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EFFECT OF CLINICALLY MODELED REGIMENS ON THE GROWTH RESPONSE AND DEVELOPMENT OF RESISTANCE IN HUMAN COLON CARCINOMA CELL LINES

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Abstract—Two human colon cell lines, HCT-8 and HT-29, were exposed to 5-fluorouracil (FUra) under conditions similar to the human plasma pharmacokinetic profile achieved by a single bolus dose or a sustained i.v. infusion. The bolus treatment for 5 days caused a substantial cell kill; however, only a moderate inhibition in cell growth was obtained with sustained exposure to the clinically relevant level of 2 μM. To achieve a cell kill equivalent to the bolus method, a sustained concentration of 10 μM was required. This would constitute a 60% increase in the total area under the curve (AUC) compared with the bolus treatment. After three courses of therapy with each of the schedules, emerging cell lines displayed a similar degree of resistance. HT-29 resistant cell lines returned to the original sensitivity within a few weeks, and most of the enzymes involved in the metabolic activation of FUra returned to their pretreatment activities. However, resistance and enzymatic modifications remained in the HCT-8 line for at least 3 months. In the HCT-8 cell line derived from bolus treatment, resistance was associated with a 50–60% reduction in uridine kinase activity. In the line derived from continuous exposure, there was a 35–40% reduction in uridine kinase in addition to a greater reduction in the activity of orotate phosphoribosyltransferase. These changes in both resistant cell lines resulted in a decreased incorporation of [^{3}H]FUra into nucleic acids and a reduced formation of di- and triphosphate nucleotides of FUra.

Key words: 5-fluorouracil; cell lines; colon carcinoma; drug resistance; drug administration; enzymes

The limited therapeutic index of FUra† in advanced gastrointestinal cancer, as well as other solid tumors, has encouraged many modifications of the schedule of administration to reduce toxicity and retain antitumor effects. However, the relative merit of rapid i.v. bolus and prolonged i.v. infusion remains unclear. In 1977, Ansfield *et al.* [1] compared four different regimens of FUra. FUra bolus injections, daily for 5 days, showed a response rate of 33% compared with an average of 13% for the other schedules with colorectal tumors. A study by Moertel *et al.* [2] on large bowel cancer reported the same rate of objective response, 12% at 10 weeks for both schedules, and 23 versus 30% of symptomatic response for infusion and rapid injections, respectively. This study used a short-term infusion rather than a prolonged one. In a clinical trial of colorectal adenocarcinomas, Seifert *et al.* [3] observed a 40% objective response rate in patients infused continuously for 120 hr and 23% in patients treated with i.v. bolus. These authors attributed the difference in response rate to unequal distribution between the two groups of patients with different characteristics. More recently, Lokich [4] compared i.v. bolus with a very protracted infusion for about 10 weeks in a randomized trial. Patients receiving infusion therapy responded better than those receiving the bolus dose, but the overall survival was comparable.

However, a very distinct difference in the dose-limiting toxicity has been demonstrated between bolus injection and infusion. Myelosuppression is predominant after i.v. bolus therapy, with stomatitis and diarrhea also being frequent side-effects. Gastrointestinal toxicity, particularly mucositis and stomatitis, is the dose-limiting toxicity with slow i.v. infusion and is often associated with dermatologic toxicity (hand–foot syndrome) [5].

To date, studies attempting to simulate different dosage regimens *in vitro* have been limited. Most have employed either continuous exposure to a range of drug concentrations over a period of several days or exposure to the drug for short periods of time, followed by washout and subsequent determination of the clonogenic potential in soft agar. A recent paper from Bertino and coworkers [6] partially addressed this issue, reporting an impaired polyglutamylation of the folate cofactor for thymidylate synthetase activity in the cell line obtained after continous exposure to FUra and a decreased incorporation into RNA for the subline exposed to a high-dose pulse of FUra. However, the kinetics of FUra disappearance from plasma after an i.v. bolus dose, the most common mode

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† Abbreviations: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluorodeoxyuridine; dUrd, deoxyuridine; DTT, dithiothreitol; OPRTase, orotate phosphoribosyltransferase; and TCA, trichloroacetic acid.

of therapy, was not well-modeled, and the concentrations of FUra utilized during the selection period were well above the clinically achievable levels. To our knowledge, the model employed in this report has no precedent and may create a drug-concentration profile that differentially affects enzymes with high and low affinity for a therapeutic agent in a manner that better approximates the situation in patients.

