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MOLECULAR EFFECTS OF 2′,2′-DIFLUORODEOXYCYTIDINE (GEMCITABINE) ON DNA REPLICATION IN INTACT HL-60 CELLS*

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Abstract—The ability of pH-step alkaline elution to isolate different size species of nascent DNA (nDNA) from intact cells was utilized to study the effects of 2′,2′-difluorodeoxycytidine (dFdC) on DNA replication in HL-60 cells. Preincubation with dFdC caused a concentration-dependent decrease in overall [^{3}H]thymidine incorporation into DNA, accompanied by an increase in the proportion of radiolabel accumulated in small nDNA fragments. Twenty-four hours following removal of dFdC, radiolabel progressed from smaller to larger fragments and into genomic-length DNA. At initial concentrations of exposures to dFdC or cytosine arabinoside (ara-C) that caused 50% lethality (LC_{50}) to HL-60 cells (40 and 50 nM, respectively), slower and less complete transit of nDNA from small subreplicon-length fragments through larger intermediates to genomic-length DNA was observed for nDNA fragments containing incorporated [^{3}H]dFdC than for fragments containing [^{3}H]ara-C. This was accomplished with less [^{3}H]dFdC incorporated into DNA than [^{3}H]ara-C at these extracellular concentrations of drug. Pulse-chase studies, using higher concentrations of radiolabeled drug, similarly revealed that nDNA fragments containing incorporated dFdC, like those containing ara-C, progressed with respect to time into larger nDNA intermediates and ultimately into genomic-length DNA; however, such progression for nDNA fragments containing dFdC was less complete than for fragments containing ara-C. The radioactivity incorporated into DNA represented authentic dFdC, as determined by DNA degradation studies, and was stable in DNA for at least 48 hr after removal of extracellular [^{3}H]dFdC. Some of the effects of dFdC on ribonucleotide reduction in HL-60 cells were assessed by measurement of the intracellular pools of dCTP and dGTP. The drug had a greater effect on pools of dGTP than of dCTP, with transient reductions in dGTP observed at concentrations that encompass the LC_{50} for dFdC. These studies suggest that the interaction with DNA synthesis is an important component of the cytotoxicity of dFdC in HL-60 cells. Because it is incorporated progressively through nDNA compartments and ultimately into genomic-length DNA, dFdC should be categorized as an agent that slows DNA elongation in the intact cell, and not as a chain terminator in the absolute sense.

Key words: antimetabolite; Gemcitabine; ara-C; DNA synthesis; drug mechanism of action

Gemcitabine, or dFdC¶, is a fascinating new antimetabolite dCyd analogue that contains twin substitutions of fluorine at the 2′ position of deoxyribose [1]. Most promising is the activity of dFdC against solid tumors, demonstrated preclinically in human solid tumor xenografts and murine solid tumors [2–4], and clinically in patients with adenocarcinoma of the colon [5, 6], non-small cell lung cancer [5, 7, 8], small cell lung cancer [9], head and neck cancer [10], and renal, bladder and breast cancer [11–13]. Gemcitabine has also shown preclinical activity against human leukemia cells in culture [2, 3, 14]. Thus, its spectrum of antitumor activity is broader than that of the well-known deoxycytidine analogue antimetabolite, ara-C, which has a high degree of clinical effectiveness in the treatment of acute leukemia [15], but poor activity in solid tumors.

There are a number of aspects of dFdC intracellular metabolism, activation and biological effects that are shared by ara-C. For example, phosphorylation of dFdC by dCyd kinase to produce 5′-dFdCMP is required as a first step in the formation of the biologically active metabolites dFdCDP and dFdCTP [14, 16]. Both dFdCTP and ara-CTP inhibit mammalian DNA polymerases (e.g. pol α and ε), competitive with the natural substrate, dCTP [17–

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¶Abbreviations: dFdC, 2′,2′-difluorodeoxycytidine (Gemcitabine); dFdU, 2′,2′-difluorodeoxyuridine; dFdCMP, dFdCDP and dFdCTP, the 5′ mono-, di- and triphosphates of dFdC; ara-C, cytosine arabinoside (1-β-D-arabinofuranosylcytosine); dCyd, deoxycytidine; dThd, thymidine; ara-U, uracil arabinoside (1-β-D-arabinofuranosyluracil); nDNA, nascent DNA; HU, hydroxyurea; LC_{50}, initial concentration causing 50% decrease in cell survival; dNTP, deoxynucleoside 5′-triphosphate; $T_{\frac{1}{2}}$, half-life; AUC, area under the concentration × time curve.

21], and hence inhibit cellular DNA synthesis. Both dFdC and ara-C are incorporated into DNA [18, 21]. The cytotoxic effects of dFdC and ara-C are strongly correlated with their incorporation into DNA [21–23], with reversal of cytotoxicity accomplished by exogenous dCyd [16].

In contrast to the above, there are a number of biologic properties of dFdC that are distinctly different from those of ara-C: (1) Intracellular pools of dNTP (particularly dCTP) are reduced in cells exposed to dFdC, which is thought to be a consequence of inhibition of ribonucleotide reductase [14, 24, 25]. This may contribute, along with inhibition of DNA polymerases, to the inhibitory effect of dFdC on cellular DNA synthesis. The dFdC metabolite thought to be responsible for this inhibition is dFdCDP [24]. Although initially it was suggested that ara-C diminished intracellular pools of dNTP [26], subsequent work suggests that it does not [20]. (2) At any given concentration of extracellular drug, intracellular accumulation of dFdCTP is found to be much greater than accumulation of ara-CTP [16]; similarly, the intracellular $T_{\frac{1}{2}}$ of dFdCTP is much longer than that of ara-CTP [16]. These effects may be due, at least in part, to the ability of dFdC metabolites to reduce the endogenous intracellular pool of dCTP, which would, in turn, enhance the formation of dFdCTP [14]. (3) In a cell-free DNA primer elongation assay, using purified DNA polymerases, marked inhibition of chain elongation (virtual chain termination) occurs immediately after the addition of an ara-C residue to the 3′ end of the primer, whereas following the addition of a dFdC residue, one more deoxynucleotide is added and then virtual chain termination is observed [21]. (4) Incorporated dFdC is not degraded by the 3′→5′ exonuclease activity of DNA polymerase ε, whereas incorporated ara-C is susceptible to removal by this enzyme [21]. The above comparative and contrasting information is important, because delineation of the differences in biological effects of ara-C and dFdC not only may aid in understanding the molecular mechanism of cytotoxicity of dFdC, but also may facilitate comprehension of its activity against a broader spectrum of neoplasms.

The technique of pH-step alkaline elution provides a facile way to isolate different discrete size species of nDNA from intact cells [27, 28]. The method involves exposing cells in culture to a variety of radiolabeled DNA precursors, and then collecting the cells on a membrane filter. The cells are lysed on the filter, and then the various size fragments of nDNA (range from Okazaki and larger subreplicon-length fragments to very large, but subgenomic-length DNA) are eluted by stepwise increments of pH (11.0, 11.3, 11.5, 12.1) within the transition zone for DNA denaturation [27]. An increase in the size of the fragment eluted occurs with increasing pH. Genomic-length DNA is retained on the filter. Accordingly, kinetic studies can be done to follow the transit of a radiolabeled precursor from incorporation into newly formed short nDNA fragments, through larger replication intermediates, and ultimately, into genomic DNA (which is retained on the filter). Hence, pH-step alkaline elution is a superb way to investigate the effects of pharmaceuticals on the processes of initiation, elongation and ligation in intact cells.

