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THE EFFECT OF 5-FLUOROURACIL ON DNA CHAIN ELONGATION IN INTACT BONE MARROW CELLS

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The effect of 5-fluorouracil (FUra) on DNA elongation was assessed in intact bone marrow cells that had been pulsed for 1 hr with [^{3}H]-dThd in the absence or presence of FUra, chased in fresh media from 0 to 3 hr, and then analyzed on alkaline sucrose gradients. While DNA from control cells elongated at an average rate of 86 nucleotides per sec over a 3 hr interval, DNA from FUra-treated cells did not elongate and in contrast decreased in size over the same interval. In a parallel study to examine what happens to the FUra that was incorporated into DNA, bone marrow cells were pulsed for 1 hr with 50 μM [^{3}H]-FUra, and then chased in fresh media from 0 to 2 hr. An aliquot of cells from each time point was lysed on an alkaline sucrose gradient to assess the size of [^{3}H]-FUra-containing DNA, while another aliquot of cells from each time point was analyzed for radioactivity remaining in total DNA. The percentage of replicon-size DNA (≥ 100S) containing radiolabel decreased over the 2 hr chase while the percentage of small molecular weight DNA (≤ 7.2S) increased over the same interval. These changes in DNA size were accompanied by a decrease in radioactivity in total DNA. These studies suggest that excision of FUra from nascent DNA chains may prevent further elongation of DNA.

The biochemicals event associated with cytotoxicity of the antineoplastic drug FUra[2] have been intensively investigated since its introduction in 1957 (1,2). While most of these studies have focused on the resultant inhibition of thymidylate synthase (2-4) or incorporation of FUra in RNA (5-7), recent studies have demonstrated that [6-^{3}H]-FUra and its nucleoside [6-^{3}H]-FdUrd can be incorporated into DNA (8-16). However, the actual amount of FUra detected in DNA is rather small (8). This has been attributed to the presence of enzymes which minimize the amount of FUra deoxyribonucleotide that is available to be incorporated (17), and other enzymes which can remove FUra that has been incorporated into DNA (18-19). One effect of the incorporation of FUra into

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[2] Abbreviations: FUra, 5-fluorouracil; FdUrd, 5-fluorodeoxyuridine; AP, Apurinic/Apyrimidinic; dThd, thymidine; α-MEM, α-minimal essential media.

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DNA is the formation of DNA fragments (11,13,16) thought to be secondary to DNA repair in which FUra is initially removed from DNA by uracil glycosylase followed by cleavage of the AP site by an AP endonuclease (11). In the present study we attempt to clarify what happens to DNA that has been synthesized in the presence of FUra (at a concentration that results in incorporation of FUra into DNA) and specifically to determine whether this DNA can elongate.

MATERIALS AND METHODS

Chemicals. [6-^{3}H]-FUra (18 Ci/mmol) and [methyl-^{3}H]-dThd (50 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Prior to using, each isotope was chromatographed by HPLC and purified if necessary to be certain of the absence of contaminants such as other pyrimidines (or metabolites) or free tritium. All other chemicals used were of the highest quality commercially available.

Preparation of Cells. Studies were performed using single cell suspensions of marrow cells isolated from the femurs and tibias of male CF-1 mice (21-22 gm) as previously described (12). Cell viability, as determined by trypan blue dye exclusion, was 85% or greater during these experiments. Cells were suspended in α-MEM (Gibco #410-2000, Grand Island, NY) containing no deoxyribonucleosides or ribonucleosides.

Pulse Labelling DNA with [6-^{3}H]-dThd. [6-^{3}H]-dThd (10 μCi/ml) was added to a single cell suspension for 1 hr at 37°C in the absence or presence of 50 μM FUra. Following the 1 hr pulse, the radioactive media was removed and the cells were washed with fresh α-MEM at 0°C. The cells were then chased for 0 to 3 hr in α-MEM containing 10 μM (non-labelled) dThd. The chase was terminated by removing the cells and placing them in an ice-cold water bath. An aliquot of control and FUra-treated cells at each time point was then analyzed on an alkaline sucrose gradient as described below.

Labelling DNA with [6-^{3}H]-FUra. DNA was labelled with [6-^{3}H]-FUra in accordance with earlier studies in bone marrow cells that had demonstrated that FUra was incorporated into DNA at pharmacologically-relevant cytotoxic concentrations of FUra (12) and that the FUra was incorporated as primarily 3'-FdUMP indicating localization within internucleotide linkages and not at the chain termini (16). 50 μM [6-^{3}H]-FUra (800 μCi/mmol) was added to a single cell suspension in α-MEM for 1 hr at 37°C. The radioactive media was then removed and the cells were washed with fresh α-MEM at 0°C. The cells were subsequently chased for 0, 1, or 2 hr in α-MEM containing 50 μM (non-labelled) FUra. The chase was terminated by removing the cells and placing them in an ice cold water bath. An aliquot of cells from each time point was analyzed on an alkaline sucrose gradient as described below, while a second aliquot was analyzed for radioactivity in total DNA (see below).

