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Modulation of Fluorouracil Cytotoxicity by Interferon-α and -γ

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ABSTRACT

Because interferons (IFN)-α and -γ individually have increased fluorouracil (FUra) cytotoxicity in several *in vitro* models, we studied the effects of FUra combined with IFN-α + γ in HT29 colon cancer cells. A 96-hr exposure to IFN-α (500 units/ml) plus IFN-γ (10 units/ml) and a 72-hr exposure to 0.25–1 μM FUra (hr 24–96) inhibited cell growth and colony formation in an additive or more-than-additive fashion. When cells were exposed to IFN-α + γ and FUra, free FdUMP levels became detectable, whereas [^{3}H]FUra-RNA incorporation decreased. Exposure to IFN-α + γ, FUra, or the combination decreased dTTP pools to 58%, 43%, and 17% of control, respectively. A marked increase in the dATP to dTTP ratio was seen with FUra with or without IFN-α + γ. Thymidylate synthase catalytic activity was reduced to 28% and 24% of control with FUra with or without IFN-α + γ, suggesting that the enhanced dTTP depletion must be due to another mechanism. FUra-mediated thymidylate synthase inhibition was accompanied by a 124-fold increase in total deoxyuridylate immunoreactivity and a 31-fold increase in dUTP pools, but the addition of IFN-α + γ attenuated the accumulation. Treatment with IFN-α + γ and FUra individually interfered with nascent DNA chain elongation, whereas the three-drug combination produced the most striking effects. IFN-α + γ plus FUra produced the greatest amount of single-strand breaks in nascent DNA and dramatically decreased net DNA synthesis. IFN-α + γ with or without FUra produced double-strand breaks in parental DNA. These results suggest that dTTP depletion, dATP/dTTP imbalance, pronounced inhibition of DNA synthesis, and damage to nascent and parental DNA contribute to the enhanced cytotoxicity with the triple combination.

IFNs are a family of proteins that regulate a wide spectrum of cell functions and modulate host responses to infection and malignancy (Kalvakolanu and Borden, 1996). The binding of type I (α and β) and type II (γ) IFN to high affinity cell surface receptors activates a postreceptor-signaling mechanism that leads to changes in gene transcription, protein expression, enzyme activities, nucleotide pools, and cell cycle distribution. The *in vivo* immunomodulatory consequences include effects on natural killer cells, T cells, macrophages, and induction of other cytokines.

Several *in vitro* studies have shown that IFN-α, -β, and -γ may interact with fluoropyrimidines in a more-than-additive fashion to produce cytotoxicity in a variety of human cancer cell lines (Elias and Crissman, 1988; Elias and Sandoval, 1989; Neefe and Glass, 1991; Chu *et al.*, 1993; Eda *et al.*, 1993a, 1993b; Houghton *et al.*, 1993; Schwartz *et al.*, 1994). Extended concurrent exposure to FUra and IFN seems to be optimal in preclinical studies. The type of IFN that maximally enhances fluoropyrimidine cytotoxicity differs among cell lines and may be explained by the different cellular receptors for IFN-α and IFN-γ (Branca and Baglioni, 1981; Brunda and Wright, 1986). The apparent basis for the potentiation of fluoropyrimidine cytotoxicity also varies with the type of IFN used, specific cell lines studied, and duration of drug exposure. Potential mechanisms include increased formation of the active metabolite FdUMP, increased DNA damage, and abrogation of the induction of TS protein expression during FUra exposure. In addition to biochemical and molecular mechanisms, immunomodulatory and pharmacological effects may be operative *in vivo*. The combination of IFN-α and IFN-γ has been reported to produce synergistic cytotoxicity in some cancer cell lines (Fleishman, 1982; Schiller *et al.*, 1986). We therefore wanted to study the effects of the combination of IFN-α and IFN-γ on FUra cytotoxicity in a human colon cancer cell line and the apparent basis for the interaction. Several investigators have reported that preexposure to IFN followed by FUra exposure produces greater cytotoxic effects than the reverse sequence (Elias and Criss-

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ABBREVIATIONS: IFN, interferon; FUra, 5-fluorouracil; HPLC, high performance liquid chromatography; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; TS, thymidylate synthase; TCA, trichloroacetic acid; FI, fractional inhibition.

man, 1988; Elias and Sandoval, 1989; Neefe and Glass, 1991). This observation may in part be explained by the fact that IFN-mediated cellular effects require active cellular RNA and protein synthesis, and a lag time of 12–24 hr is needed to detect increased expression of IFN stimulation genes (Schiller *et al.*, 1990; Kalvakolanu and Borden, 1996). We therefore delayed administration of FUra for 24 hr after the addition of IFN for the experiments reported herein.

Experimental Procedures

Materials. Unless otherwise stated, chemicals were purchased from Sigma Chemical (St. Louis, MO). The clinical formulations of IFN-α-2a (Hoffman-La Roche, Nutley, NJ) and IFN-γ-1b (Genentech, South San Francisco, CA) were used in these *in vitro* studies. [6-^{3}H]FUra (specific activity, 20 Ci/mmol), [^{3}H]adenosine (specific activity, 20 Ci/mmol), [*methyl*-^{3}H]dThd (specific activity, 25 Ci/mmol), [*methyl*-^{14}C]dThd (specific activity, 50 mCi/mmol), [2,8-^{3}H]dATP (specific activity, 28.5 Ci/mmol), and [*methyl*-^{3}H]dThd (specific activity, 82.9 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Isotonic PBS without calcium and magnesium was obtained from Digene Diagnostics (Beltsville, MD).

Cell culture. HT29 colon cancer cells obtained from American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 medium supplemented with 8% dialyzed fetal bovine serum and 2 mM L-glutamine (GIBCO, Grand Island, NY) at 37° in a humidified 5% CO_2 atmosphere. To assess effects on cell growth, 40,000 cells were replicately plated onto six-well tissue culture plates. Under the experimental conditions, there was a lag time of ~24 hr from the time of plating until the cells enter exponential growth phase; the population doubling time for the HT29 cells thereafter was 22 hr. Diluent, IFN-α, IFN-γ, or IFN-α + γ were added (both types of IFN at final concentrations ranging from 1 to 1000 units/ml) 24 hr after plating. Twenty-four hours later, FUra (at concentrations ranging from 0.1 to 1 μM) or diluent (PBS) was added. The adherent cell number was then determined at 96 hr in a Coulter Multisizer II (Miami, FL). For clonogenic assays, 500-1000 exponentially growing cells were replicately plated onto six-well plates. The after day, IFN-α, IFN-γ, or the combination was added, and FUra was added 24 hr later. After an additional 24-hr incubation, the medium was aspirated, and the cells were gently washed twice and then incubated in drug-free medium for an additional 7 days. Colonies were enumerated after staining. The control colony number averaged 399 ± 50. The interactions of IFN-α and IFN-γ with FUra were classified by the fractional inhibition method as follows: i_1 and i_2 represent the fractional inhibition of cell growth or colony formation with each drug individually; $i_1i_2 = i_1$ multiplied by i_2; $i_{1,2}$ = the fractional inhibition observed with the combination (Grem and Allegra, 1991). Additive inhibition produced by both inhibitors occurs when $i_{1,2} = i_1 + i_2 - i_1i_2$, synergism occurs when $i_{1,2} > i_1 + i_2 - i_1i_2$, and antagonism when $i_{1,2} < i_1 + i_2 - i_1i_2$. The combination index for cell growth data was calculated using CalcuSyn Windows Software for Dose Effect Analysis (T. C. Chou and M. P. Hayball, BioSoft, Ferguson, MO).