We recently completed comprehensive studies of the effects of ara-C on DNA synthesis in HL-60 human myeloid leukemia cells using pH-step alkaline elution [28, 29]. Because of our experience with ara-C and HL-60 cells, we now apply pH-step alkaline elution to study the molecular effects of dFdC on DNA synthesis in intact HL-60 cells. Although detailed studies of the molecular consequences of dFdC on DNA replication have been done in cell-free systems wherein dFdCTP was reported to cause chain termination [21], little is known as to whether dFdCTP causes such an effect on DNA synthesis in the complex molecular and biochemical environment of the intact cell. Hence, pH-step alkaline elution was used to provide clearer understanding of the action of dFdC on DNA replication in intact cells. Alterations observed in DNA synthesis were related to dFdC effects on cytotoxicity and on intracellular pools of dCTP and dGTP.

MATERIALS AND METHODS

Materials. [5-^{3}H]Ara-C (sp. act. 30 Ci/mmol) and [5-*methyl*-^{3}H]dThd (sp. act. 86 Ci/mmol) were obtained from the Amersham Corp., Arlington Heights, IL. [5-^{3}H]dFdC (sp. act. 21.2 Ci/mmol) and dFdU were obtained as a gift from the Lilly Research Laboratories, Indianapolis, IN. Reverse-phase HPLC analysis of [^{3}H]dFdC revealed that >95% of radiolabel co-chromatographed with authentic non-radiolabeled dFdC, which was used as internal standard, and which was detected by absorption at 254 nm. Non-radiolabeled dFdC was obtained as gifts from both the Merrell-Dow Research Institute, Cincinnati, OH, and from the Lilly Research Laboratories. Non-radiolabeled ara-C was obtained as a gift from the Upjohn Co., Kalamazoo, MI. HU and dThd were obtained from the Sigma Chemical Co., St Louis, MO. Drugs or radiolabeled compounds were added to cultures of HL-60 cells as described in Results or in the figure legends.

Cell culture. HL-60 human leukemia cells (passage 30–80) were cultured in RPMI 1640 medium, precisely as described previously [30]. Cells in logarithmic growth phase were used in all studies. The HL-60 cells used in these studies were tested to assure absence of contamination by *Mycoplasma* (Gen Probe, San Diego, CA).

Cytotoxicity assay. A sensitive flow cytometric method that we developed for determining the number of cells surviving in suspension culture was used [31]. Briefly, at the time of determination of the number of surviving cells in culture, fluorescein diacetate (FDA) and propidium iodide (PI) in isotonic solution are added to the cells in culture to achieve final concentrations of 0.5 and 50 μg/mL, respectively. Viable cells are identified as those that display a bright green fluorescence induced by the intracellular metabolism of FDA to fluorescein, and a low red fluorescence indicating cellular exclusion of PI. In this assay, only intact viable cells are counted. Cellular debris and dead cells are ignored.

The number of viable cells per milliliter of culture medium sample is determined by a timed count and knowledge of the flow cytometer sample flow rate, as previously described [31]. The analyses were performed with a FACStar Plus™ flow cytometer (Becton Dickinson Immunocytometry Systems, Mountainview, CA).

pH-Step alkaline elution. This procedure was performed exactly as described previously [27–29]. Briefly, HL-60 cells labeled with the appropriate radioactive DNA precursor were deposited on Nucleopore™ filters (25 mm, 3.0 μm pore size, Costar Corp., Cambridge, MA) held in a "smoke-stack" type filter funnel apparatus, washed with ice-cold saline, and then lysed with 5 mL of a buffer consisting of 2.0 M NaCl, 0.02 M Na_2EDTA, 0.3% (v/v) Sarkosyl detergent, pH 7.0. Following this, the lysed cells were washed with 3 mL of a solution containing 20 mM Na_2EDTA, pH 9.0, to remove the NaCl. Then, the exit tubing from the filter funnel was connected to a peristaltic pump (Minipuls III; Gilson Instrument Corp., Middleton, WI), and a solution containing 20 mM EDTA (free acid form) and sufficient tetrapropyl ammonium hydroxide (RSA Corp., Ardsley, NY) to make the pH 11.0 was added to the filter funnel and was pumped through the filter at a rate of 0.08 mL/min. After 1 hr, the solution in the filter funnel was changed to one at pH 11.3, the elution was continued for another hour, and so on, for successive elutions with pH 11.5 and 12.1. During elution, fractions were collected every 12 min, and radioactivity in these fractions and that retained on the filters were determined by liquid scintillation counting (LS 6000IC, Beckman Instruments, Fullerton, CA), as described previously [28, 29].

To assure that the alkaline elution filter funnels were absolutely free of radiolabel from previous experiments, following each use, and prior to reuse, each funnel was washed extensively with detergent, and then a "mock" lysis, wash and pH-step procedure was done (using the appropriate buffers, but no cells). The radioactivity of the eluate of each step of this "mock" procedure was counted. Funnels were used only if the counts were equal to background, which for our liquid scintillation counter average 26 ± 10 dpm for the ^3H window.

DNA purification and digestion. Following exposure to [^3H]dFdC, as indicated below in Results, cells ($1–3 \times 10^6$) were placed on ice, washed once with ice-cold PBS, then suspended in 1.0 mL of lysis buffer (1 mg/mL proteinase K, 10 mM EDTA, 150 mM NaCl, 0.4% SDS, 10 mM Tris, pH 7.4), and incubated at 65° for 15 min, followed by incubation at 37° for 4 hr. DNA was then purified by extraction with phenol, chloroform and isoamyl alcohol followed by precipitation with 0.3 M sodium acetate and ethanol, as described previously [32]. Purified cellular DNA was digested to free nucleosides by dissolving in 0.5 mL of nuclease buffer containing 0.1 mM tetrahydrouridine (Sigma). Then, 100 U of DNAse I (EC 3.1.21.1, Sigma) was added, the mixture was incubated (60 min, 37°), 0.2 U of phosphodiesterase I (EC 3.1.4.1, Sigma) was added, and the incubation was continued for a further 30 min. At this point, 0.2 U of alkaline phosphatase (*Escherichia coli*, EC 3.1.3.1, Sigma) was added, and the mixture was incubated overnight at 37°. Protein was removed from the digest by Centrifree™ filtration, as above, and the filtrate was analysed by HPLC.

HPLC analysis of incorporated [^3H]*dFdC, and the fate of* [^3H]*dFdC or* [^3H]*ara-C in the culture medium.* HPLC was performed as described previously [29]. Briefly, a reverse-phase C18 column (Waters Associates, Milford, MA) was used, with linear gradient elution conditions and a flow rate of 1.5 mL/min. Initial conditions were 100% buffer A (0.01 M KH_2PO_4, pH 5.5). Final conditions, achieved after 30 min, were 15% buffer B (80% methanol in water) in buffer A. For each run of unknown DNA digest, or deproteinated (Centrifree™ filtration) cell culture medium supernatant, the appropriate authentic standards (e.g. dCyd, dFdC, dFdU, ara-C, ara-U) were injected simultaneously. Retention times for ara-C, ara-U, dFdC, dFdU and dCyd (mean ± SD) were 8.6 ± 0.2, 11.6 ± 0.2, 15.3 ± 0.9, 18.5 ± 1.1 and 11.2 ± 0.3 min, respectively. Radioactivity eluted from HPLC was detected by collecting fractions at 30-sec intervals, and then counting in a liquid scintillation counter. Detection of the authentic standards was by absorbance at 254 nm. For pharmacokinetic analyses, AUCs were calculated by the trapezoidal rule, terminal $T_{\frac{1}{2}}$ by weighted least squares.