Similarly, most studies of FUra resistance have been based on exposure of cell lines to increasing concentrations of drugs until a substantial resistance is achieved. In these lines, the anticipated decrease in FUra activation or alteration of the target thymidylate synthase has been reported [7–11]. There are no reports that document biochemical changes associated with FUra concentrations that are within the achievable clinical range, particularly using an exposure protocol that more directly mimics the clinical regimens.

MATERIALS AND METHODS

Chemicals. FUra and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{14}C]FUra (55 μCi/mmol), [^{14}C]FUrd (50 μCi/mmol), [^{14}C]FdUrd (56 μCi/mmol), [^{14}C]dUrd (59 μCi/mmol) and [^{3}H]FUra (26 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). Medium, sera and antibiotics for tissue culture were purchased from the Grand Island Biological Co. (Grand Island, NY). Plasticware was from Corning Glass Works (Corning, NY) and Costar (Cambridge, MA).

Cell lines. The two human colon carcinoma cell lines utilized were HCT-8 [12] and HT-29 [13]. Cultures were maintained as monolayers in RPMI 1640 supplemented with 10% horse serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37° in a 5% CO_2 atmosphere.

Kinetic model of bolus FUra exposure. A stepwise dilution of medium after addition of FUra was timed to approximate clinical clearance kinetics. Cells were seeded in 150 cm flasks at a concentration of 1×10^6 cells/flask. After 20–24 hr when the cells were attached, medium was changed, and 1 mL of 10^{-2} M FUra was added to 19 mL of fresh complete medium to obtain a concentration of 500 μM. Two minutes later, the concentration was diminished to 250 μM by adding 20 mL of fresh medium and maintained for 15 min.

Twenty milliliters of this medium was discarded and replaced with 30 mL of new medium, to achieve a concentration of 100 μM for 30 min. The concentration was reduced further by a similar dilution to 20 μM for 1 hr and 2 μM for 3 hr. Finally, a 1:4 dilution was used to give 0.5 μM for 24 hr.

Development of resistance in HCT-8 and HT-29. Cells were treated with FUra at the indicated concentrations for 5 days by either continuous exposure or the bolus technique (one pulse daily, as described above, for 5 days). These regimens were repeated three times at 14-day intervals for HCT-8 cells and at 21-day intervals for HT-29 cells; in both cases, the intervals corresponded to 18 doubling times.

Between periods of drug exposure, the cells were in drug-free medium to permit recovery from the previous exposure. New cultures were reseeded at 10^6 cells/flask for each successive course. To assess sensitivity to FUra, monolayers of cells in 25 cm^2 flasks were exposed to various concentrations of FUra for 72 hr, the medium was removed, and the cell layer was washed twice with PBS and trypsinized. Appropriate dilutions of cell suspension were counted using a ZB Coulter Counter (Coulter Electronics, Hialeah, FL).

Enzyme analysis. Cell extracts were obtained by sonication in 50 mM Tris–HCl, pH 7.4, 3 mM sodium fluoride and 4 μM DTT, and centrifugation at 100,000 *g* for 60 min. Uridine and thymidine phosphorylase activities were determined by incubating 10–50 μL of cell extract with 2 mM inorganic phosphate, a 20 μM concentration of the appropriate ^{14}C substrate, 2 mM ATP and 2 mM $MgCl_2$ in a final volume of 100 μL. Uridine and thymidine kinase activities were measured by incubating the Tris–HCl cell extract (5–20 μL) with 2 mM ATP, 2 mM $MgCl_2$ and 20 μM [^{14}C]FUrd or [^{14}C]FdUrd in a final volume of 100 μL. OPRTase activity was assayed with 200 μM phosphoribosyl phosphate, 2 mM $MgCl_2$ and 20 μM [^{14}C]FUra. Reactions were initiated by the addition of cell extract and incubated at 37° for 30 min; then 10 μL was removed, applied to TLC plates (silica gel with 60 F 256 fluorescent indicator; Merck), and immediately dried at 80° to stop the reaction. The plates were developed in chloroform:methanol:acetic acid (85:15:5) [14] with appropriate markers and localized by UV. Radioactivity in appropriate regions of the plates was determined in 0.5 mL of methanol and scintillation fluid, using a Beckman liquid scintillation counter.