FUra metabolism. Approximately 1–1.5 million HT29 cells were replicately plated in 75-cm^2 cell culture flasks. After overnight incubation, the cells were preexposed to 500 units/ml IFN-α, 10 units/ml IFN-γ, or the combination for 24 hr. FUra (1 μM) was added, and [^{3}H]FUra (final concentration, 2 μCi/20 nmol) was added during the final 4 hr of a 24-hr exposure. The medium was aspirated, the cells were washed three times with iced PBS and then extracted with 0.5 N PCA; and the soluble fraction was neutralized and lyophilized. The radioactivity in an aliquot of the reconstituted residue was determined by liquid scintillation counting, and another aliquot was analyzed by ion-pairing reversed-phase HPLC (Grem and Fischer, 1986). The distribution of [^{3}H]FUra metabolites was identified by an in-line scintillation detector, and the total amount of each type of FUra metabolites was determined by multiplying the total pmol of FUra metabolites/1 million cells by the percent of total radioactivity eluting with each peak. The retention times were as follows: FUra, 4–5 min; FdUMP, 28 min; FUDP-sugars, 33–36 min; FUDP, 48 min; and FUTP, 55 min.

FUra RNA incorporation. HT29 cells were replicately plated onto 175-cm^2 tissue culture flasks. After overnight incubation, the cells were preexposed to diluent (500 units/ml IFN-α, 10 units/ml IFN-γ, or the combination) for 24 hr, after which [^{3}H]FUra was added (1 μM, 4 μCi/30 nmol). After 72 hr, RNA was isolated and purified with the RNA Stat-30 kit (Tel-Test "B", Friendswood, TX) containing RNAzol B as recommended by the manufacturer. The RNA pellet was dissolved with 1 ml of 10 mM Tris/1 mM EDTA, pH 8.0, and quantified by UV absorbance at 260/280 nm. The RNA then was precipitated with TCA and filtered through 0.45-μm HA filters (Millipore, Bedford, MA) held in a vacuum manifold. The filters were washed three times with 5% TCA and once with 70% ethanol. The residual radioactivity on the filter was determined with a liquid scintillation counter.

Measurement of dATP, dTTP, dUMP, and dUTP pools. Exponentially growing cells were exposed to diluent or IFN-α (500 units/ml) and IFN-γ (10 units/ml) for 48 hr; 1 μM FUra or diluent was added for the final 24. The cells were extracted with 0.5 N PCA, neutralized, and lyophilized. The samples were stored at −70° until the time of analysis. dTTP and dATP pools were determined by an enzymatic assay (Sherman and Fyfe, 1989). Three synthetic oligonucleotides were purchased from Genosys Biotechnologies (The Woodlands, TX). The template for the dTTP assay was 5′-TTA-TTA-TTA-TTA-TTA-TTA-GGC-GGT-GGA-GGC-GG-3′; the template for the dATP assay was 5′-AAA-TAA-ATA-AAT-AAA-TAA-ATC-CCG-GTG-GAG-GCG-G-3′; and the primer for both assays was 5′-CCG-CCT-CCA-CCG-CC-3′. The addition of [^{3}H]dATP through the Klenow fragment of bacterial DNA polymerase to the growing primer strand was directed by the template sequence and limited by the amount of dTTP in the reaction mixture. Conversely, incorporation of [^{3}H]dTTP into the template/primer was limited by the amount of dATP in the reaction mixture. Standard curves were linear between 0.5 and 40 pmol.

A radioimmunoassay was used to measure dTTP, immunoreactive dUMP, and dUTP pools sizes (Piall *et al.*, 1989; Aherne *et al.*, 1996). Ribonucleotides first were eliminated by incubation with sodium periodate, followed by methylamine cleavage, and deoxyribonucleotide triphosphates were separated from the monophosphates and diphosphates by QAE Sephadex chromatography. Two different antisera were used: anti-dTTP and anti-dUTP. Immunoreactive dUMP pools were measured using a portion of the cell extract that was not subjected to chromatography. Total immunoreactivity was calculated by comparison with a standard curve constructed with [^{3}H]dUMP as the radiolabel. The antiserum has a much higher affinity for the triphosphate and diphosphate compared with the monophosphate. Immunoreactive dUMP pools thus reflect the presence of all three deoxyuridine nucleotides, and the contribution of each nucleotide cannot be estimated accurately.

TS assays. A cellular lysate was prepared after drug exposure, and equal amounts of protein (200 μg) were resolved by 15% polyacrylamide gel electrophoresis as described previously (Johnston *et al.*, 1992). Western immunoblot analysis was performed using TS106 monoclonal antibody as the primary antibody; the secondary antibody was goat anti-mouse horseradish peroxidase conjugate (Bio-Rad, Richmond, CA). The protein bands were developed with the enhanced chemiluminescence kit (ECL Kit; Amersham, Buckinghamshire, UK). The quantity of TS was analyzed by densitometry using Sigmagel for Windows version 1.0 (Jandel Scientific, San Rafael, CA).

TS catalytic activity in cellular lysates was determined by tritium release from [5-^{3}H]dUMP (Allegra *et al.*, 1985; Grem *et al.*, 1996). The cell pellets were sonicated in 200 μl of buffer, and an aliquot (25–40 μl) was added to a mixture containing 2000 pmol of dUMP,

200,000 dpm of [^{3}H]dUMP, 200 pmol of 5,10-methylene-tetrahydrofolate, and 20 μmol of 2-β-mercaptoethanol in a final volume of 200 μl. Approximately 1.3 million control cells were present when the cells were harvested; the cell numbers (percent of control) for IFN-α + γ, FUra, and all three drugs were 76 ± 7%, 80 ± 6%, and 52 ± 8%, respectively. The percentage of the total cellular lysate used in the TS assays averaged 19 ± 4%. Protein was quantified with the Bio-Rad protein assay kit.

RNA and DNA synthesis. After a 30-min exposure to [^{3}H]adenosine, the macromolecules were precipitated with 0.5 N PCA. The pellet was collected by centrifugation, and the acid-precipitable material was washed three times with 1 ml of 0.5 N PCA. In initial experiments, the samples were dissolved in 0.5 N sodium hydroxide at 37° and divided into two portions. One half was neutralized, and the radioactivity was determined. The other half was reacidified, incubated on ice for 20 min, and then centrifuged. The radioactivity in the alkali-labile and -stable fractions were determined. Under these conditions, >90% of the counts localized in the RNA fraction. Thereafter, the radioactivity in the total acid-precipitable material was taken to reflect [^{3}H]adenosine/RNA incorporation. DNA synthesis was estimated by incorporation of [^{3}H]thymidine (1 hr) into acid-precipitable material.

To correct for any possible effects of FUra and IFN on the relevant nucleotide pools, we determined endogenous dTTP, ATP, and [^{3}H]dTTP pools. Control and drug-treated cells were rinsed with ice-cold PBS and extracted with 0.5 N PCA, and the soluble fraction was neutralized and lyophilized. The residue was resuspended in distilled water before analysis. Endogenous ATP pools and [^{3}H]dTTP formation were quantified by an anion-exchange HPLC method and in-line scintillation detection (Grem *et al.*, 1995). The total pool size of endogenous dTTP as determined above and [^{3}H]dTTP then were used to correct for radiolabeled thymidine incorporation into DNA as follows: (total dpm incorporated into DNA) × (1 μCi/2.2 million dpm) × (total pmol dTTP/total μCi added per flask).

Because the endogenous ATP pools (control, 56 nmol/10^6 cells) were several orders of magnitude higher than that contributed by the [^{3}H]ATP (1 μCi represents <0.1 nmol), the endogenous ATP pool size was considered equivalent to the total pool size. The synthetic rate was defined as the pmol of adenosine or fmol of thymidine incorporated into RNA or DNA/1 million cells/hr (Grem *et al.*, 1996).

Effects on nascent DNA. Exponentially growing HT29 cells were exposed to diluent, IFN-α (500 units/ml), or IFN-γ (10 units/ml) for 48 hr; 1 μM FUra or diluent was added at hr 24. The cells were pulse-labeled with [^{3}H]thymidine (10 μCi, 25 Ci/mmol) for the final 2 hr. The pH step alkaline elution profile of nascent DNA was determined as described previously (Grem *et al.*, 1994, 1996). Total radioactivity was defined as the sum of the DPM in each elution fraction and that retained on the filter (less background).

