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LOSS OF MURINE TUMOR THYMIDINE KINASE ACTIVITY *IN VIVO* FOLLOWING 5-FLUOROURACIL (FUra) TREATMENT BY INCORPORATION OF FUra INTO RNA

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Abstract—The effects of 5-fluorouracil (FUra) treatment on thymidine kinase (TKase) activity were examined *in vivo* in $CD8F_1$ mice bearing first generation $CD8F_1$ mouse mammary tumors. TKase activity was not affected by low dose $FUra_{25}$ (25 mg/kg), a dose which substantially inhibited thymidylate synthase (TSase), but was severely inhibited 24 hr following treatment with $FUra_{100}$, a weekly maximally tolerated dose, as judged by activity measurements and labeling of DNA with [^{3}H]thymidine. The amount of (FU)RNA was increased markedly with increasing FUra dose from 0.4 nmol/mg DNA at $FUra_{25}$ to 2.2 nmol/mg DNA at $FUra_{100}$. At $FUra_{100}$, TKase activity gradually declined over 24 hr to < 10% of the control value, remained low for a further 48 hr, and then was gradually restored to control levels by 168 hr. The loss of TKase activity followed the incorporation of FUra into RNA which peaked at 4–5 hr. TKase activity was not restored by removal of endogenous inhibitors but was restored by treatment with uridine. TKase activity was not inhibited by therapeutic levels of methotrexate (300 mg/kg). TKase from murine colon 38 carcinoma was also severely inhibited, but the activity from colon 26 was only partially (50%) inhibited. Ornithine decarboxylase was also inhibited by $FUra_{100}$ treatment in the $CD8F_1$ tumor. These results demonstrate that certain short-lived, proliferation-related enzymes are affected by FUra doses higher than those required for TSase inhibition, and this effect appears to correlate with incorporation of FUra into RNA. Thus, in some tumors high doses of FUra can inhibit salvage as well as *de novo* synthesis of thymidylate providing an increased block of DNA synthesis and increased therapeutic advantage.

5-Fluorouracil (FUra†) has been widely used as an antitumor agent and is currently used in the treatment of cancers of the colon, breast and lung. FUra produces antitumor effects by at least two mechanisms; inhibition of thymidylate synthase (TSase) and/or incorporation of FUra into RNA, (FU)RNA. FdUMP, a metabolite of FUra, provides potent inhibition of TSase and inhibits *de novo* formation of thymidylate and DNA synthesis. Where both parameters have been measured, it appears that TSase is inhibited at lower concentrations of FUra whereas the (FU)RNA effect(s) occurs at higher doses of FUra [1–7]. In contrast to the discrete effects of TSase inhibition by FdUMP, the mechanism of antitumor activity via incorporation of FUra into RNA has not been studied as extensively due to the lack of specific measureable effects. However, with recent advances in understanding the mechanisms involved in the transcription and processing of RNA, the effects of (FU)RNA are being explored in greater detail [8, 9]. The effects of FUra incorporation into various RNA species have been reviewed recently [7].

The $CD8F_1$ mouse mammary tumor is a model in which the increased antitumor effects of FUra correlate with incorporation of FUra into RNA. Using this model, our group previously reported [10–12] the inhibition of thymidine kinase (TKase) activity *in vivo* in mice treated with FUra; however, the basis for this inhibition was not established.

In the present report we have investigated the time courses of incorporation of FUra into RNA and depletion of TKase activity. We present data which are consistent with TKase activity being specifically depleted by incorporation of FUra into RNA.

MATERIALS AND METHODS

Materials. [5-C^3H_3]Thymidine (63 Ci/mmol), [6-^{3}H]deoxyuridine (20 Ci/mmol), [6-^{3}H]fluorouracil (20 Ci/mmol), [1-^{14}C]ornithine (56 mCi/mmol) and [5-^{3}H]deoxyuridine monophosphate (20 Ci/mmol) were purchased from Moravek. NAP-25 columns were purchased from Pharmacia.