Thymidylate synthase activity was determined by the method of Roberts [15]. Briefly, 100 μM [^{3}H]deoxyuridylate, 1 mM (±)-L-tetrahydrofolate, 0.9 mM formaldehyde, 62.5 mM 2-mercaptoethanol, 50 mM NaF, 130 mM phosphate buffer, pH 7.5, and extract (5–10 μL) were incubated for 60 min at 37° in a final volume of 40 μL. The reaction was stopped by adding 200 μL of a 10% activated charcoal suspension (Norit) in 4% TCA. After centrifugation, 100 μL of supernatant was counted for radioactivity.

Protein concentration was determined by the method of Bradford [16].

[^{3}H]FUra metabolites and incorporation into nucleic acids. Cells were incubated, as indicated, with 10^{-5} M or 4×10^{-6} M FUra containing [6-^{3}H]-FUra at 2 μCi/mL, washed twice with ice-cold PBS, trypsinized, and treated with 500 μL of ice-cold 5% TCA. After centrifugation, total radioactivity in the supernatant was counted, and the remainder was saved for FUra metabolite determination. The pellet was washed twice with 5% TCA, and incorporated radioactivity was determined after digestion with NCS tissue solubilizer (Amersham, Arlington Heights, IL).

To assay FUra metabolites, TCA cell extracts were neutralized with Freon/trioctylamine extraction and chromatographed, at room temperature, on a 250 × 4.6 mm i.d. Spherisorb SAX 5 μm column, equilibrated with 20 mM sodium phosphate, pH 3.3.

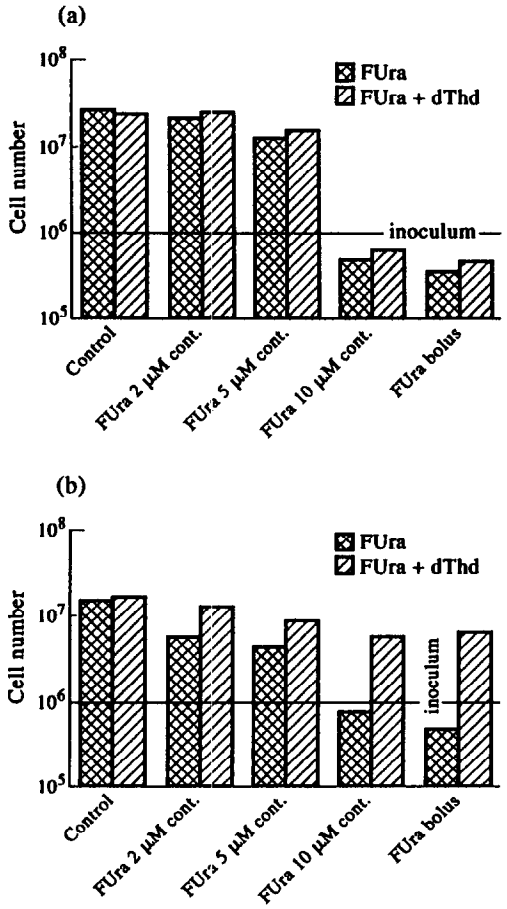

Fig. 1. Effect of different FUra schedules on HCT-8 and HT-29 cell growth. Cells were cultured at an initial concentration of 1×10^6 cells/flask, and treated with different regimens of FUra either with or without 100 μM dThd; the effect on cell growth was determined as described in Materials and Methods. (a) HCT-8; (b) HT-29.