In separate experiments to determine the effects of FUra, IFN, and the combination on the integrity of newly synthesized DNA, cells were exposed to [^{14}C]dThd (0.05 μCi/ml, 15 ml/flask) for the final 8 hr of incubation. The cells were harvested, deposited on Nuclepore filters, and lysed, and the DNA was eluted with 20 mM EDTA adjusted to pH 12.1 with 1 M tetrapropylammonium hydroxide at a rate of 2.4 ml/hr as described previously (Grem *et al.*, 1995, 1996). Fractions were collected at 1.5-hr intervals for 15 hr. To distinguish [^{14}C]DNA from any unincorporated [^{14}C]dTTP or [^{14}C]thymidine, an aliquot of the eluate from the lysis and wash fractions was treated with 20% cold TCA to precipitate the DNA and then filtered through HA filters. The remaining elution fractions and the filter were processed as described previously(Grem *et al.*, 1995, 1996).

Effects on parental DNA. Parental DNA fragmentation was analyzed by a filter binding assay under nondeproteinizing, non-DNA-denaturing conditions (Grem *et al.*, 1995, 1996). HT29 cells were prelabeled with [^{14}C]dThd (0.05 μCi/ml, 15 ml/flask) for 24–48 hr. After a 6–24-hr "chase" period, 500 units/ml IFN-α and 10 units/ml IFN-γ were added. FUra (1 μM) was added either concurrently with IFN-α + γ or after a 24-hr period. At the desired time, the cells were harvested and deposited on a Metricel membrane (0.8-μm pore, 25-mm diameter; PALL Gelman Sciences, Ann Arbor, MI) held in an alkaline elution funnel. The total radioactivity was determined by combining the counts from the eluting fractions (loading, wash, lysis, and EDTA wash) plus that retained on the filter. DNA fragmentation was calculated by dividing the dpm in the eluting fractions by the total dpm.

For conventional agarose electrophoresis, nucleic acids were purified by a salting-out method (Miller *et al.*, 1988). The extract was resuspended in 10 mM Tris/1 mM EDTA, pH 8.0, and incubated with DNase-free RNase for 60 min at 37°. The samples were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v), and the DNA was precipitated with ammonium acetate and ethanol. The purified DNA was resuspended in 10 mM Tris/1 mM EDTA, pH 8.0. Equal amounts of DNA (2–3 μg) were electrophoresed through a 1.5% agarose gel at 70–90 V for 3–4 hr. The gels then were stained with 0.5 μg/ml ethidium bromide, destained, and photographed under UV illumination.

For pulsed-field electrophoresis, intact cells were embedded in 0.5% low-melting-point agarose plugs (100,000 cells/plug); digested for 48 hr with 5 volumes of a buffer containing 0.5 M EDTA, pH 9.0, 1% sodium lauroyl sarcosine, and 0.5 mg/ml Proteinase K at 50°; washed; and stored at 4° as recommended by the manufacturer (BioRad). Individual plugs were placed on the teeth of a gel comb, and a 1.3% chromosomal grade agarose gel (160 ml) was cast. A Chef Mapper (BioRad) multistate program was used as described previously (Grem *et al.*, 1996). Tris-borate-EDTA buffer (0.5×, 4 liters) was recirculated at 14° during electrophoresis, and the gels subsequently were stained.

Graphical and statistical analysis. The scientific graphs were prepared using SigmaPlot for Windows version 3.03 (Jandel Scientific). Statistical analysis of the differences between control cells and drug-treated cells was performed using SigmaStat for Windows version 2.0 (Jandel Scientific).

Results

Enhancement of FUra cytotoxicity by IFN-α, IFN-γ, and IFN-α + γ. A 24-hr preexposure to IFN was used to allow sufficient time for any possible IFN-mediated effects in gene and/or protein expression to occur. In preliminary experiments, the cytotoxicity was determined of each agent given individually. In cell growth experiments, the 50% inhibitory concentration (IC_{50}) for a 72-hr exposure to FUra was ~1 μM. A 96-hr exposure to IFN-γ inhibited cell growth in a concentration-dependent manner, with an IC_{50} value of ~100 units/ml. In contrast, a similar exposure to 1000 units/ml IFN-α produced only minor (~20%) inhibition of cell growth. The ability of several concentrations of IFN-α (100, 500, and 1000 units/ml) and IFN-γ (1, 10, and 100 units/ml) to enhance FUra-mediated growth inhibition was examined. Optimal results were observed with 500 units/ml IFN-α and10 units/ml IFN-γ (data not shown), and these concentrations were used for the remainder of the experiments.

A 96-hr exposure to the combination of 500 units/ml IFN-α and 10 units/ml IFN-γ seemed to produce more-than-additive growth inhibition (Fig. 1). The observed fractional inhibition (FI) value that was seen with exposure to IFN-α and IFN-γ was slightly greater than the expected value (0.43 versus 0.37), suggesting at least additive toxicity when cells were treated with the combination of IFN-α + γ. A ratio of the expected to the observed FI of 1, <1, or >1 would reflect additive, more-than-additive, and less-than-additive effects, respectively. The median ratios (±half range) of the expected to observed FI value for the various combinations of IFN-for

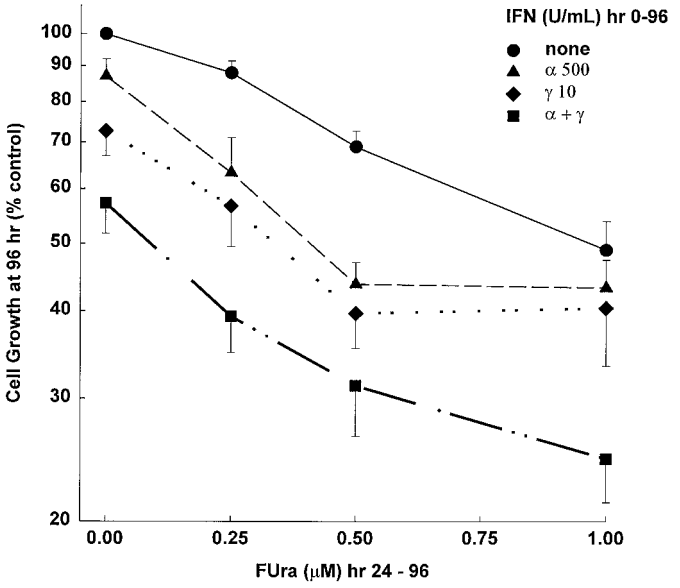

Fig. 1. Augmentation of FUra cytotoxicity by IFN-α, IFN-γ, and IFN-α + γ. HT29 cells were exposed to diluent, 500 units/ml IFN-α, 10 units/ml IFN-γ, or the combination for 24 hr, after which FUra was added at the indicated concentrations. The number of adherent cells was determined at 96 hr (72 hr after the addition of FUra). The data, presented as the percent of control cell number (mean ± standard error), are from three to eight separate experiments, each performed in replicate. The control cell number at 96 hr averaged $3.6 \pm 0.8 \times 10^5$. The actual cell numbers after 1 μM FUra, IFN-α + γ, and the three-drug combination were significantly different from control ($p < 0.01$, signed rank test), and the cell numbers for the three drug combination also were significantly different from FUra alone and IFN-α + γ alone ($p < 0.01$).

96 hr with FUra (0.25, 0.5, and 1.0 μM) for the final 72 hr were as follows: IFN-α, 0.78 ± 0.18; IFN-γ, 0.83 ± 0.12; and IFN-α + γ, 0.88 ± 0.06. Thus, preexposure of HT29 cells to IFN-α, IFN-γ, or the combination for 24 hr followed by concurrent exposure to FUra for an additional 72 hr resulted in more-than-additive cytotoxicity. Because nonconstant drug ratios were used, classic median effect analysis could not be performed. However, combination index values for each data point of the three drug ratios were calculated; the values ranged from 0.3 to 0.6, indicating more-than-additive effects. Finally, when the actual cell numbers were compared for the experiments involving 1 μM FUra with or without IFN-α + γ, the median values for FUra, IFN-α + γ, and the combination were significantly different from that of control. Moreover, the median cell numbers after exposure to the three drug combination were significantly lower than those for FUra and IFN-α + γ given alone ($p < 0.01$, signed rank test).