Intracellular pools of dCTP and dGTP. A modification of the DNA polymerase method of Hunting and Henderson [33] was used, as described previously in detail [34]. Briefly, pellets containing $1–2 \times 10^7$ cells were extracted for 30 min on ice in 0.4 M perchloric acid. The extracts were buffered with 1 M Tris–HCl (pH 8.0), and adjusted to near neutrality by the addition of 8 N KOH. After centrifugation to remove perchlorate salts, the extracts were stored at −20° until used. The reaction mixture for measuring dCTP and dGTP pools contained 0.01 OD_{260} U of poly[d(IC)] (Pharmacia, Piscataway, NJ), 1.8 μmol dAMP, 1.8 μmol $MgCl_2$ and 18 μmol HEPES, pH 7.4. The dCTP assay contained 187 pmol (1.8 μCi) [^3H]dGTP (Amersham) and 1.0 Richardson U of *E. coli* DNA polymerase I (EC 2.7.7.7, Boehringer Mannheim, Indianapolis, IN). The dGTP assay contained 100 pmol (2.2 μCi) [^3H]dCTP (Amersham) and 1.2 U DNA polymerase I. The reactions were run at 37° for 30 min. Aliquots were then spotted on Whatman 3MM filter discs, washed extensively with 5% TCA, then ethanol-rinsed and counted for acid-insoluble radioactivity. Standard curves were constructed from samples with known amounts of dNTPs included in each experiment. Calculation of pool sizes was by the equation given by Hunting and Henderson [33].

RESULTS

Stability and fate of dFdC or ara-C in HL-60 cell cultures. Ara-C was relatively stable in the cultures, with a $T_{\frac{1}{2}}$ of 55.3 hr. The loss in ara-C was accompanied by a stoichiometric rise in its major metabolite, ara-U. In contrast, dFdC displayed a $T_{\frac{1}{2}}$ in culture of only 3.2 hr. Its disappearance was accompanied by a stoichiometric increase in dFdU.

Table 1. Cytotoxic effects of ara-C, dFdC or HU to HL-60 cells

Exposure time (hr)	LC_{50} (nM)		
	ara-C	dFdC	HU
72*	50 ± 10	41 ± 5	115,500 ± 24,700
16†	82 ± 13	39 ± 10	860,000 ± 180,000

* HL-60 cells in log growth phase were placed in cultures containing a concentration range of drug (initial concentrations of 0.001–10,000 nM for dFdC or ara-C, 10–10,000 μM for HU). After 72 hr, the number of cells surviving in culture were counted using flow cytometry ([29], see Materials and Methods). Data shown are the means ± SEM of 4 separate experiments, done on different days.

† Cells were placed in cultures containing the initial concentrations of dFdC, ara-C or HU indicated above. After 16 hr, they were washed free of drug and placed back in culture for an additional 72 hr, at which time cell survival was measured as described in the footnote above. Data shown are the means ± SEM of 3 separate experiments, done on different days.

In culture medium alone (without HL-60 cells), no degradation of dFdC or ara-C was noted within a 24-hr period.

Effects on cytotoxicity. In view of the different $T_{\frac{1}{2}}$ values of dFdC and ara-C, for convenience we define the LC_{50} as the initial concentration of drug in a given exposure that results in lethality to 50% of the cells. The LC_{50} values for ara-C and dFdC against HL-60 cells are presented in Table 1. In addition, we studied the cytotoxic effects of HU, a known inhibitor of ribonucleotide reductase. If survival was measured 72 hr after placing the cells in medium containing either drug, the LC_{50} for ara-C and dFdC was 50 and 41 nM, respectively. If these were the initial concentrations in HL-60 culture, given the drug stability data above, the total AUC (extrapolated to infinity) was 180 nM·hr for dFdC, and 4000 nM·hr for ara-C. This represents over a 20-fold greater exposure of cells to ara-C than to dFdC. The 72-hr exposure LC_{50} for HU (115.5 μM) was approximately 2000-fold higher than those of ara-C and dFdC (Table 1). If drug was removed from the medium after 16 hr by washing the cells and then returning them to culture for an additional 72 hr, the LC_{50} of ara-C increased, whereas that of dFdC remained the same as the 72-hr value (16-hr LC_{50} was 82 and 39 nM for ara-C and dFdC,

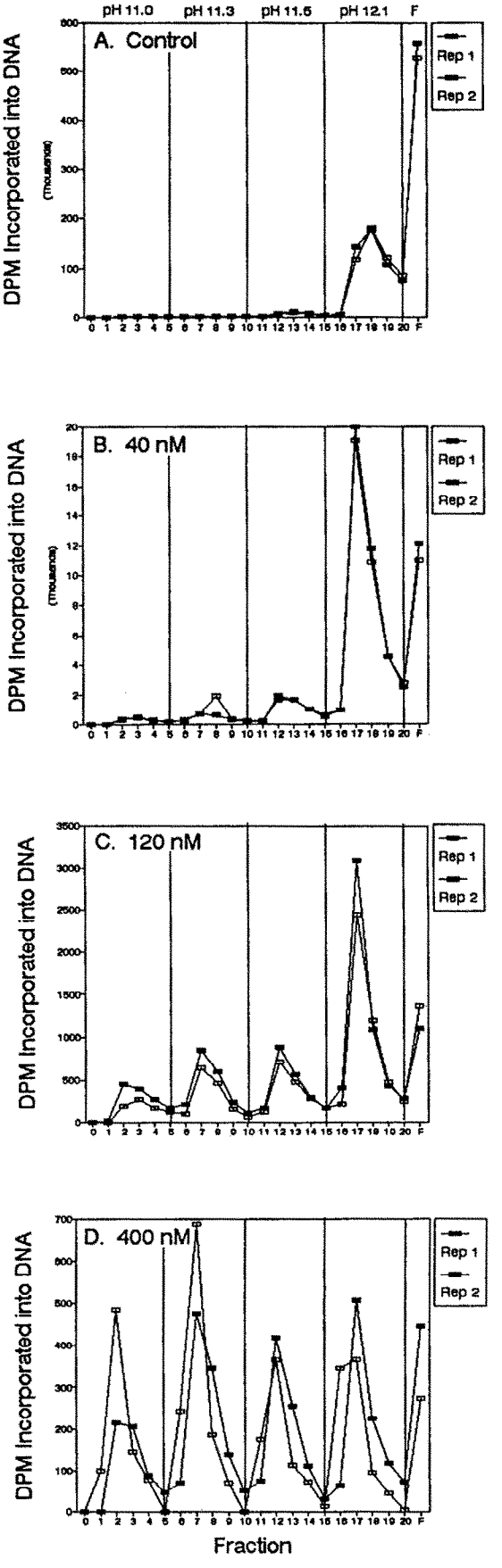

Fig. 1. Effects of dFdC on [^{3}H]dThd incorporation into the various compartments of HL-60 cell nDNA, as determined by pH-step alkaline elution. Cells in culture in logarithmic growth were pre-exposed to 0 nM (control, panel A), 40 nM (panel B), 120 nM (panel C), or 400 nM (panel D) dFdC for 90 min, after which [^{3}H]dThd (85 Ci/mmol, 2 μCi/mL culture) was added for an additional hour in the continued presence of dFdC. Then, pH-step alkaline elution was performed as described in Materials and Methods. In this experiment, 1×10^6 cells were used per filter funnel. Data shown are dpm recovered in a particular fraction or on the filter. Rep 1 and Rep 2 signify replicates 1 and 2, respectively; F = filter.

respectively). The short $T_{\frac{1}{2}}$ of dFdC may account for the lack of change in the LC_{50} of dFdC in the two exposures used above. The 16-hr LC_{50} for HU showed a rather dramatic increase to 860 μM.

pH-Step alkaline elution analysis of dFdC effects on [^{3}H]dThd incorporation into DNA. HL-60 cells in logarithmic growth phase were exposed to 0 (control), 40, 120 or 400 nM dFdC in culture for 90 min, and then [^{3}H]dThd (85 Ci/mmol, 2 μCi/mL culture) was added to the cultures for an additional hour. At this point, aliquots of the cultures were subjected to pH-step alkaline elution analysis, as described in Materials and Methods. Elution profiles obtained in these studies are shown in Fig. 1A–D. The ordinate of Fig. 1 displays the radioactivity obtained in each fraction or retained on the filter, for duplicate determinations. Note that in control cultures (Fig. 1A), radiolabel was found predominantly in the population of nDNA of very large size that elutes at pH 12.1, and on the filter (i.e. genomic-length DNA). Preincubation of cells with increasing concentrations of dFdC caused a concentration-dependent decrease in the proportion of radiolabel retained on the filter, or eluted at higher pH, and an increase in the proportion of radiolabel eluting at a lower pH (i.e. smaller fragments of nDNA) (Fig. 1B–D). These data are consistent with the effects of an agent that inhibits DNA elongation or that causes chain termination. Using total incorporation values and linear regression analysis, the concentration of dFdC causing 50% inhibition of [^{3}H]dThd incorporation (IC_{50}) was found to be 3.3 nM (slope 1.203 ± 0.113, $R = 0.983$).