Animals. $CD8F_1$ mice bearing first-generation subcutaneous transplants of the $CD8F_1$ spontaneous murine mammary carcinoma were used [13, 14]. C26 or C38 colon carcinoma were grown by subcutaneous

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† Abbreviations: FUra, 5-fluorouracil; FUdR, 5-fluorodeoxyuridine; (FU)RNA, 5-fluorouracil incorporated into RNA; dThd, thymidine; dUrd, deoxyuridine; Urd, uridine; TSase, thymidylate synthase (EC 2.1.1.45); TKase, thymidine kinase (EC 2.7.1.21); ODCase, ornithine decarboxylase (EC 4.1.1.17); DFMO, α-difluoromethylornithine; and MTD, maximum tolerated dose (the dose yielding an LD_{10} over 3–4 weekly courses of treatment).

implantation into BALB/c or C57BL mice, respectively. Food and water were consumed *ad lib.* All drugs were prepared in 0.85% NaCl solution and were injected intraperitoneally. $FUra_{100}$ (100 mg/kg) is the weekly maximum tolerated dose (MTD) in this murine tumor-host system.

Enzyme assays. Tissues were homogenized (Potter–Elvehjem homogenizer) as a 20% (w/v) solution in Tris–Cl (100 mM, pH 7.6), 2-mercaptoethanol (20 mM) and sodium fluoride (100 mM) for TSase and TKase or Tris–Cl (25 mM, pH 7.5), EDTA (0.1 mM) and dithiothreitol (2.5 mM) for ornithine decarboxylase (ODCase). Homogenates were centrifuged at 4° (100,000 *g*, 60 min or 10,000 *g*, 30 min) and the supernatant fractions retained on ice. Enzyme assays were performed either on individual samples or on pooled tissues from three animals. Tumors were between 300 and 500 mg. Thymidine kinase was measured immediately after cytosol preparation by means of a DE81-filter-binding assay [15]. The assay mixture contained Tris–Cl (100 mM, pH 7.6), ATP (5 mM), $MgCl_2$ (1 mM), [5-C^3H_3]thymidine (25 μM, 1.0 Ci/mmol) and cytosolic protein in a total volume of 50 μL. Ornithine decarboxylase activity was measured by the release of $^{14}CO_2$ from L-[1-^{14}C]-ornithine as described by Seely and Pegg [16]. Assay mixtures consisted of 150 μL of cytosolic fraction, pyridoxal phosphate (0.25 mM), [1-^{14}C]ornithine (400 μM; 3 mCi/mmol) in a total volume of 400 μL. Thymidylate synthase activity was measured by the release of tritium from [5-^{3}H]deoxyuridine [17, 18]. Reaction mixtures (50 μL) contained [5-^{3}H]dUMP (10 μM, 1.0 Ci/mmol), CH_2-H_4PteGlu (100 μM) and cytosolic fraction (25 μL). Reactions were terminated by the addition of perchloric acid (10 μL, 0.7 M). Protein was determined by the method of Lowry *et al.* [19].

Removal of small molecules from the cytosolic fraction. NAP-25 columns were washed with 20 vol. of homogenization buffer. The cytosolic fraction was applied and the protein was collected in 0.5-mL fractions. In a trial run, the columns effectively separated a tracer amount of [6-^{3}H]deoxyuridine from the protein fraction.

Incorporation of precursors into RNA and DNA. Radioactive precursors were injected intraperitoneally in saline. [6-^{3}H]FUra (1 mCi/mmol) was used to measure the time course of incorporation into nucleic acid. Tissues were first homogenized in TNE buffer: Tris–Cl (10 mM, pH 7.6), NaCl (150 mM), EDTA (1 mM). The homogenate was treated with sodium dodecyl sulfate (SDS), sonicated and digested with Pronase for 60 min at 37° (20 μg/mL; predigested for 2 hr at 37°), and extracted with chloroform/isoamyl alcohol (24:1, v/v). Nucleic acid was precipitated with 10% trichloroacetic acid (TCA) to determine total incorporated radioactivity. Other samples were first treated with alkali (0.4 M NaOH, for 90 min at 37°) to determine alkali-stable acid-precipitable activity. DNA was measured by the diphenylamine color reaction [20]. The difference between the total and alkali-stable radioactivity was assumed to represent radioactivity in RNA. Insoluble material was filtered onto GF-C filters that were then washed twice with ice-cold 5% TCA and heated for 30 min at 50° in a scintillation vial with 1 mL of BCS scintillation fluid (Amersham)/1 M hyamine hydroxide in MeOH (ICN) (9:1, v/v). BCS fluid was added (10 mL) and the radioactivity was determined.