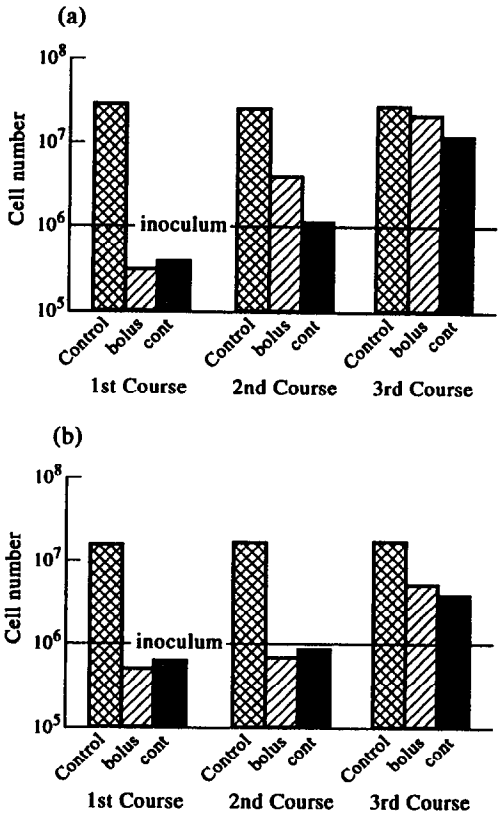

Fig. 2. Development of FUra resistance in HCT-8 and HT-29 cell lines. Cells (1×10^6) were treated with two different schedules of FUra, and at the end of each 5-day course, the number of cells was determined as described in Materials and Methods. (a) HCT-8; (b) HT-29.

The column was eluted with a gradient of sodium phosphate, pH 3.3, from 0.02 to 0.30 M for 40 min at 0.7 mL/min, and the effluent was collected in 1-min fractions. Scintillation fluid was added to determine radioactivity. Unlabeled standards were added to locate FUra nucleotides.

RESULTS

We have used two human colon carcinoma cell lines, HCT-8 and HT-29, to model the response of clinical bolus and i.v. infusion regimens. These cell lines have different sensitivities to FUra (IDC_{50} for HCT-8 = 4×10^{-6} M and for HT-29 = 0.9×10^{-6} M after continuous exposure for 72 hr). The bolus protocol provides a drug concentration profile similar to that obtained in patients after a 600 mg/m^2 dose of FUra [17], and the continuous exposure to a constant concentration of 1–2 μM is in the range produced clinically by continuous i.v. administration of 1100 mg/m^2/day [5].

The daily bolus for 5 days caused 60–70% killing of the inoculum (10^6 cells) of HCT-8 cells. The same cell line treated for 5 days by continuous exposure at a clinically relevant concentration of 2 μM or even at 5 μM was inhibited only minimally in its growth (Fig. 1A). To achieve a cell killing equivalent to the bolus treatment, it was necessary to elevate the continuous exposure concentration to 10 μM. This level would correspond to an area under the curve (AUC) 60% higher than that achieved by the bolus exposure. When a single pulse of FUra was administered on day 1 immediately followed by 4 days of continuous exposure to 2 μM, it caused 20–30% cell kill, with an AUC that was only 45% of the daily bolus treatment. The addition of 10^{-4} M dThd did not prevent FUra cytotoxicity on HCT-8 during either bolus or continuous exposure.

The HT-29 cell line was more sensitive to FUra at 2 and 5 μM but, again, to achieve an equivalent cell kill continuous exposure to 10 μM was required. With this cell line, dThd could partially prevent FUra cytotoxicity (Fig. 1B).

Using the same models of clinical bolus and continuous exposure, we examined the development of FUra-resistant subpopulations in these cell lines.

The parent cell lines, HCT-8 or HT-29, were treated with three courses of FUra, by the daily

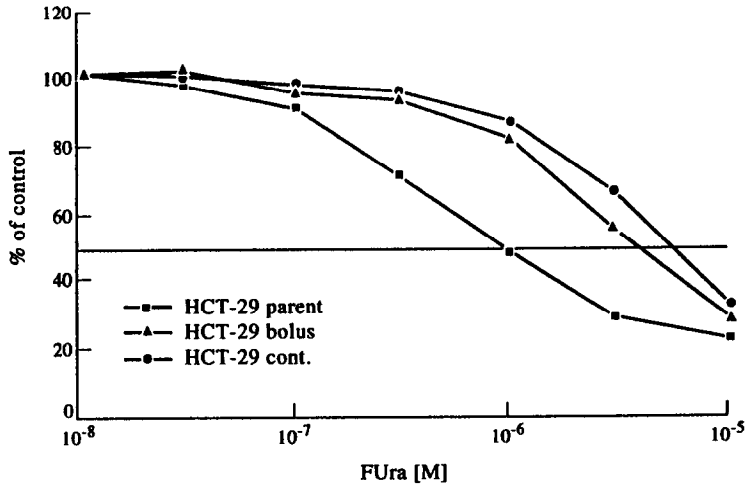

Fig. 3. Inhibitory effect of FUra on proliferation of sensitive and resistant HT-29 lines obtained after three courses of bolus treatment or continuous exposure. Cells (2–4 × 10^5 cells/flask) were exposed at the indicated concentrations of FUra for 72 hr and then counted (see Materials and Methods). Data are from an experiment conducted in triplicate (SD < 10%).