In clonogenic assays, a 72-hr exposure to 1 μM FUra reduced colony formation to 66 ± 10% of control, and a 96-hr exposure to 500 units/ml IFN-α and 10 units/ml IFN-γ reduced the colony number to 80 ± 8% and 72 ± 8% of control, respectively (mean ± standard error, four experiments). The combination of FUra and IFN-α, IFN-γ, and IFN-α + γ reduced colony formation to 52 ± 13%, 47 ± 3%, and 30 ± 4% of control, respectively (mean ± half range, two experiments). These results suggested additive lethality by the FI method. The degree of cytotoxicity for FUra-treated cells was slightly less than that observed in the cell growth assays, but the relative effects versus FUra alone were comparable (growth versus colony): FUra + IFN-α, 0.85 versus 0.77; FUra + IFN-γ, 0.80 versus 0.71; and FUra + IFN-α + γ, 0.49 versus 0.45. Thus, IFN- and FUra-mediated effects on cell growth provided a reasonable reflection of cytotoxicity.

FUra metabolism and RNA incorporation. Additional studies were performed to elucidate the underlying mechanism or mechanisms of interaction. HPLC analysis of FUra metabolites after a 24-hr exposure to 1 μM ([^{3}H]FUra for the final 4 hr) indicated that FUTP was the predominant metabolite, accounting for 68% of the total intracellular metabolites (Table 1). Parent drug, FUDP and FUDP-sugars represented ~20%, ~4%, and ~6% of the total metabolites; breakdown products eluting with the solvent front accounted for the balance (~2%). Free FdUMP was not detected, presumably because the available [^{3}H]FdUMP was complexed with TS. Because FUTP and 5-fluoro-2′-deoxyuridine-5′-triphosphate elute from the HPLC column within several minutes of each other, an aliquot of the cellular residue was treated with sodium periodate and methylamine to destroy the ribonucleotide metabolites of FUra (Grem and Fischer, 1986). However, 5-fluoro-2′-deoxyuridine-5′-triphosphate still was not evident.

A 24-hr preexposure to500 units/ml IFN-α did not appreciably alter FUra-ribonucleotide formation; free FdUMP was detected in two of eight samples. In contrast, FUra-ribonucleotide formation seemed to be diminished by ≥40% after a 24-hr preexposure to 10 units/ml IFN-γ. With the combination of IFN-α + γ, free FdUMP was detected in seven of eight samples, whereas FUTP levels were intermediate between that seen with IFN-α and IFN-γ individually.

After a 72-hr exposure to 1 μM [^{3}H]FUra (hr 24–96), FUra-RNA levels averaged 402 ± 65 fmol/μg (mean ± standard error, four experiments, each performed in duplicate). In the presence of IFN-α (500 units/ml, hr 0–96), IFN-γ (10 units/ml, hr 0–96), or the combination of IFN-α + γ, FUra RNA levels were decreased by an average of 10%, 24%, and 32%, respectively, to 364 ± 54 (α), 304 ± 81 (γ), and 272 ± 82 (α + γ) fmol/μg. Thus, IFN-γ produced a slight decrease in FUra RNA incorporation.

TABLE 1

FUra metabolism and RNA incorporation

HT29 cells were preexposed to diluent, IFN-α (500 units/ml), IFN-γ (10 units/ml), or the combination for 24 hr, after which 1 μM FUra was added. [^{3}H]FUra (final concentration, 2 μCi/20 nmol) was added for the last 4 hr, after which the cells were extracted with 0.5 N perchloric acid. The soluble fraction was neutralized and lyophilized, and an aliquot of the reconstituted residue was analyzed with ion-pairing HPLC. For RNA incorporation, cells were preexposed to IFN as indicated for 24 hr; [^{3}H]FUra then was added (final concentration, 4 μCi/31 nmol). After 72 hr, the RNA was extracted and purified as outlined in the text. The metabolite and RNA data are shown as mean ± standard error and are from four separate experiments, each performed in duplicate. FdUTP was not detected under any of the four conditions.

Condition	free FdUMP	FUDP sugars	FUDP	FUTP	FUra RNA
		pmol / 10^6 cells			*fmol / μg*
FUra	Not detected	2.0 ± 0.4	1.5 ± 0.3	26.4 ± 4.0	402 ± 93
FUra + IFN-α	0.1 ± 0.1	1.9 ± 0.4	0.9 ± 0.2	28.1 ± 6.5	364 ± 75
FUra + IFN-γ	Not detected	0.6 ± 0.2	0.6 ± 0.2	16.2 ± 2.3	354 ± 70
FUra + IFN-α + γ	1.6 ± 0.7	1.2 ± 0.6	1.8 ± 1.1	20.4 ± 7.1	272 ± 70

Biochemical effects of IFN-and FUra. For all experiments, the cells for all conditions were plated at the same time and at the same density, and the control cells were harvested at the same time as the drug-treated cells. FUra-mediated TS inhibition is expected to result in dTTP pool depletion. After a 4-hr exposure to 1 μM FUra, dTTP pools (pmol/10^6 cells) were not affected (78 ± 9) compared with control (70 ± 6, mean ± standard error, four experiments). Similarly, dTTP pools were only minimally affected immediately after a 24-hr exposure to IFN-α + γ: 63 ± 18 pmol/10^6 cells, 90% of control. When FUra was added to IFN-α + γ during the final 4 hr of IFN exposure, however, dTTP pools were diminished to 46 ± 14 pmol/10^6 cells (66% of control). When the durations of exposure to IFN-α + γ (48 hr) and FUra (24 hr) were extended, more pronounced depletion of dTTP was evident (Fig. 2, *left*). dTTP pools were lowered to 59% and 43% of control with IFN-α + γ or FUra given individually but were reduced to 17% of control with the three-drug combination. Because the effects on dTTP pools were more prominent after a 24-hr versus a 4-hr exposure to FUra (with or without IFN preexposure), subsequent experiments focused on a 24-hr exposure to FUra (and a 48-hr exposure to IFN).

The accumulation of deoxyuridine nucleotides provides an indirect reflection of TS blockade. FUra exposure was accompanied by a 124-fold increase in immunoreactive dUMP (Fig. 2, *middle*), which reflects contributions from dUMP, dUDP, and dUTP. Although IFN-α + γ alone produced a 2.2-fold increase in dUMP pools, the addition to FUra attenuated the accumulation (16-fold higher than control). The specific measurement of dUTP pools also showed a 30-fold increase in FUra-treated cells compared with controls (Fig. 2, *right*). However, this effect was largely negated by the addition of IFN-α + γ. The ratio of immunoreactive dUMP to dTTP increased in drug-treated cells from 0.7 (control) to 2.6 (IFN-α + γ), 195 (FUra), and 62 (IFN-α + γ plus FUra). The ratio of dUTP to dTTP increased from 0.05 in control cells to 0.12 (IFN-α + γ), 3.6 (FUra), and 0.40 (IFN-α + γ plus FUra).

As shown in Table 2 (*far right*), a 48-hr exposure to IFN-α + γ was accompanied by a slight decrease in dATP pools (78% of control). In contrast, dATP pools were expanded by ~1.4-fold over control after a 24-hr exposure to FUra without and with IFN-α + γ. The ratios of dATP to dTTP were as follows: control, 2.8; IFN-α + γ hr 0–48, 3.5; FUra hr 24–48, 9.3; IFN-α + γ plus FUra, 23.2, indicating a profound deoxyribonucleotide imbalance in FUra-treated cells; the greatest effect occurred with IFN-α + γ combined with FUra.

By Western immunoblot analysis, the relative change in total TS protein content was estimated by comparing the sum of the bound and free TS bands in drug-treated cells with the free TS band in control cells. Total TS protein content increased ~1.4-fold after a 24-hr exposure to 1 μM FUra (Fig. 3, *left*). A 48-hr exposure to either 500 units/ml IFN-α, 10 units/ml IFN-γ, or the combination did not appreciably affect TS content when given alone and did not abrogate the FUra-associated increase in TS content. TS catalytic activity in cell lysates was decreased to 28% of control after a 24-hr exposure to 1 μM FUra, but the addition of IFN-α, IFN-γ, or IFN-α + γ (hr 0–48) did not seem to potentiate TS inhibition (Fig. 3, *right*).