After aliquots were taken for the studies described in Fig. 1, the remaining cells were washed free of [^{3}H]dThd and extracellular drug, and placed back in culture for an additional 24 hr; then pH-step alkaline elution was performed (Fig. 2A–D). In this 24-hr interval, for control and for all experimental conditions, radiolabel initially associated with subgenomic-length DNA fragments became associated with a nDNA fragment of greater size, as seen for 120 and 400 nM dFdC, or with full-length DNA (retained on the filter) as seen for control, 40, and 120 nM dFdC. There was no decrease in cell number after the 24-hr interval in any culture. These data indicate that the nDNA fragments accumulated in the presence of dFdC are assembled into larger fragments and genomic-length DNA following the removal of drug. Although this suggests that inhibition of DNA elongation is occurring, and not chain termination (where persistence of the distribution of radiolabel in subgenomic-length fragments would be observed), we performed studies with radiolabeled dFdC to clarify this issue.

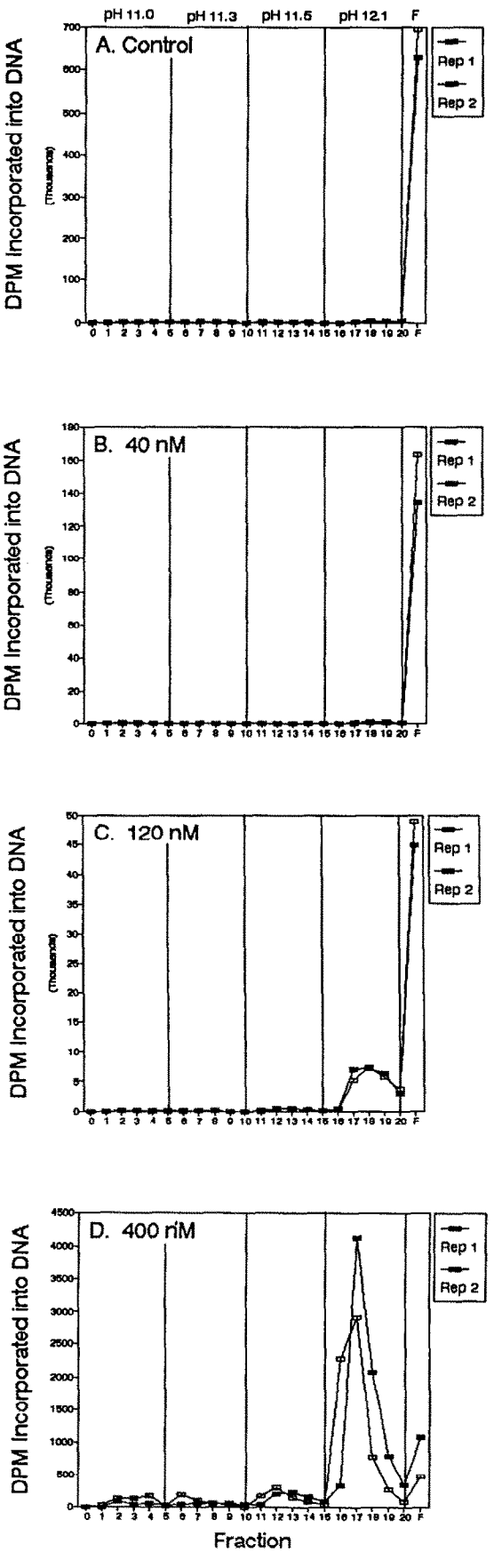

Fig. 2. A 24-hr chase of cells exposed as described in the legend of Fig. 1 in dFdC- and [^{3}H]dThd-free medium. Aliquots of the cultures described in Fig. 1 above were washed free of dFdC and [^{3}H]dThd, then placed back in culture for an additional 24 hr, after which pH-step alkaline elution was done. Panel A, control (0 nM dFdC); panel B, 40 nM dFdC; panel C, 120 nM dFdC; panel D, 400 nM dFdC. Data shown are dpm recovered in fraction or filter. Rep 1 and Rep 2 represent replicates 1 and 2, respectively; F = filter. There was no significant decrease in intact cell number in this 24-hr interval.

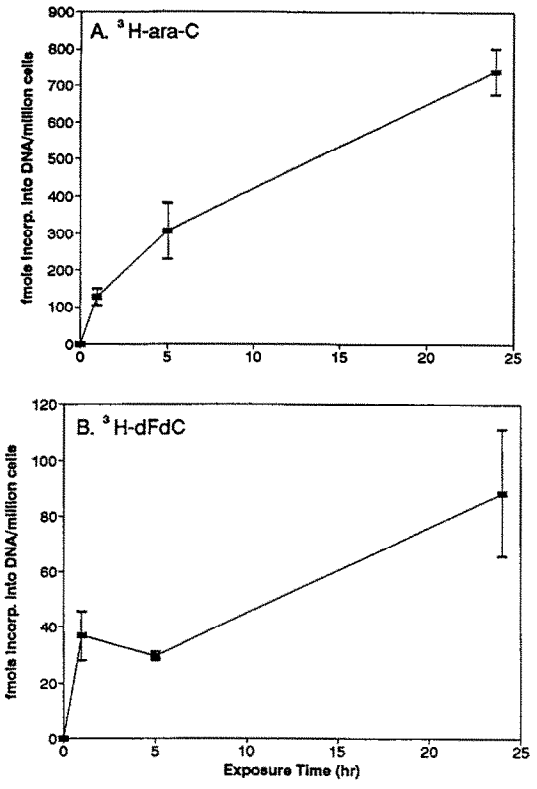

Fig. 3. Incorporation of [^{3}H]ara-C (A) or [^{3}H]dFdC (B) into HL-60 DNA. Cells in log growth phase were exposed to 50 nM [^{3}H]ara-C (sp. act. 30 Ci/mmol, 1.5 μCi/mL culture) or 40 nM [^{3}H]dFdC (sp. act. 21.2 Ci/mmol, 0.85 μCi/mL culture) for 1, 5 or 24 hr, after which incorporation of radiolabel into DNA was determined by pH-step alkaline elution (1.5×10^6 cells per filter funnel), as described in Materials and Methods. The data in this figure are based on the total radioactivity incorporated into DNA from pH-step alkaline elution analysis (sum of incorporation in all pH-step fractions plus radioactivity retained on the filter). Values are means ± SD of three experiments. For dFdC, total radioactivity (per 10^6 cells) was 1740, 1380 and 4150 dpm for 1, 5 and 24 hr, respectively. For ara-C, total radioactivity at 1, 5, or 24 hr was 8450, 20,220 and 52,820 dpm, respectively. The distribution of incorporation shown in this figure into the various pH-step alkaline elution nDNA compartments is presented in Fig. 4.