tapered pulse for 5 days or with a continuous exposure to 10 μM FUra at 14- and 21-day intervals. The development and maintenance of resistance was monitored carefully (Fig. 2). In both cell lines, the first course produced about 60% cell kill. The second cycle only caused growth inhibition (75%) in the HCT-8 cultures treated with pulses of FUra and no cell growth with continuous exposure. In the third cycle of treatment of the HT-29 line, sensitivity to both regimens was reduced. After the third course of both regimens with HCT-8, the surviving subpopulations experienced very minimal growth inhibitions.

Three weeks after the last exposure to FUra by either bolus or continuous exposure, HT-29 cells remained about 5 to 7-fold resistant as assessed by growth in the presence of drug for 72 hr (Fig. 3). In HT-29 cells derived from the continuous exposure regimen, all relevant enzyme activities were decreased when compared with the untreated control line, except for thymidylate synthase, which was increased to 130% of the control values (Fig. 4A). The HT-29 population selected after bolus treatment showed a much greater decrease in both kinase activities and thymidine phosphorylase. Thymidylate synthase activity was increased to 160% of control values (Fig. 4B). Enzyme activities in the two HT-29 subpopulations were also assayed 10 weeks after the last exposure at which time the sensitivity to growth inhibition was essentially the same as that of the parent line. Most of the activities returned to control levels. However, OPRTase in both cell lines remained at about 150% of the control, and the increase in thymidylate synthase was maintained.

A sharply different pattern of enzyme activity changes was seen with the HCT-8 subpopulations 3 and 10 weeks after the last exposure to FUra. Three weeks after the last treatment with the EC_{50} of FUra was 2 × 10^{-5} M (Fig. 5), about 4-fold greater than

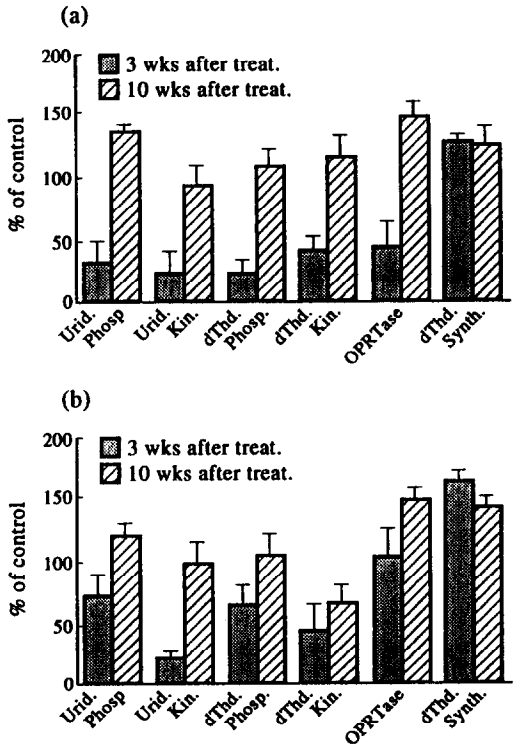

Fig. 4. Alterations in the activities of the enzymes involved in pyrimidine metabolism in HT-29 cells resistant to FUra. Enzyme determinations were conducted 3 and 10 weeks after the end of the last exposure to the drug. Reaction conditions are described in Materials and Methods. (a) Cells derived from continuous exposure. (b) Cells derived from bolus treatment. Results are the means ± SD of two experiments conducted in quadruplicate. See the preceding paper for the enzyme levels in the parental cell lines.