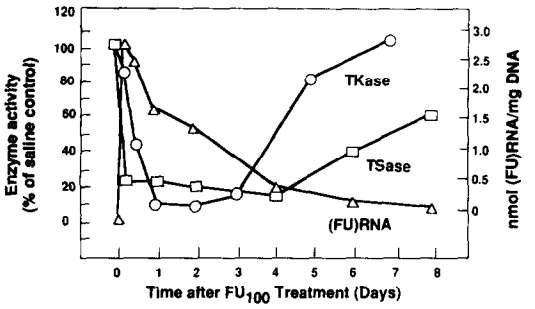

Fig. 1. Comparison of the *in vivo* incorporation of FUra into RNA, the inhibition of TSase activity, and the depletion of TKase activity over time. $CD8F_1$ mice bearing mammary tumors were injected i.p. with 100 mg/kg of FUra for determination of TKase and TSase activities. A separate group of mice was injected with 100 mg/kg FUra (1.0 mCi/mmol) for determination of (FU)RNA. Each determination is the value from three pooled tumors. Control values for TSase and TKase were 21.0 and 27.4 pmol/min/mg protein, respectively.

The rate of DNA synthesis via the *de novo* pathway (thymidylate synthase) or the salvage pathway (thymidine kinase) was measured by intraperitoneal injection of [^{3}H]dUrd or [^{3}H]dThd at 1.5 μCi/g of body weight and observation of the incorporation of radioactivity into the alkaline-stable acid-precipitable fraction as described above.

RESULTS

Comparison of the time courses of (FU)RNA, thymidylate synthase inhibition and thymidine kinase depletion (Fig. 1). After injection of 5-[6-^{3}H]-fluorouracil (1 mCi/mmol) at 100 mg/kg, the time course of incorporation and disappearance of (FU)RNA was observed. Incorporation was gradual with a peak at 4–5 hr followed by a decrease to 60% of the peak value by 24 hr. From 24 to 96 hr the decrease in RNA-associated radioactivity was more gradual with residual counts lasting up to 8 days. The activity of TKase was reduced to less than 10% of the control value over 24 hr by a single bolus treatment with $FUra_{100}$, was inhibited a further 48 hr, and was then gradually restored to control values by 168 hr. In contrast, the activity of TSase was inhibited at the earliest time point (6 hr) by $FUra_{100}$ and this level of inhibition persisted for 4 days with a gradual restoration of activity up to 60% of the control value by day 8.

TKase, TSase activities and DNA synthesis versus FUra dose. Twenty-four hours after treatment, a low (25 mg/kg) dose of FUra inhibited TSase activity 77% but had little effect on TKase activity (Fig. 2A). At the maximum tolerated dose (MTD) of $FUra_{100}$, however, TSase was inhibited a further 5% and, in addition, TKase was also inhibited 90%. For

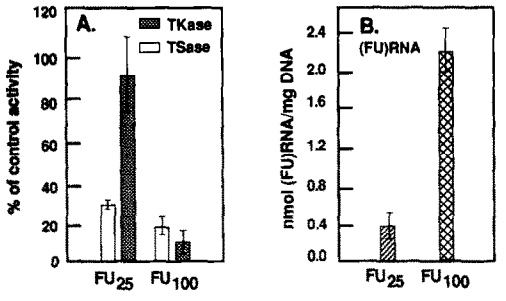

Fig. 2. Effect of i.p. $FUra_{25}$ or $FUra_{100}$ on TSase and TKase activities and (FU)RNA in $CD8F_1$ mammary tumors. (A) TKase and TSase activities 24 hr after treatment with FUra. Saline-treated control activities (±SD), expressed in pmol/min/mg protein, were 25.6 ± 7.1 for TSase (N = 4) and 20.0 ± 3.7 for TKase (N = 4). (B) Incorporation of FUra into RNA 6 hr after treatment with [6-^{3}H]FUra (1.0 mCi/mmol) (N = 5). Values are means ± SD.