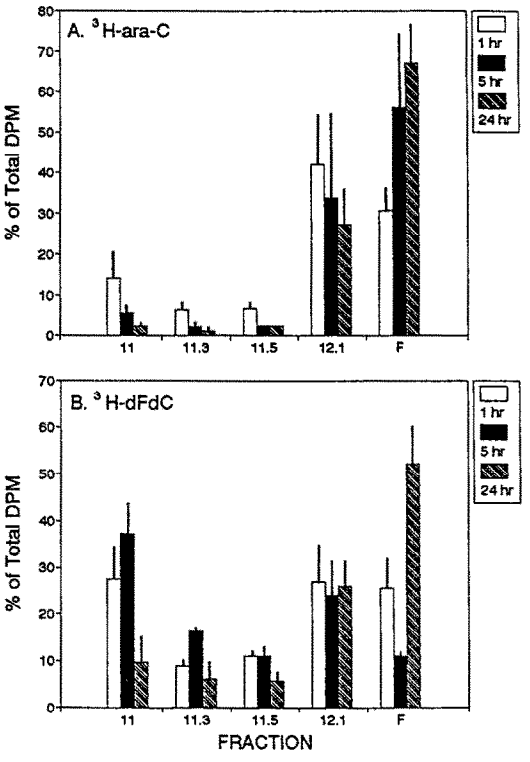

Fig. 4. Distribution of incorporation of [^{3}H]ara-C (A) or [^{3}H]dFdC (B) among the various compartments of nDNA identified by pH-step alkaline elution, during continuous exposure of cells to an LC_{50} concentration of drug. The experimental conditions used are described in the legend to Fig. 3. In this bar graph presentation, the bars represent the sum of radioactivity eluted at each pH, or retained on the filter. Vertical lines represent standard deviations (or range): 1 hr = three experiments; 5 hr = two experiments; and 24 hr = four experiments.

pH-Step alkaline elution analysis of [^{3}H]*dFdC or* [^{3}H]*ara-C incorporation into DNA.* Based on the cytotoxicity studies of ara-C and dFdC (above), we chose 50 and 40 nM, respectively, as the initial concentrations of exposures that are equally toxic (cause a 50% decrease in cell survival) to HL-60 cells. Figure 3 displays the accumulation of radiolabel in DNA of cells placed in medium containing 40 nM [^{3}H]dFdC or 50 nM [^{3}H]ara-C for 1, 5 or 24 hr. At these equally toxic drug exposures, the accumulation of radiolabel from [^{3}H]dFdC in DNA was much less than that from [^{3}H]ara-C (Fig. 3), even after short exposure times (1 or 5 hr). Although the incorporation of dFdC was low in these studies, it was well within the limits of detection in terms of radioactivity (see legend to Fig. 3). Moreover, digestion of DNA from cells so treated with [^{3}H]-dFdC recovered authentic dFdC (see below).

Analysis by pH-step alkaline elution of the incorporation of [^{3}H]ara-C or [^{3}H]dFdC into DNA shown in Fig. 3 is presented in Fig. 4. To aid in the presentation of data, the radioactivity eluted at each pH step or retained on the filter, expressed as the percent of total radioactivity, is shown in bar graph form, rather than presenting the entire pH-step profile, as was done in Figs 1 and 2. After 1 hr, the bulk of radiolabel from [^{3}H]ara-C (Fig. 4A) was found in nDNA replication intermediates (primarily the large intermediates that elute at pH 12.1), with <40% of label residing in DNA retained on the filter. With increasing time of exposure to [^{3}H]ara-C, transit of radiolabel out of the replication intermediate compartment, and into genomic-length DNA (retained on the filter) was observed. Even after 5 hr, a decrease in the proportion of label eluting at pH 11.0, 11.3 and 11.5 was apparent, with a concomitant increase in the proportion of labeled DNA retained on the filter. In pH-step alkaline elution studies of cells exposed to [^{3}H]dFdC (Fig. 4B), like [^{3}H]ara-C, the majority of radiolabel was found in subgenomic-size DNA replication

intermediates after 1 hr of exposure. However, a greater proportion of this radiolabel was found in the lower pH-step fractions (particularly the pH 11.0 fraction) than was the case with [^{3}H]ara-C. Unlike the [^{3}H]ara-C studies where progression of label to larger nDNA intermediates and to genomic-length DNA was seen, radioactivity remained in subgenomic-length fragments with [^{3}H]dFdC after 5 hr, indicating a slower rate of elongation of nDNA fragments containing dFdC compared with ara-C. However, by 24 hr, approximately 50% of radiolabel from [^{3}H]dFdC was found associated with DNA retained on the filter (genomic-length DNA), accompanied by a fall in radiolabeled DNA eluting at pH 11.0. There was no significant decrease in cell number following a 24-hr exposure to these concentrations (data not shown).

To test whether the radiolabel observed in DNA as depicted in Figs 3B and 4B actually represented incorporated authentic dFdC, and not exchange of radiolabel with a natural DNA precursor, HL-60 cells were placed in medium containing 40 nM [^{3}H]-dFdC. After 24 hr the cells were washed, and DNA was isolated and digested to nucleosides as described in Materials and Methods. HPLC analysis revealed that >94% of the radiolabel in the DNA was found to be in material that co-chromatographed with an authentic non-radiolabeled dFdC standard, indicating that the radiolabel we observed in the pH-step alkaline elution studies using [^{3}H]dFdC represented dFdC incorporated into DNA.

To determine whether the incorporation of dFdC into DNA depicted in Figs 3 and 4 represented incorporation into nDNA, and was not a consequence of DNA fragmentation caused by dFdC or subsequent incorporation of dFdC after fragmentation by means of DNA repair, the effects of dFdC on template DNA was studied. Template DNA was labeled by exposing recently passaged HL-60 cells to 2 μCi/mL [^{3}H]dThd for 30 min, followed by washing and then returning the cells to culture in fresh medium for 24 hr. At this time, the cells were exposed to 40 nM dFdC for 1, 5 or 24 hr, after which pH-step analysis was performed. Control studies (no exposure to dFdC) were performed concomitant with the 1-hr and 24-hr dFdC exposure studies. In control and under each experimental condition, greater than 95% of the radiolabel remained associated with genomic-length DNA (retained on the filter), indicating that the integrity of the DNA was maintained and that fragmentation of the DNA did not occur in any experimental condition. Total radioactivity incorporated into DNA did not change significantly from control in any of the experimental conditions.

We next investigated the effects of extracellular concentration on the incorporation of [^{3}H]ara-C or [^{3}H]dFdC into DNA, as analysed by pH-step alkaline elution. For these studies, we selected concentrations that were 1, 3, 5 and 10 times the LC_{50} concentrations for ara-C or dFdC. Hence, for ara-C, we used 50, 150, 250 and 500 nM; for dFdC, 40, 120, 200 and 400 nM were used. Cells were suspended in culture medium containing these concentrations for 2 hr, and then pH-step alkaline elution was performed. Figure 5 shows the total incorporation of dFdC or ara-C into DNA in response to these concentrations. Again, note that the incorporation of dFdC into DNA was considerably less than that of ara-C. It is unlikely that the low incorporation of dFdC observed was the result of its short $T_{\frac{1}{2}}$ in the cultures since the 2-hr interval AUC, calculated from the data in Table 1, was 70.1 nM·hr for dFdC and 98.5 nM·hr for ara-C, which represents only a 1.4-fold greater exposure to ara-C during this 2-hr period. A representative pH-step alkaline elution analysis of this incorporation is presented in Fig. 6. For both dFdC and ara-C, increasing concentrations caused a slowing of transit of radiolabel through the various nDNA compartments into genomic-length DNA during the 2-hr interval. This is illustrated by a decrease in the proportion of radiolabeled DNA that eluted at pH 12.1 or was retained on the filter, and an increase in the proportion of radiolabel that eluted at pH 11.0, 11.3 or 11.5 (for dFdC) or at pH 11.3 and 11.5 (for ara-C), with increasing concentrations of drug. As observed previously with the timed incorporation studies (Fig. 4), radiolabel from [^{3}H]dFdC was found to be distributed in greater proportion among smaller fragments of nDNA than was [^{3}H]ara-C.