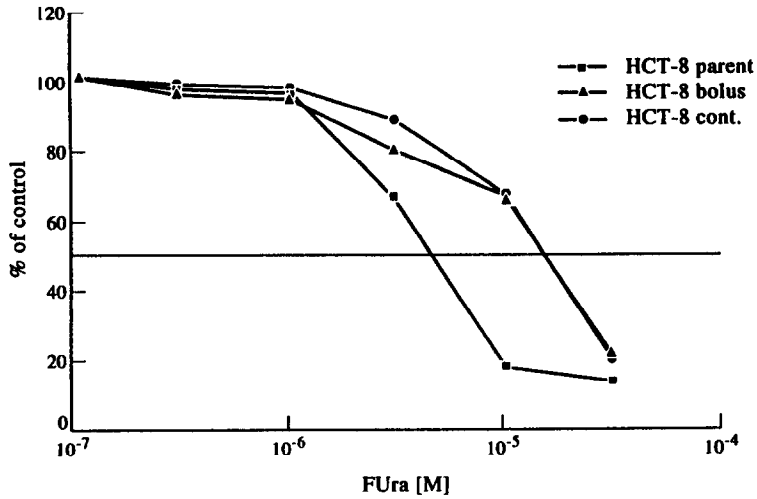

Fig. 5. Growth inhibition on proliferation of sensitive and resistant HCT-8 lines by FUra. Cells (2–4 × 10^5 cells/flask) were exposed at the indicated concentrations of FUra for 72 hr and counted (see Materials and Methods). Data are from a single experiment conducted in triplicate.

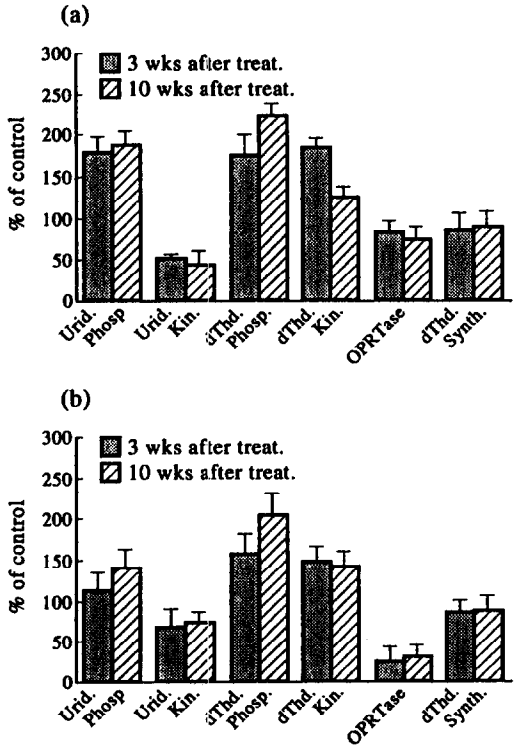

Fig. 6. Alterations of pyrimidine metabolic enzymes involved in resistant HCT-8 cells. Enzyme determinations were conducted 3 and 10 weeks after the last exposure to FUra. Reaction conditions are described in Materials and Methods. (a) Cells derived from bolus treatment. (b) Cells derived from continuous exposure. Results are the means ± SD of two experiments conducted in quadruplicate. See the preceding paper for the enzyme levels in the parental cell lines.

in the parent line. Although both treatments decreased the sensitivity to growth inhibition about 4-fold at 3 weeks, the changes in enzyme activities in the HCT-8 line were quite different from those seen with HT-29. Bolus treatment elevated both phosphorylases and thymidine kinase activities, while uridine kinase activity was greatly reduced (Fig. 6A). The subpopulation derived from continuous exposure had elevated thymidine kinase and phosphorylase activity, reduced uridine kinase, and a marked reduction of OPRTase activity. No significant difference was shown for thymidylate synthase (Fig. 6B). Unlike the resistant HT-29 cultures, the two resistant HCT-8s retained the same degree of resistance to growth inhibition 10 weeks after therapy as was seen immediately after drug exposure. Furthermore, the changes in enzyme activities remained substantially the same as those seen at 3 weeks.

To correlate the consequences of these changes in pyrimidine enzyme activities on the activation of FUra, the incorporation of [^{3}H]FUra into acid-soluble nucleotide derivatives and nucleic acids in both HCT-8 sublines was determined and compared with that of the parent cell line at a FUra concentration of 4 μM, the EC_{50} for the parental cells. Incorporation into acid-soluble FUra nucleotides and nucleic acids was reduced by 50–60% in both resistant populations (Fig. 7).