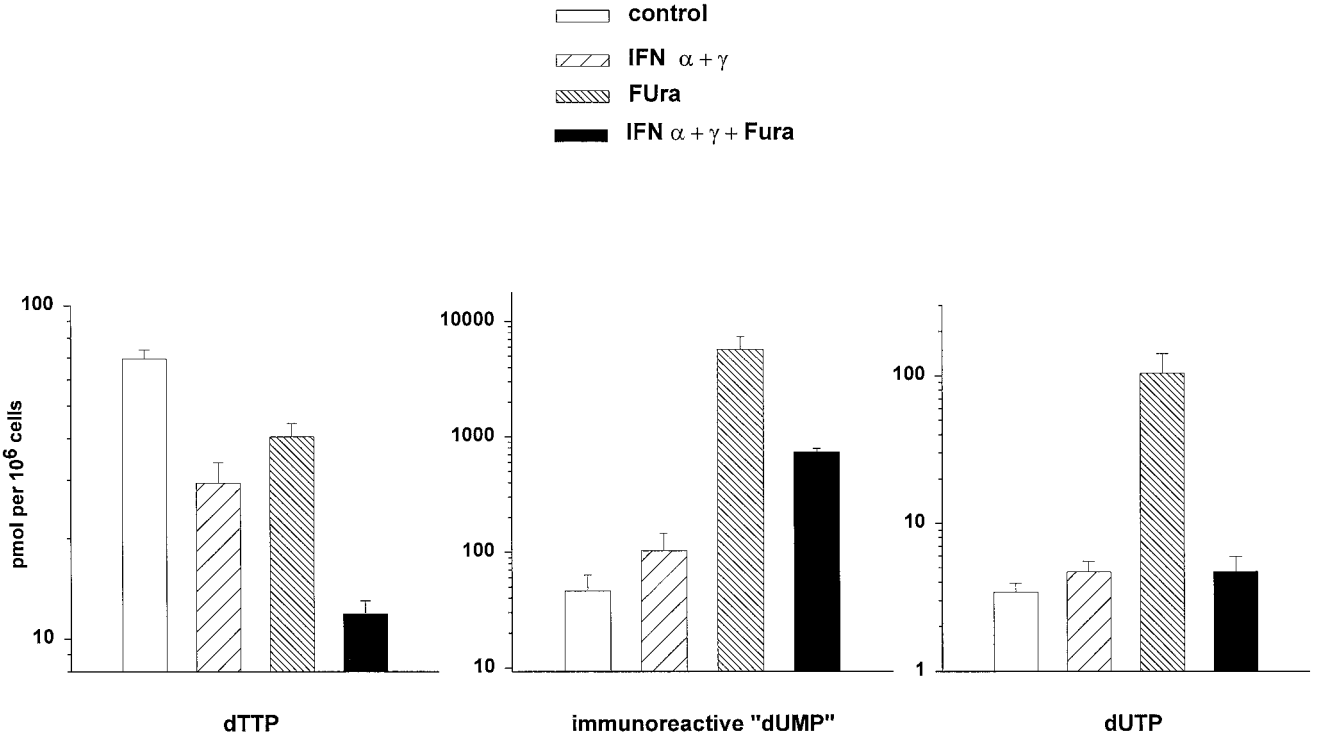

Fig. 2. Changes in deoxynucleotide pools during drug exposure. HT29 cells were exposed to 500 units/ml IFN-α and 10 units/ml IFN-γ for 48 hr; 1 μM FUra was added for the final 24 hr. dTTP pools were measured by both the DNA polymerase assay (seven experiments) and radioimmunoassay (seven experiments). Because the results were comparable for both assays, the data have been combined. Immunoreactive dUMP and dUTP pools were determined by radioimmunoassay (seven experiments). Data are presented as the mean ± standard error. *Left*, dTTP pools were significantly different from control for all three conditions ($p < 0.001$, signed rank test). *Middle*, total dUMP immunoreactivity in cells treated with FUra alone or with IFN-α + γ were significantly higher than control ($p < 0.02$). *Right*, dUTP pools in cells treated with FUra alone were significantly higher than control ($p < 0.02$).

TABLE 2

Effect of FUra and IFN-α + γ on ribonucleotide triphosphate and dATP pools

HT29 cells were preexposed to diluent, IFN-α (500 units/ml), IFN-γ (10 units/ml), or the combination for 24 hr, after which 1 μM FUra was added. After 24 hr, the cells were extracted as described in Table 1. Ribonucleotide triphosphate pools were determined with anion exchange HPLC, and the data are given as mean ± standard error (n = 4). dATP pools were determined by a bacterial DNA polymerase assay using synthetic oligonucleotides as template/primers (Sherman and Fyfe, 1989), and the data are given as mean ± standard error (n = 5).

Condition	UTP	CTP	GTP	ATP	dATP
	nmol/10^6 cells (-fold of control)				*pmol/10^6 cells (-fold of control)*
Control	2.53 ± 0.24	0.55 ± 0.08	1.61 ± 0.36	56.2 ± 3.4	194 ± 47
FUra	3.62 ± 0.22 (1.43)	0.81 ± 0.14 (1.47)	2.33 ± 0.49 (1.45)	79.9 ± 1.7 (1.42)	273 ± 56 (1.41)
IFN-α + γ	2.32 ± 0.10 (0.92)	0.61 ± 0.11 (1.11)	1.33 ± 0.28 (0.83)	55.2 ± 0.5 (0.98)	142 ± 44 (0.73)
FUra + IFN-α + γ	2.42 ± 0.09 (0.96)	0.54 ± 0.11 (0.98)	1.17 ± 0.43 (0.73)	62.1 ± 3.4 (1.10)	277 ± 68 (1.43)

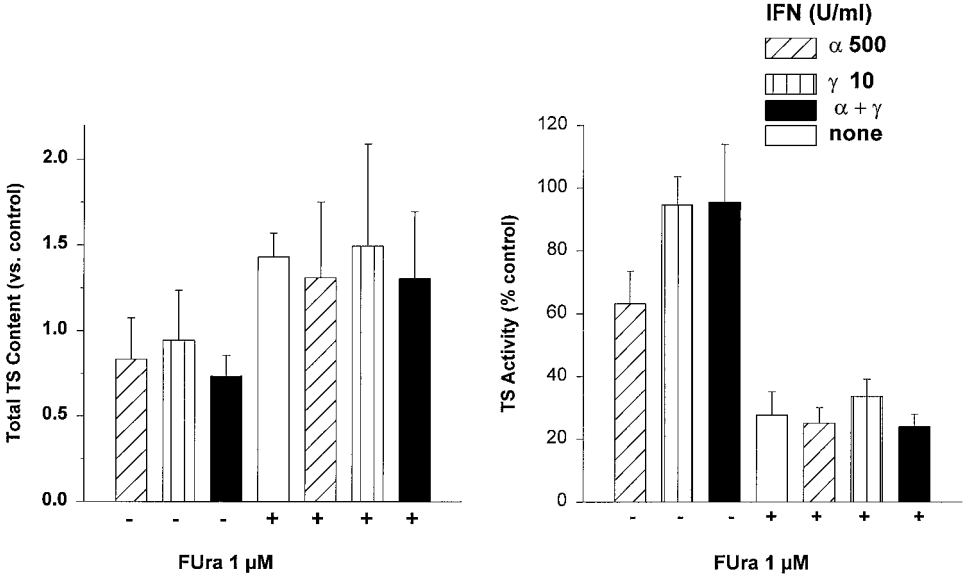

Fig. 3. Effect of FUra and IFN on thymidylate synthase content and catalytic activity. HT29 cells were exposed to 500 units/ml IFN-α, 10 units/ml IFN-γ, or the combination for 48 hr. FUra (1 μM) was added for the final 24 hr. TS protein content (*left*) was determined by Western immunoblot analysis; in the FUra-treated cells, data represent the sum of the free and bound TS signals, are expressed as a fraction of the densitometric signal for the control sample (0.74 ± 0.27 arbitrary units), and represent the mean ± standard deviation of three experiments. TS catalytic activity (*right*) in cellular lysates was determined by tritium release from [5-^{3}H]dUMP. Data, shown as the percent of control activity, represents the mean ± standard error from seven experiments, each performed in duplicate. TS activity in control cells was 40.6 ± 14.3 pmol/min/mg of protein.