To study further the progression of radiolabel through the various nDNA compartments, pulse-chase experiments were performed (Fig. 7). Cells were exposed to 250 nM [^{3}H]ara-C or 200 nM [^{3}H]-dFdC (5 times the LC_{50} concentration) for 2 hr, then washed free of radiolabeled drug and returned to the incubator in fresh drug-free culture medium.

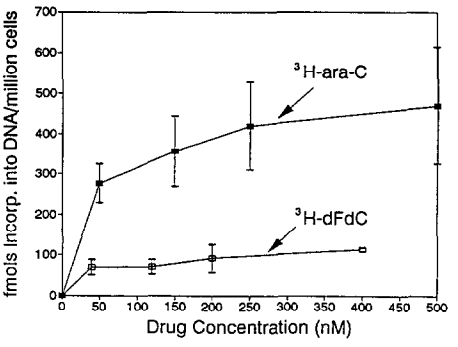

Fig. 5. Effects of extracellular concentration on incorporation of [^{3}H]ara-C or [^{3}H]dFdC into HL-60 DNA. Cells in log growth phase were exposed to 50, 150, 250 or 500 nM [^{3}H]ara-C (sp. act. 30 Ci/mmol) or 40, 120, 200 or 400 nM [^{3}H]dFdC (sp. act. 21.2 Ci/mmol) for 2 hr, after which the incorporation of radiolabel into DNA was determined by pH-step alkaline elution, as described in Materials and Methods. The data shown in this figure are based on the total radioactivity incorporated into DNA (1.5×10^6 cells) obtained from pH-step alkaline elution analysis (sum of incorporation in all pH-step fractions plus radioactivity retained on the filter). Vertical bars represent the range of the means of two experiments. Radioactivity (mean) incorporated per million cells for dFdC was 3300, 3300, 4300 and 5300 dpm for 40, 120, 200 and 400 nM dFdC, respectively. For 50, 150, 250 or 500 nM ara-C, total radioactivity incorporated was 18,300, 23,600, 27,700 and 31,000 dpm, respectively. The pH-step alkaline elution distribution of the incorporation shown in this figure are presented in Fig. 6.

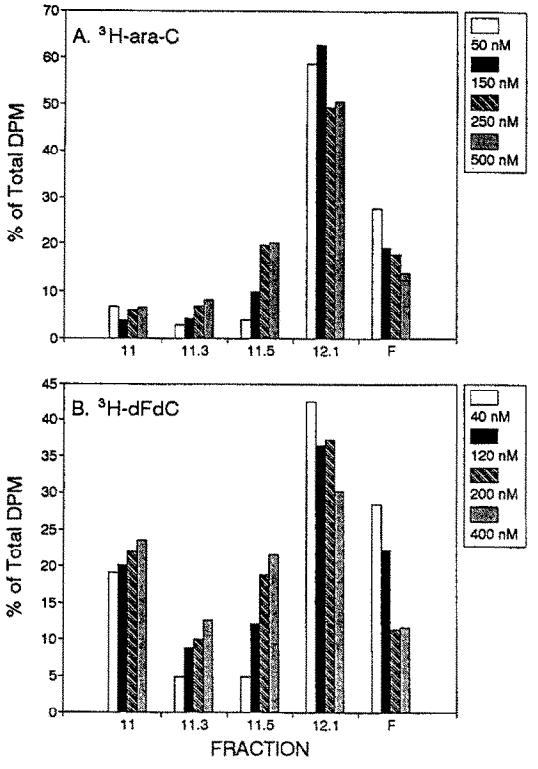

Fig. 6. Distribution of incorporation of [³H]ara-C (A) or [³H]dFdC (B) among the various compartments of nDNA identified by pH-step alkaline elution, in response to exposure of cells to 1, 3, 5 or 10 times the LC_{50} concentration of ara-C or dFdC. The experimental conditions used are described in the legend to Fig. 5.

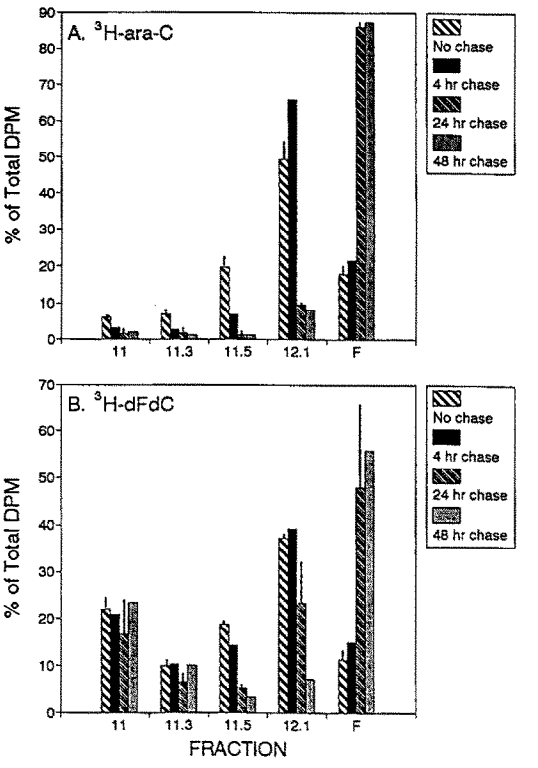

Fig. 7. [³H]Ara-C or [³H]dFdC pulse-chase experiment. Cells (10^6/mL, initial concentration) in logarithmic growth were placed in medium containing 250 nM [³H]ara-C (A) or 200 nM [³H]dFdC (B) (5 times the LC_{50} concentration) for 2 hr (pulse), then placed on ice, washed free of radiolabeled drug with ice-cold PBS, and returned to cell culture conditions in drug-free medium (chase). Aliquots of 1.0 mL were removed following 0 and 24 hr of chase for pH-step alkaline elution analysis. Vertical lines for 0 hr (no chase) and 24 hr represent the range of the means of two experiments, done on different days. One of these experiments also included 4-hr or 48-hr chase measurements. Total incorporation of [³H]ara-C into DNA was the following for each chase time: 0 hr, 420 ± 110 fmol (27,720 dpm); 4 hr, 490 fmol (32,340 dpm); 24 hr, 1640 ± 320 fmol (108,240 dpm); and 48 hr, 2340 fmol (154,440 dpm). For [³H]dFdC, total incorporation into DNA for each chase time was: 0 hr, 90 ± 30 fmol (4230 dpm); 4 hr, 40 fmol (1830 dpm); 24 hr, 100 ± 20 fmol (4700 dpm); 48 hr, 100 fmol (4700 dpm).

Aliquots were removed from each culture immediately after washing (no chase), or at 4, 24 or 48 hr. This 2-hr exposure caused no decline in the number of intact cells at 24 or 48 hr. Immediately following the 2-hr exposure, the greatest proportion (>80%) of DNA labeled with [³H]ara-C or [³H]dFdC was subgenomic in length (i.e. was not retained on the filter; Fig. 7), consistent with data shown previously for these concentrations of drug (Fig. 6). Figure 7A demonstrates a transit, within 24 hr, of nDNA labeled with [³H]ara-C from subgenomic-length fragments to genomic-length DNA, with >80% of radiolabeled nDNA being retained on the filter after 24 hr of chase. In contrast, the transit of subgenomic-length nDNA labeled with [³H]dFdC to genomic-length nDNA was less complete (Fig. 7B), with only approximately 50–60% of radiolabeled DNA retained on the filter at 24 or 48 hr. Even after 24 or 48 hr, a substantial portion of nDNA containing [³H]dFdC remained in the subgenomic-length compartments, particularly the compartment of small nDNA fragments that eluted at pH 11.0 (Fig. 7B). For these pulse-chase studies, we found that [³H]dFdC incorporated into cellular DNA was stable throughout the entire 48 hr of chase, with our measurements being 90 ± 30 fmol/10^6 cells at 24 hr, and 100 fmol/10^6 cells at 48 hr.

