the continued presence of HU? Presumably the breaks are formed in only the small population of DNA molecules that were engaged in replication when the deoxynucleoside triphosphate pool reached a critically low concentration. After that very few DNA molecules would participate actively in replication.

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