DISCUSSION

Our study demonstrates that the exposure of human colon carcinoma cell lines to clinically relevant and non-escalating concentrations of 5-fluorouracil results in the selection of resistant cell populations after only three cycles of treatment. Our simulation *in vitro* of two common clinical schedules of treatment revealed that both lines were particularly sensitive to "bolus" administration of FUra. To

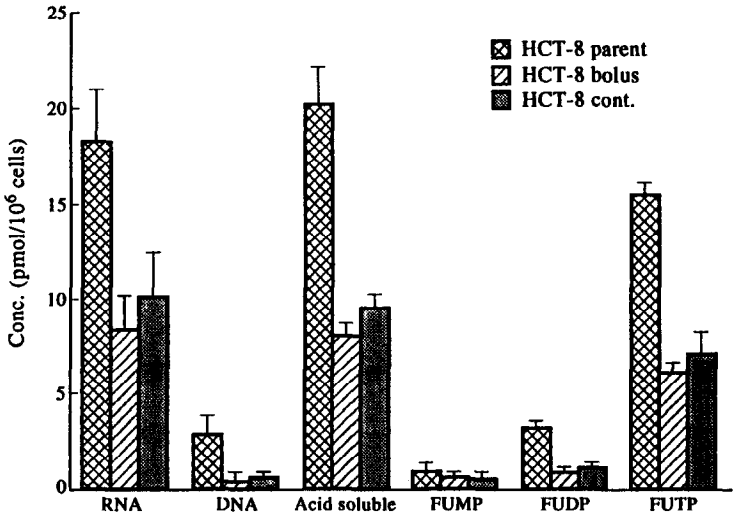

Fig. 7. RNA incorporation and FUra nucleotide content of sensitive and resistant HCT-8 cell lines. Cells were incubated for 4 hr with 4 μM [^{3}H]FUra, and then were washed with ice-cold PBS; next 10% TCA was added to the monolayer. The cell extract was analyzed as described in Materials and Methods. Results are the means ± SD of at least two experiments conducted in duplicat.

cause extensive cell kill by continuous infusion, the concentration of FUra had to be increased to 10 μM, concentrations that are 5 to 10-fold higher than the usual tolerated plasma concentration in the clinic.

The bolus and continuous exposure protocols also caused a somewhat different evolution of resistance. Even though this very limited selective pressure induced only 4- to 6-fold resistance, the procedure is much more likely to uncover mechanisms of resistance encountered in human therapy than increasing concentrations of drug with many passages in the presence of drug.

After continuous exposure, both HT-29 and HCT-8 resistant cell populations exhibited major reduction of ORPTase, the primary enzyme responsible for the metabolism of FUra, showing probably a different mechanism of activation related to each schedule. An elevation of thymidylate synthase occurred in both HT-29 subpopulations and was maintained even after the two cell lines were no longer resistant to FUra. This alteration suggests that thymidylate synthase represents a principal target for FUra chemotherapy in HT-29 cells (see preceding paper). However, the increased enzyme level, retained even after the cell lines lost resistance to growth inhibition, was not able, in itself, to alter significantly the sensitivity to FUra.

The HCT-8 cell line, though intrinsically less sensitive to FUra, acquired only a 4-fold resistance to FUra, but this change was maintained even 4 months after the last exposure to drug. Unlike the HT-29 sublines, both resistant subpopulations of HCT-8 showed an altered pattern of enzyme activities. The decrease in uridine kinase, the enzyme involved in the phosphorylation of fluorouridine, correlated with the decreased incorporation of [^{3}H]-FUra into nucleotides and nucleic acids. The even greater reductions in the concentrations of FUDP and FUTP suggest a more specific reduction of pyrimidine nucleoside monophosphate kinase activity, as described for a P388 FUra-resistant mutant by Ardalan *et al.* [18].

The HCT-8 cell line selected by continuous exposure to FUra presented a decrease in both OPRTase and uridine kinase activities; particularly dramatic was the effect on OPRTase. Also in this case, FUra incorporation into the formation of nucleic acids and nucleotides was compromised. When we compare the effect on nucleotide synthesis in this cell line with that in the other mutant, where only uridine kinase was affected, it seems unclear what role OPRTase plays in FUra activation.

The capability of thymidine to rescue the two cell lines from FUra cytotoxicity further emphasizes the differences in the activation of FUra and the target for cell kill as discussed in the preceding paper. Though thymidine was incapable of rescuing HCT-8 cells but was able to protect the HT-29 line, no differences were apparent after exposure to different regimens. The schedules of administration may determine the mode of activation for FUra.

The modest but significant resistance achieved with these two treatments and the rapid emergence within the first three cycles of treatment further emphasize the dramatic importance of this problem in the management of cancer patients. These data appear to model directly the clinical circumstance in which an intolerable increase in the dose of chemotherapy would be needed to achieve the same cytotoxic effect after the first few courses of therapy.

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