Impact of IFN-α + γ and FUra on macromolecular synthesis. Incorporation of [^{3}H]nucleoside precursors was used to estimate RNA and DNA synthesis. By HPLC analysis, exposure to 1 μM FUra from hr 24–48 was associated with a 1.42-fold increase in ATP pools compared with control (Table 2). A 48-hr exposure to IFN-α + γ had no effect on ATP pools, but their addition to FUra attenuated the ATP pool. Net RNA synthesis was calculated, taking into account the endogenous ATP pools. RNA synthesis seemed to be greater than control in cells treated with FUra alone or in combination with IFN-α + γ; however, the differences were not significant (Table 3, *left*). Because IFN-α + γ clearly did not inhibit net RNA synthesis relative to control, the IFN-γ-mediated decrease in FUra-RNA incorporation is most likely due to decreased FUTP formation rather than inhibition of RNA synthesis.

FUra exposure also was accompanied by a 1.43- to 1.47-fold increase in UTP, CTP, and GTP pools. IFN-α + γ did not affect the ribonucleotide triphosphate pools, suggesting that ribonucleotide synthetic pathways were not disturbed. The addition of IFN-α + γ to FUra, however, negated the increase in ribonucleotide triphosphate pools.

Because of the profound changes in dTTP pools in drug-treated cells, our calculations of DNA synthetic rate by [^{3}H]thymidine incorporation accounted for the total dTTP pool (endogenous and exogenous [^{3}H]dTTP). DNA synthesis was reduced to 69% and 51% of control with FUra alone and IFN-α + γ, respectively (Table 3, *middle*). With the combination of IFN-α + γ and FUra, however, DNA synthesis was reduced to 18% of control, which is clearly greater than expected on an additive basis.

Inhibition of thymidine salvage might be a possible explanation for the IFN-mediated depletion of dTTP pools. Comparison of the amount of [^{3}H]thymidine nucleotides formed during a 1-hr exposure permits an assessment of thymidine kinase activity in intact cells (Table 3, *right*); however, the differences in the median values among the treatment groups were not significant. Thus, the profound depletion of dTTP in cells treated with IFN-α + γ plus FUra could not be attributed to decreased thymidine salvage.

Interference with nascent DNA. The effect of FUra with or without IFN-α, IFN-γ, and IFN-α + γ on nascent DNA replication intermediates was examined by pH step alkaline elution. Over the pH transition zone, the size of the DNA strand influences its rate of denaturation to single-strand species. Interference with chain elongation is characterized by an increased proportion of the nascent DNA eluting with sequential 1-hr exposures to buffer with progressively more alkaline pH. IFN-α and IFN-γ produced minimal effects compared with control (Fig. 4). Exposure to either IFN-α + γ or FUra led to greater interference with DNA chain elongation; the effect on nascent DNA was more

TABLE 3

Effect of IFN-α + γ and FUra on net RNA and DNA synthesis

HT29 cells were treated with diluent, IFN-α + γ (500 units/ml and 10 units/ml), FUra (1 μM), or the combination as indicated. The cells then were incubated with [^{3}H]adenosine (1 μCi) for 30–60 min or [^{3}H]thymidine (1–2 μCi) for 1 hr. Incorporation into acid-precipitable material was determined. The data have been corrected for the total ATP and dTTP pool sizes (both endogenous and [^{3}H]triphosphate pools). The data for RNA synthesis are presented as mean ± standard deviation (n = 3). [^{3}H]Thymidine nucleotide formation (dTMP, dTDP, and dTTP) is shown as the mean ± standard error (n = 4). The data for DNA synthesis are presented as mean ± standard error (n = 6). The percentage of control values are shown in parentheses. The differences in the median values among the treatment groups for RNA synthesis and [^{3}H]thymidine nucleotide formation are not significant (p = 0.313 and p = 0.375, respectively; Kruskal-Wallis one-way analysis of variance on ranks). However, the differences in the median values for DNA synthesis among the treatment groups are significant (p = 0.018), and pairwise multiple comparisons indicated that control and the three-drug combination were significantly different ($p < 0.05$)

Condition	Adenosine incorporated into RNA	Thymidine incorporated into DNA	[^{3}H]Thymidine nucleotides formed during the 1-hr pulse
	pmol/10^6 cells/hr	*fmol/10^6 cells/hr*	*fmol/10^6 cells*
Control	460 ± 149	547 ± 182	78 ± 17
IFN-α + γ hr −048	622 ± 60 (135%)	277 ± 109 (50.6%)	85 ± 18 (109%)
FUra hr 24 to 48	649 ± 54 (141%)	377 ± 97 (68.9%)	55 ± 17 (71%)
IFN-α + γ plus FUra	651 ± 50 (142%)	98 ± 36 (17.9%)	63 ± 19 (81%)

pronounced with the combination of FUra plus either IFN-α or IFN-γ: ~17% DNA retained (38% of control, $p < 0.01$). The triple combination produced the most dramatic interference with nascent replication intermediates, with only 11% of DNA retained (24% of control, $p < 0.001$).

The impact of FUra and IFN-α + γ on the fragility of nascent DNA was examined by extended alkaline elution at a fixed pH of 12.1. As shown in Fig. 5, both FUra alone and IFN-α + γ were associated with an increase in the proportion of single-strand breaks relative to control, and the three-drug combination had the greatest effect (p = 0.006).

Impact of IFN-α + γ and FUra on parental DNA fragmentation. The effects of IFN-α + γ alone and in combination with FUra on parental DNA (prelabeled with [^{14}C]thymidine) was assessed by a filter binding assay (Fig. 6). A 24-hr exposure to IFN-α + γ was associated with a slight excess (1.7-fold) of double-strand breaks relative to control, and the effect was more pronounced when the duration of exposure to IFN-α + γ was extended to 48-hr (2.5-fold greater than control). A 24-hr exposure to 1 μM FUra was not associated with induction of double-strand breaks, nor did the addition of FUra to IFN-α + γ further increase DNA fragmentation over that seen with IFN-α + γ alone. Under these conditions, oligonucleosomal DNA laddering was not seen with conventional agarose gel electrophoresis (data not shown).

Although the filter binding assay detects double-stranded breaks in DNA, high-molecular-weight DNA fragments might not be detected because size influences the ability to traverse the filter. We therefore analyzed the effects of FUra and IFN-α + γ alone and in combination on parental DNA using pulsed field gel electrophoresis (Fig. 7). High-molecular-weight DNA fragmentation was not evident in either control or FUra-treated cells but was detected in cells treated with IFN-α + γ with or without FUra.