Alkaline Sucrose Gradients. Alkaline sucrose gradients were prepared essentially as described by Rawles and Collins (20) and spun in a Spinco SW 41Ti rotor at 17,500 rpm. Briefly, gradients were made up with a bottom layer consisting of 1.7 ml of 60% sucrose in 0.1 N NaOH, overlayed with a 10 ml linear (5 to 20%) sucrose gradient in 0.1 N NaOH, 0.9 M NaCl, and 10 mM EDTA. A 0.5 ml layer of a cell lysing solution containing 0.45 N NaOH, 0.55 M NaCl, 10 mM EDTA and 1% sodium N-lauroyl sarcosinate was gently pipetted onto the top of the gradient following which 0.5 ml of the cell suspension (approximately 4 x 10^6 cells representing a maximum of 20 μg DNA) was layered over the lysis solution. By lysing the cells directly on the alkaline sucrose gradients in

the dark, potential mechanical shearing of the DNA secondary to DNA isolation techniques was minimized. The gradients were incubated for 5 hrs at 2°C in the dark. From the data of Davison (21) it can be calculated that the size of the largest single strand of DNA able to denature and unwind after 5 hr in alkali is on the order of 3.13×10^9 daltons. Centrifugation at 17,500 rpm for 180 min at 2°C resolved material up to 245S, while centrifugation for 340 min resolved material up to 130S.

Gradient Fractionation. Following centrifugation of the [methyl-^{3}H]-dThd labelled cell lysate, the gradients were punctured from the bottom and fractions were collected onto Whatman 3 MM filter discs with quantitation of radioactivity as previously described (12). Following centrifugation of the [6-^{3}H]-FUra labelled cell lysate, the gradients were punctured from the bottom and fractions were collected into separate 12 mm by 75 mm tubes. To each fraction 0.3 N NaOH was added. These samples were incubated for 18 hr at 37°C to hydrolyze RNA. Subsequently each fraction was pipetted onto a Whatman 3 MM disc, dried, washed twice with 5% (w/v) trichloroacetic acid, ether extracted, followed by evaporation with subsequent quantitation of the radioactivity (12).

Determination of S Values, Molecular Weights, Nucleotides/DNA Chain. Gradients were calibrated with R6K plasmid [^{3}H] DNA having 39S and 51S components (22). Sedimentation constants of radioactive DNA from these cells were estimated by linear interpolation according to the method of Martin and Ames (23). A weighted average of the various DNAs were calculated from the sum of the fractional S values using Studier's equation, S = 0.028(M) 0.400, where M is the molecular weight and S is the sedimentation coefficient (24). The number of nucleotides per DNA chain were calculated by the following equation: M/330, where M is the weight-averaged molecular weight of the DNA determined above and 330 is the average molecular weight of a DNA base.

Analysis of Radioactivity in DNA. The cells (5×10^6 cells) which had been incubated with [6-^{3}H]-FUra and then chased for 0 to 2 hrs were analyzed for radioactivity in total extracted DNA. DNA was extracted as described previously (12). DNA was quantitated using the diphenylamine reaction as modified by Burton (25).

Measurement of the Effect of FUra on Total DNA Synthesis. This was assessed as described previously (12) measuring radioactivity from [methyl-^{3}H]-dThd incorporated into total DNA. Percent DNA synthesis = (cpm/g of DNA of FUra-treated)/(cpm/g of DNA of control) x 100.

RESULTS AND DISCUSSION

Figure 1 compares the size and subsequent elongation of tritium-labelled DNA that had been pulsed for 1 hr with [methyl-^{3}H]-dThd in the absence (control) or presence of 50 μM FUra. In control, the longest chain of DNA increased from 73S (approximately 1.06×10^5 nucleotides) at the beginning of the chase (Fig. 1A) to 93S (approximately 1.93×10^6 nucleotides) at the end of a 3 hr chase (Fig. 1B). The average rate of DNA chain elongation in these control cells was approximately 86 nucleotides/sec, consistent with previously reported values for mammalian cells (26-28).

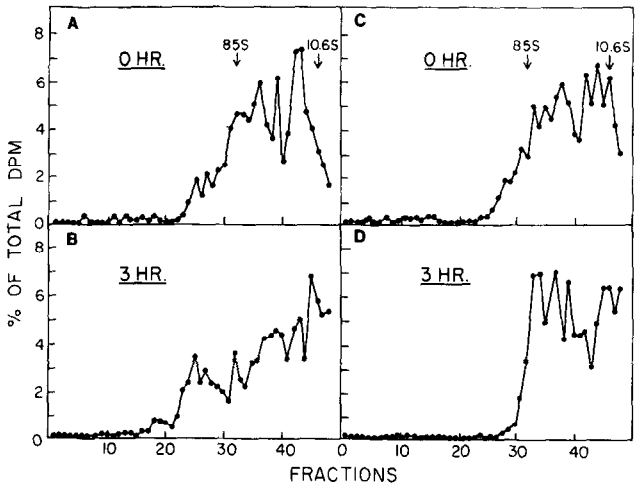
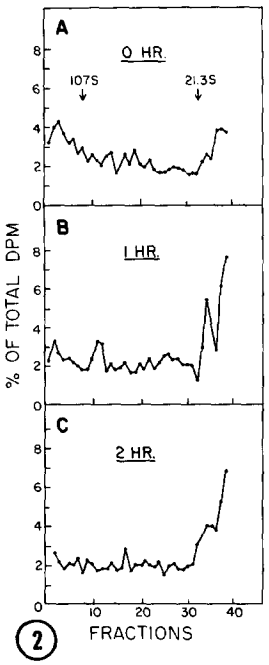