Effects of dFdC on intracellular pools of dCTP and dGTP. The endogenous intracellular pools of dCTP and dGTP were determined using an enzymatic (DNA polymerase) assay (Fig. 8). Cells were placed in medium containing the indicated concentrations of dFdC for 3 or 16 hr, at which time pools of dCTP or dGTP were determined. The control values (see legend, Fig. 8) obtained for intracellular dCTP and dGTP were within the range reported previously for HL-60 cells [35, 36]. A greater reduction of intracellular dCTP or dGTP was seen when determinations were made after 3 hr than after 16 hr. This paradox can be explained by the short $T_{\frac{1}{2}}$ of dFdC in HL-60 culture (3.2 hr), which may allow some degree of recovery of the dNTP pool size by

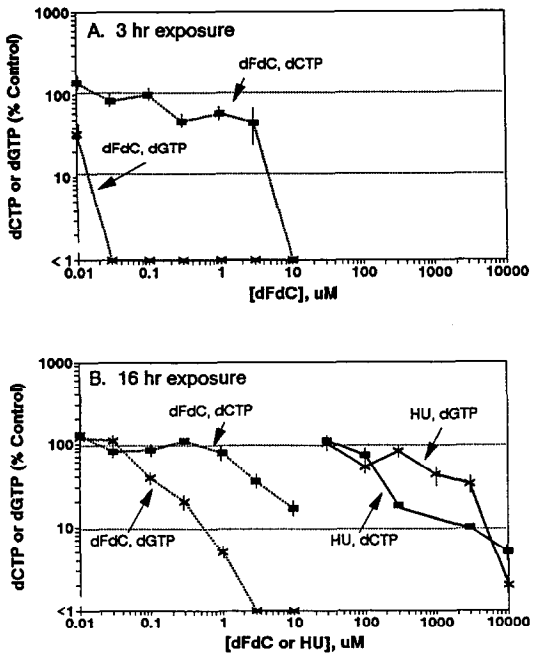

Fig. 8. Effects of dFdC or HU on intracellular pools of dCTP or dGTP. Cells were exposed to the indicated concentrations of drug for 3 hr (dFdC only) or 16 hr. Intracellular dCTP or dGTP pools were determined as described in Materials and Methods. Overall control values (no drug) representing the mean (±SEM) of three separate experiments done on different days were 102 ± 26 and 86 ± 18 pmol/10^7 cells for dCTP and dGTP, respectively. Vertical bars in the figure represent the range of duplicate determinations.

16 hr. The most pronounced effects of dFdC were on dGTP, with intracellular pools being reduced to 35% of control with 0.01 μM dFdC and to non-detectable levels in response to 0.03 μM or higher concentrations after a 3-hr exposure (Fig. 8A). After 16 hr, dGTP pools had recovered completely for cells placed in medium initially containing 0.01 or 0.03 μM dFdC, and recovered partially for cells in medium containing 0.1, 0.3 or 1.0 μM dFdC (Fig. 8B). Hence, concentrations of dFdC that encompass the LC_{50} (30 and 100 nM) caused profound but transient reduction in dGTP, with full or partial recovery seen by 16 hr. On the other hand, concentrations of dFdC in excess of the LC_{50} value were required to cause sustained reduction of pools of dGTP or dCTP (Fig. 8B). A 16-hr exposure of cells to HU caused substantial reduction of both the dGTP and dCTP pools (Fig. 8B), with >50% reduction seen in response to concentrations that encompass the 16 hr LC_{50} for HU (860 μM).

DISCUSSION

Among the differences between ara-C and dFdC is the inability of patients to tolerate prolonged continuous infusion of dFdC. Consequently, ara-C is usually given by 7-day continuous infusion, whereas dFdC is typically administered as a 30-min to 3-hr infusion given weekly [5]. In plasma, dFdC is rapidly deaminated to dFdU (plasma terminal $T_{\frac{1}{2}}$ of dFdC is 8 min) [5]. In view of the above, the rapid loss of dFdC in the HL-60 cell cultures compared with ara-C is fortuitous, since it enables us to emulate exposures of neoplastic cells to ara-C or dFdC that are found in the clinical setting. In this way, comparisons of the extent of biochemical alterations resulting from equally toxic exposures of HL-60 cells to ara-C or dFdC may more accurately reflect events associated with neoplastic cytotoxicity of these agents *in vivo*. The more rapid rate of production of dFdU than of ara-U is interesting, since one would expect both dFdU and ara-U to be products of the same enzymes, namely cytidine deaminase (EC 3.5.4.5) and deoxycytidylate deaminase (EC 3.5.4.b) [37]. This implies that in intact cells dFdC may be a better substrate for these enzymes than ara-C. Hence, the cytotoxic potency of dFdC against HL-60 cells may be much greater than that of ara-C when the difference in stability in HL-60 culture is taken into account. This is illustrated by the 2-fold greater potency of dFdC compared with ara-C with the 16-hr exposure schedule (Table 1). Furthermore, Heinemann *et al.* [16] found that dFdC exhibits significantly greater cytotoxic potency than ara-C against wild-type Chinese hamster ovary cells when deamination by cytidine/deoxycytidine deaminase is blocked by tetrahydrouridine.

The effects of dFdC on [^{3}H]dThd incorporation into nDNA or the patterns of incorporation of [^{3}H]-dFdC itself into nDNA can be understood better from a review of the kinetics of incorporation of [^{3}H]dThd into nDNA in the absence of a DNA synthesis inhibitor, as measured by pH-step alkaline elution [27, 28]. When radiolabeled precursor such as [^{3}H]dThd is added to asynchronously growing cells for a brief period of time (e.g. 30 sec to a few minutes), pH-step alkaline elution reveals accumulation of the radiolabel predominantly in small nDNA fragments (e.g. those that elute at pH 11.0 or 11.3). With increasing time of exposure, the absolute amount of radiolabel in a given pH-step compartment reaches a plateau, where the rate of influx of radiolabel into the compartment equals the rate of efflux to a larger compartment, whereas the accumulation in the genomic-length nDNA compartment (material retained on the filter) continues indefinitely [27]. Hence, with increasing time of exposure, the radiolabel will be associated proportionally with larger nDNA fragments, and ultimately with genomic-length nDNA. Thus, in Fig. 1A, where cells were exposed to [^{3}H]dThd for 1 hr, the greatest proportion of radiolabel was associated with nDNA that eluted at pH 12.1 or was retained on the filter. Had the exposure been for only 2 min, previous work demonstrated that the greatest proportion of radiolabel would be associated with nDNA that elutes at pH 11.0 and 11.3 [27, 28]. The addition of an inhibitor of DNA synthesis puts the above process in slow motion. Hence, the concentration-dependent increase in the proportion of radiolabel eluting at lower pH seen in Fig. 1B–D reflects a slowing of the DNA synthetic rate by dFdC, either by slowing chain elongation or in the extreme case, by causing absolute chain termination. The pattern of incorporation of [^{3}H]dFdC or [^{3}H]-

ara-C into DNA can be understood in the above context as well, with slow but progressive transit of radiolabel into incrementally larger nDNA compartments observed with time (Figs 4 and 7), and with the concentration-dependent decrease in the overall size of labeled nDNA eluted (Fig. 6).