Discussion

We found that IFN-α and IFN-γ individually and in combination increased FUra-mediated growth inhibition and di-

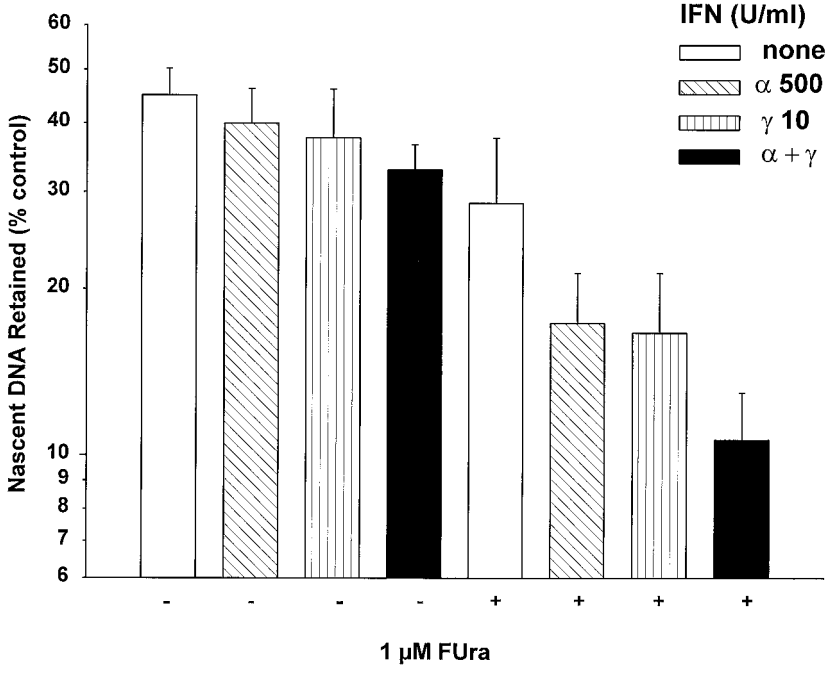

Fig. 4. Interference with nascent DNA replication intermediates. HT29 cells were exposed to 500 units/ml IFN-α, 10 units/ml IFN-γ, or the combination for 48 hr; 1 μM FUra was added for the final 24 hr. The cells were pulse-labeled with [^{3}H]thymidine (10 μCi/flask) for the final 2 hr, and the pH step alkaline elution profile was determined. Data are shown as the percent of total DPM retained on the filter (mean ± standard deviation from three experiments). The differences in the mean values among the treatment group are greater than would be expected by chance ($p < 0.001$, Kruskal-Wallis one-way analysis of variance); multiple comparisons with the control group indicated that the differences in the mean values were significant ($p < 0.05$) for FUra plus IFN-α, FUra plus IFN-γ, and FUra plus IFN-α + γ.

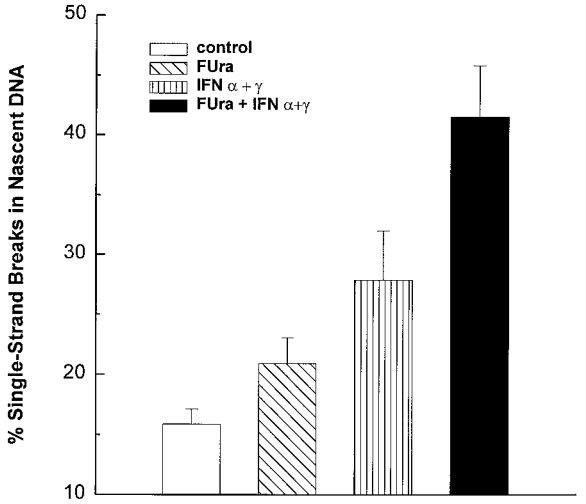

Fig. 5. Single-strand breaks associated with FUra and IFN-α + γ exposure. HT29 cells treated with diluent, 1 μM FUra, IFN-α + γ (500 and 10 units/ml), or the combination were labeled with [^{14}C]thymidine (0.75 μCi) for the final 8 hr of drug exposure, and the DNA was eluted at a fixed alkaline pH (12.1) over a 15-hr period. The proportion of [^{14}C]DNA that eluted relative to the total [^{14}C]DNA for each condition is presented as the mean ± standard error (5–10 separate experiments). The p values for the differences between the median values for the drug-treated versus control were as follows: FUra, $p = 0.077$; IFN-α + γ, $p = 0.011$; FUra plus IFN-α + γ, $p = 0.006$, rank sum test).

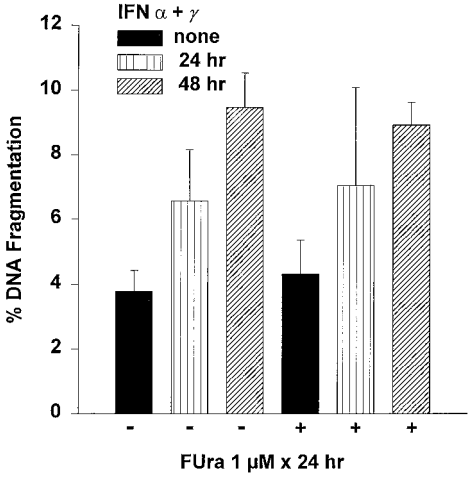

Fig. 6. Induction of parental DNA fragmentation by IFN-α + γ. HT29 cells were prelabeled with [^{14}C]thymidine for 24–48 hr. After a 6-hr chase period, the cells were exposed to 500 units/ml IFN-α plus 10 units/ml IFN-γ for 24–48 hr. FUra (1 μM) was added concurrently with IFN or after a 24-hr preincubation. At the indicated times, adherent and nonadherent cells were collected and lysed under nondeproteinizing, non-DNA-denaturing conditions on Gelman Metricel membranes as described in the text. DNA fragmentation was determined as the percentage of [^{14}C]DNA in the lysis fraction divided by total cellular [^{14}C]DNA. Data, presented as either mean ± half-range (24-hr IFN-α + γ plus FUra, two experiments) or mean ± standard error (remaining conditions, five experiments), are expressed as the percent fragmented DNA. The results for IFN-α + γ for 48-hr with or without FUra were significantly different from control ($p < 0.001$, rank sum test).

minished viability in at least an additive fashion. The observation that FUra RNA incorporation was decreased by IFN-α + γ argues against a primary role for RNA-directed cytotoxicity. The following data support the contention that DNA-directed effects were important. The combination of FUra and IFN-α + γ produced significantly greater depletion of dTTP compared with either condition alone. FUra and IFN-α

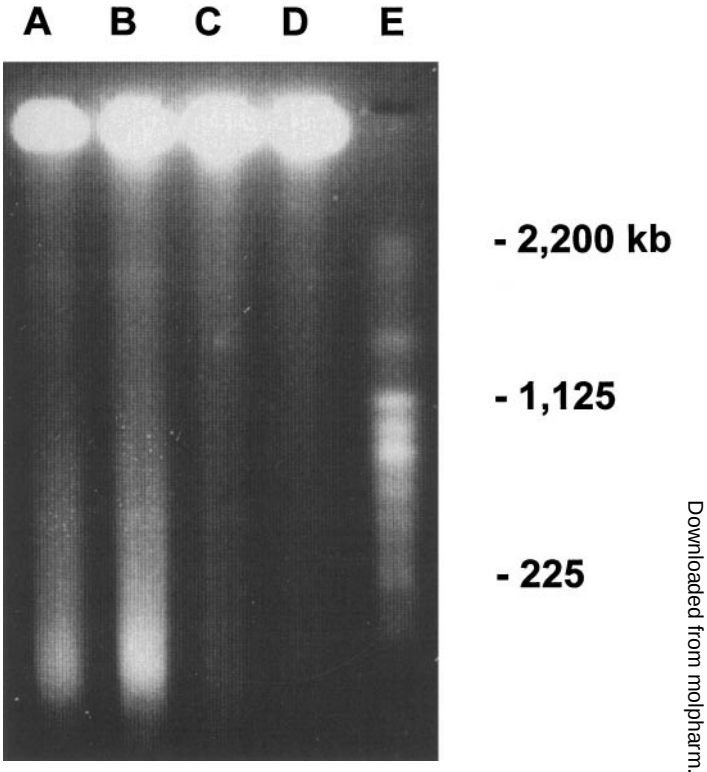

Fig. 7. Induction of high-molecular-weight DNA fragmentation by IFN-α + γ. Control cells and those treated with IFN-α (500 units/ml) and IFN-γ (10 units/ml) (hr 0–48), 1 μM FUra (hr 24–48), or the combination were embedded in agarose, digested *in situ*, and subjected to pulsed-field gel electrophoresis. *Lane E*, *Saccharomyces cerevisiae* DNA. *Lane D*, control. *Lane C*, FUra alone. *Lane B*, Fura plus IFN-α + γ. *Lane A*, IFN-α + γ. Similar results were obtained in different experiments.