Fig. 1. Comparison of the size and subsequent elongation of tritium-labelled DNA that had been pulsed for 1 hr with [methyl-^{3}H]-dThd in the absence (A and B) or presence (C and D) of 50 μM FUra. A and C show the distribution of tritium in fractions before the chase (0 hr) while B and D show the distribution after the 3 hr chase. The location of 85S and 10.6S markers is shown.

It should be noted that a different pattern of distribution of label is seen in the FUra-treated cells (Fig. 1C) compared to control (Fig. 1A) at time 0. The average DNA chain length in the FUra-treated cells was 7.0×10^4 nucleotides compared to control which was 1.06×10^5 nucleotides. DNA synthesis in the FUra-treated cells (assessed at time 0) was 67% of control.

In contrast to control cells, there was no DNA elongation in FUra-treated cells following a 3 hr chase (Fig. 1D) with the average DNA chain length decreasing to 5.0×10^4 nucleotides. This value was 87% of that observed in FUra-treated cells at time 0 (Fig. 1C) and is consistent with DNA fragmentation. These findings are compatible with earlier studies that have demonstrated an increase in DNA fragments after incubation of cells with FUra (9,11). Our studies were carried out at a pharmacologically relevant concentration of FUra known to produce cytotoxicity (12) and resulted in detectable changes in DNA after a much shorter exposure to FUra [3 hr in contrast to the 24 hr incubation used by Cheng and Nakayama (11)] and at a much lower concentration of FUra [50 μM instead of 1 mM used by Lonn and Lonn (9)].

In order to better characterize the apparent decrease in DNA size seen after the chase in Fig. 1D, studies were undertaken with [6-^{3}H]-Fura. The effect of increasing chase on size of [6-^{3}H]-FUra labelled DNA is shown in Figure 2 which demonstrates that initially approximately 33% of the DNA chains sediment at greater than 100S, while after a 2 hr chase less than 17% of the DNA chains sediment at greater than 100S. If one calculates the average DNA chain length, it can be seen that the DNA chain size decreases from approximately 1.7 x 10^5 nucleotides at time 0 (Fig. 2A) to 1.04 x 10^5 nucleotides at 2 hr (Fig. 2C). Thus, with increasing chase, the label is associated with a greater proportion of smaller DNA. Over this same interval there was a 39% decrease in tritium in total DNA suggesting removal of FUra from DNA (Fig. 3A), accompanying the changes in DNA size (Fig. 2 and Fig. 3B).



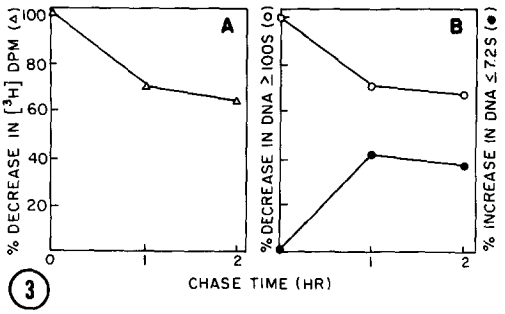


Fig. 2. Effect of increasing chase on size of [6-^{3}H]-FUra-labelled DNA resulting from a 1 hr exposure of bone marrow cells to 50 μM [6-^{3}H]-FUra and subsequently chased with fresh α-MEM for: A, 0 hr; B, 1 hr; C, 3 hr. The location of 107S and 21.3S markers are shown.

Fig. 3. Relationship over a 2 hr chase between: A. the decrease in tritium in total DNA labelled with [6-^{3}H]-FUra and B. the change in the size distribution of this DNA, assessed here as both the percent decrease in large molecular weight DNA (≥ 100S) and the percent increase in small molecular weight DNA (≤ 7.2S).

This study suggests a temporal relationship between removal of FUra from DNA and change in DNA size with a decrease in the percentage of replicon size (≥ 100S) DNA (28) containing [6-^{3}H]-FUra being associated with an accompanying increase in the percentage of low molecular weight (≤ 7.2S) DNA containing [6-^{3}H]-FUra. Thus, the increase in small molecular weight DNA fragments appears to be due to degradation of large molecular weight DNA secondary to alteration of DNA structure following excision of FUra and not due to inhibition or stalling of synthesis of DNA at the replication fork.

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