A nucleoside antimetabolite drug whose incorporation causes DNA chain termination would be expected to accumulate in small nDNA fragments, as discussed above. However, in the continued presence of the drug or in pulse-chase exposure, the distribution of radiolabel in small nDNA fragments should not change, since no further elongation or assembly into larger fragments could occur. The ability of the small nDNA fragments accumulated during exposure to dFdC to assemble into larger fragments during a chase in drug-free medium (Figs 1 and 2) and the eventual incorporation of [^{3}H]dFdC predominately into full-length DNA following an initial concentration in culture of 40 nM (Fig. 4B) or 200 nM (Fig. 7B) indicate that dFdC is not an absolute chain terminator in the intact cell. In comparison to ara-C, however, the progression of radiolabel to full-length nDNA was less complete (Figs 4A and 7A), with lower percentages of total radiolabel retained on the filter after 5 or 24 hr (Fig. 4) or 24 or 48 hr (Fig. 7) for [^{3}H]dFdC-treated cells than for [^{3}H]ara-C-treated cells. Hence, although neither agent in intact cells is a chain terminator in the absolute sense, the data suggest that dFdC may be more effective than ara-C in slowing the cellular processes of nDNA elongation, ligation and assembly of smaller nDNA fragments into larger ones. This effect may account, at least in part, for the lower incorporation of dFdC into DNA compared with ara-C at equitoxic exposures.

The lack of an absolute chain-terminating effect of dFdC in intact HL-60 cells presented here seems to conflict with data obtained from a cell-free elongation system [21], where virtual chain termination (a major pause in DNA chain elongation) occurred after extension of the primer by one base following the addition of a dFdCMP residue. This discrepancy may possibly be explained by fundamental differences between cellular and cell-free systems. There is growing evidence that DNA replication in the intact cell is mediated by a multiprotein/multienzyme complex, wherein most of the enzymes required for DNA synthesis lie in a spatial configuration that enables optimal performance [38]. Such a configuration may be disrupted in cell-free systems that utilize highly purified enzyme molecules. Hence, dFdC may be more efficiently incorporated into DNA by the cellular replication complex than in the cell-free system where its profound inhibition of elongation results in a chain-terminating effect. In the cell-free primer extension system, ara-C also causes a marked chain-terminating effect [39–41]. The data presented in this paper and reported previously by ourselves and others show clearly that ara-C is incorporated internally in DNA of intact cells, and that following exposure, incorporated ara-C exists predominantly in genomic-length DNA [18, 28, 29].

Our studies found that the amount of dFdC incorporated into DNA is much less relative to the amount of ara-C incorporation necessary to accomplish the same degree of cytotoxicity. This is particularly interesting in light of previous observations by Heinemann *et al.* [16] that intracellular dFdCTP accumulation in Chinese hamster ovary cells is up to 20-fold greater than the accumulation of ara-CTP at the same concentration of extracellular drug. Perhaps the lower degree of utilization of dFdCTP for incorporation into DNA compared with ara-CTP may account, at least in part, for the higher pools of intracellular dFdCTP than ara-CTP. We do not believe that the lower incorporation of dFdC into DNA was the result of its short $T_{\frac{1}{2}}$ relative to that of ara-C, since the incorporation of dFdC was much less than that of ara-C during brief exposures (1–5 hr), whereas overall exposure to the two drugs was similar (the 2-hr interval AUC for dFdC vs ara-C represented only a 1.4-fold greater exposure to ara-C during this 2-hr period). One consideration is that excision-repair of misincorporated dFdC may be more efficient than for misincorporated ara-C. Evidence to the contrary has been obtained by Huang *et al.* [21] who found that the 3′→5′ exonuclease of DNA polymerase ε was unable to excise an incorporated dFdCMP at the 3′ terminus of a nDNA chain. As for endonucleolytic excision-repair, our own pulse-chase studies demonstrated that once incorporated into DNA, dFdC/DNA is stable in intact cells for a period of at least 48 hr.

Our studies of the effects of dFdC on intracellular deoxynucleotide pools were aimed basically at determining dCTP pools, primarily to discern whether the self-potentiating effect of dFdC by virtue of its ability to reduce intracellular dCTP was operative at cytotoxic concentrations of dFdC such as the LC_{50}, and also because dCTP pools have been reported to be most susceptible to reduction by dFdC [14, 24]. Originally, we measured pools of dGTP simply to enable correction of the dCTP estimate for isotopic dilution of [^{3}H]dGTP by endogenous dGTP [33]. Our studies reveal a marked reduction in dGTP but not in dCTP at concentrations of dFdC that encompass the LC_{50} (e.g. 0.01 to 0.1 μM) following a 3-hr exposure to drug. This holds even if one halves the concentrations on the x-axis in Fig. 8A, which would take into consideration the 3.2-hr $T_{\frac{1}{2}}$ of dFdC in these cultures. These alterations in dGTP may indeed play a role in the cytotoxicity of dFdC at these concentrations. For HU, reductions in both dCTP and dGTP were seen at concentrations near the 16-hr LC_{50} (860 μM). More marked depletion of purine dNTP pools than pyrimidine pools by dFdC in HT-29 human colon carcinoma cells was also noted recently by Shewach *et al.* [42]. Such observations may represent the spectrum of dFdC effects among differing tissue types and cell lines.

Recently, it has been shown that ara-C and dFdC can each induce morphologic changes in cells that represent a form of programmed cell death called apoptosis [43–45]. Apoptosis is associated frequently with the appearance of an internucleosomal ladder pattern of DNA degradation [46], as a result of the activation of a cellular endonuclease [47], wherein fragments of genomic DNA in multiples of 200 bp

are produced. Recent studies by Huang and Plunkett [45] using CEM cells exposed to dFdC (1 μM, 4 hr) revealed a nucleosomal fragmentation pattern typical of apoptosis, with a predominance of DNA fragments of 1 to 5 nucleosomes in length (200–1000 bp). Such small double-stranded DNA fragments would be denatured and elute at pH 11.0 and 11.3 in the pH-step alkaline elution procedure. Larger double-stranded DNA fragments (e.g. 50–300 kb) produced by apoptosis would be denatured and elute in the pH 11.3 and 11.5 fractions, based on the size of the DNA fragment observed to elute under these conditions [27]. However, we presently feel that the pH-step alkaline elution studies reported here predominantly reflect alterations caused by dFdC in the synthesis of nDNA, and not dFdC-induced apoptosis for the following reasons: First, apoptotic double-stranded fragments should be removed by the wash at pH 9 which follows cell lysis, before any denaturing pH steps are performed (see Materials and Methods). Second, exposure of cells to the LC_{50} of dFdC did not cause fragmentation of prelabeled template DNA. Third, the timed exposure experiments (Fig. 4) and pulse-chase experiments (Figs 1, 2 and 7) argue against the DNA-fragmenting effects of apoptosis being manifested in the pH-step studies, since if they were, the fragmentation pattern should persist—i.e. radiolabel in short DNA fragments should not progress or chase into longer DNA or genomic-length DNA with time. We must note, however, that in the studies described in Fig. 7B, persistence of labeled DNA in the pH 11.0 and 11.3 compartments was observed, even after 48 hr. Although this may represent slower transit of small nDNA fragments containing dFdC to larger nDNA compartments (as discussed above), it is possible that this may represent nucleosomal fragments containing radiolabeled dFdC.

The present paper describes the interactions of dFdC with DNA synthesis in intact HL-60 cells under conditions of exposure to dFdC that are cytotoxic and that mimic exposure *in vivo*. The alterations in DNA synthesis observed may themselves be responsible for cytotoxicity, or may serve to alert the cell to trigger apoptosis. Hence, these studies serve to further our understanding of the biological effects and mechanism of action of this promising new antimetabolite in the intact cell.

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