+ γ individually interfered with elongation of nascent DNA replication intermediates as demonstrated by the pH step alkaline elution experiments, and the addition of IFN-α + γ before FUra exposure significantly magnified this effect. An increased proportion of single-strand breaks in newly synthesized DNA also was noted with the triple combination during fixed pH alkaline elution. Double-strand breaks in parental DNA were detected with IFN-α + γ using a filter binding assay, although FUra did not seem to intensify this effect. With pulsed-field gel electrophoresis, high-molecular-weight DNA damage was not seen immediately after a 24-hr exposure to FUra, but a 48-hr exposure to IFN-α + γ alone and with FUra produced DNA fragments in the 50–300-kb size range. Under these conditions, however, classic oligonucleosomal laddering was not detected. Induction of high-molecular-weight DNA fragmentation in the absence of nucleosomal laddering also has been reported in other epithelial cancer cell lines exposed to DNA-damaging agents (Oberhammer *et al.*, 1993; Grem *et al.*, 1995, 1996). The absence of nucleosomal laddering in the HT29 cells may signify that the cells either do not contain or do not activate the calcium- and magnesium-dependent endonuclease associated with oligonucleosomal DNA fragmentation under the experimental conditions used.

The locus of interaction between IFNs α + γ and FUra did not seem to directly involve TS. The magnitude of enzyme inhibition was similar in cells treated with FUra alone or in conjunction with IFN-α, IFN-γ, and IFN-α + γ. Because dTTP pools were depleted further when cells were exposed to

FUra combined with IFN-α + γ, an IFN effect distinct from TS inhibition seems to be implicated. Although IFN has been reported to inhibit thymidine transport and phosphorylation in human lymphoblastoid cells (Gewert *et al.*, 1981, 1983), we found that formation of [^{3}H]thymidine nucleotides was similar among the treatment groups in the present studies, indicating that thymidine salvage was not perturbed. Ribonucleotide triphosphate pools were comparable in control and cells treated with IFN-α + γ alone, suggesting that IFN-α + γ did not interfere with synthesis of purine and pyrimidine triphosphates. Ribonucleotide triphosphate pools were expanded by ~1.4-fold in FUra-treated cells, and the addition of IFN-α + γ seemed to negate this effect. dATP pools also were expanded by ~1.4-fold in cells treated with FUra alone or with IFN-α + γ; in concert with dTTP depletion, a pronounced increase in the ratio of dATP to dTTP was evident compared with control.

Immunoreactive dUMP and dUTP pools were increased by 124- and 30-fold, respectively, in the presence of FUra alone. HT29 cells are known to contain a relatively low activity of deoxyuridine triphosphatase activity and thus accumulate dUTP during fluoropyrimidine exposure (Canman *et al.*, 1993). The accumulation of dUTP in the current study is consistent with this prior report. The attenuation of dUTP accumulation in FUra-treated cells with the addition of IFN-α + γ was unexpected. Possible explanations include increased degradation of dUTP or, conversely, increased misincorporation of dUTP into DNA. These possibilities will be the subject of future experiments.

The determinants of sensitivity to FUra are multifactorial. The predominant mechanism of FUra action varies from one cell line to another and may even change in a given cell line depending on drug concentration and duration of exposure. Furthermore, the molecular and biochemical phenotype of the cancer cell also influences the sensitivity to an antimetabolite such as FUra. Perhaps it is not surprising that the basis for the interaction between FUra and IFN also has varied in different model systems. IFN-α augmented FUra cytotoxicity in HL-60 promyelocytic leukemia cells, and the degree of potentiation increased with increasing duration of exposure (Elias and Crissman, 1988). A 48-hr preexposure of HL-60 cells to 1000 units/ml IFN-α followed by a 5-hr exposure to 100 μM FUra led to a 10-fold increase in the formation of FdUMP, associated with increased inhibition of TS (Elias and Sandoval, 1989). dUMP pools increased 2–3-fold over control during FUra exposure; although IFN-α alone had no effect on dUMP pools, preexposure to IFN-α reduced FUra-mediated dUMP accumulation to 1.4-fold over control (Elias and Sandoval, 1989), which is in keeping with our findings. In a different model, concurrent exposure to IFN-α and FUra led to a 2-fold increase in FdUMP levels due to an IFN-mediated increase in thymidine phosphorylase activity (Schwartz *et al.*, 1994). Up-regulation of the activities of thymidine phosphorylase and uridine phosphorylase by IFN-α and IFN-γ has been confirmed by other investigators (Eda *et al.*, 1993a, 1993b). The reactions mediated by thymidine phosphorylase and uridine phosphorylase are bidirectional. Depending on the relative concentrations of substrate and end-products, the enzymes may favor formation of a particular nucleoside, with simultaneous conversion of the reciprocal nucleoside to its base form (El Khouni *et al.*, 1993). It therefore is possible that stimulation of thymidine phosphorylase activity might in part account for increased degradation of thymidine and deoxyuridine nucleotides in the current model.

The potentiation of FUra toxicity by IFN has not always been attributed to IFN-mediated changes in FdUMP pools. Resistance of NCI-H630 colon cancer cells to FUra was accounted for by a several-fold increase in TS content during FUra exposure, with partial recovery of functional TS (Chu *et al.*, 1991, 1993). Although IFN-γ had no effect on FUra metabolism, it abrogated the increase in TS content induced by FUra, thus resulting in enhanced inhibition of TS and augmentation of FUra cytotoxicity (Chu *et al.*, 1991). In GC3/cl human colon cancer cells, in contrast, the locus of interaction between IFN-α and FUra seemed to be at the level of parental DNA damage, with no effect on FUra metabolism or the extent of TS inhibition (Houghton *et al.*, 1993). Wadler *et al.* (1996) reported that although a 12-hr exposure to 500 units/ml IFN-α had no effect on dTTP pools in two different colon cancer cell lines, it produced a significant enhancement of dTTP depletion mediated by 100 μM FUra. Because TS activity was not measured in that study, however, the basis for the increased dTTP depletion was not explained. In our study, dTTP depletion also was significantly greater with the combination of IFN-α + γ and FUra, although the effect could not be attributed to greater TS inhibition.

The contribution of DNA damage to FUra-mediated cytotoxicity varies among different malignant lines, and effects on both nascent DNA and parental DNA have been described (Yoshioka *et al.*, 1987; Grem *et al.*, 1989; Canman *et al.*, 1991; Houghton *et al.*, 1995). DNA damage may result from several factors. dTTP depletion leads to interference with DNA synthesis and incomplete DNA repair. Concomitant dATP accumulation may accentuate the deoxyribonucleotide imbalance. An increase in the (F)dUTP/dTTP ratio favors incorporation of fraudulent nucleotides into DNA. Hydrolysis of the uracil- and FUra-deoxyribose glycosyl bond of dUMP and FdUMP residues present in DNA is catalyzed by uracil-DNA-glycosylase. Endonucleolytic cleavage of the base-free deoxyribose site results in a strand break, which subsequently is repaired. Human uracil-DNA-glycosylase has a much higher affinity for dUMP than for FdUMP, which leads to a slower release of FdUMP residues from DNA (Mauro *et al.*, 1993). Genotoxic stress may trigger programmed cell death pathways, resulting in double-stranded DNA fragmentation in parental DNA. In the current study, nascent DNA seemed to be the primary target of FUra, an observation that pertains to other cancer cell lines (Grem *et al.*, 1989; Jones *et al.*, 1994). The combination of IFN-α + γ seemed to damage both nascent and parental DNA. Although the precise mechanism is not clear, an effect on the content and/or activity of the enzymatic machinery involved in DNA synthesis and repair might provide an explanation. For example, IFN inhibits mammalian DNA polymerases α (involved in DNA replication) and β (involved in DNA repair) (Tanaka *et al.*, 1987; Cataldi *et al.*, 1992).

In summary, our results suggest that profound dTTP depletion, pronounced inhibition of DNA synthesis, and greater damage to nascent and parental DNA contributed to the enhanced toxicity with the combination of IFN-α + γ plus FUra. Because FUra primarily damaged nascent DNA and IFN-α + γ damaged both nascent and parental DNA, these effects seem to be complementary. Because TS activity and

content were similar with FUra alone or with IFN-α + γ, the enhanced dTTP depletion must be due to another mechanism.

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