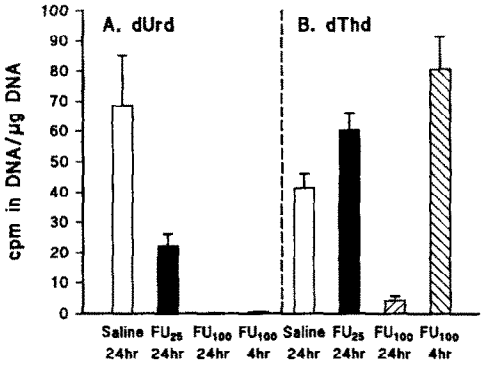

Fig. 3. Incorporation of tracer amounts of [^{3}H]dUrd or [^{3}H]dThd into breast tumor DNA after i.p. $FUra_{25}$ or $FUra_{100}$ treatment. At the indicated times after FUra treatment mice were injected with 1.5 μCi/g of body weight of (A) [6-^{3}H]deoxyuridine (20 Ci/mmol) or (B) [5-C^3H_3] thymidine (63 Ci/mmol). The labeling period was 4 hr. Each value is the mean of three determinations ± SD.

these same two doses of FUra, the amount of (FU)RNA (Fig. 2B) was considerably higher at $FUra_{100}$ (2.2 nmol/mg DNA) than at $FUra_{25}$ (0.4 nmol/mg DNA).

DNA labeling with [^{3}H]thymidine or [^{3}H]-deoxyuridine was examined to determine the extent of DNA synthesis through either the salvage pathway, TKase, or through the *de novo* pathway, TSase, under conditions where TSase alone was inhibited or where both TSase and TKase were inhibited (Fig. 3). Although TSase activity was inhibited substantially by $FUra_{25}$, DNA synthesis occurred at 32% of the control rate (Fig. 3A), and the flux through the salvage pathway was increased (Fig. 3B). At $FUra_{100}$, TSase activity was inhibited completely at the 24-hr time point and DNA synthesis via the salvage pathway was also inhibited > 90% leading to a double block of DNA synthesis. At the earlier time point of 4 hr, however, incorporation

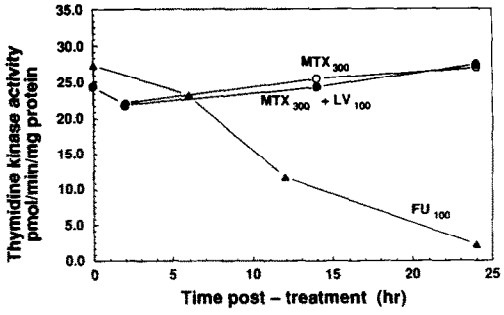

Fig. 4. Comparative effects on thymidine kinase activity after treatment with MTX_{300}, MTX_{300} + LV_{100} or $FUra_{100}$. The MTX and FUra results are from separate $CD8F_1$ breast tumor transplants. Each time point is the value from three pooled tumors.

via TSase was inhibited completely but the flux through TKase was increased to 207% of the control value.

Lack of reversal of FUra-mediated TKase inhibition by removal of inhibitors. 5-Fluorodeoxyuridine (FUdR) is the most potent inhibitor of thymidine kinase (K_i = 22 μM) [21] among the FUra metabolites and competes with thymidine (K_m = 3 μM) as an alternative substrate for thymidine kinase. The possibility that FUdR or a similar compound may be causing TKase inhibition was eliminated by separating the TKase activity of a FUra-treated homogenate (three pooled tumors) by means of a sizing column. TKase activity from the separated fraction was compared with that of an unseparated fraction. The TKase activity from the unseparated fraction was 6.8% of the control value of 39.8 pmol/min/mg protein and that of the separated fraction was 7.8%. In addition, when a heat-treated extract (100°, 60 sec) from FUra-treated tumors was mixed with a saline control extract, no inhibition of the normal enzyme activity was observed.

Effect of methotrexate on TKase activity. TKase activity is tightly regulated during the cell cycle [22]. To attribute the depletion of TKase to (FU)RNA, it was necessary to determine whether other inhibitors of DNA synthesis would cause a similar effect on the activity of TKase in the $CD8F_1$ tumor. Accordingly, the effect on TKase after treatment with MTX was evaluated (Fig. 4). MTX at a dose of 300 mg/kg had no effect on TKase activity up to the termination of the experiment at 24 hr. A chemotherapy experiment was conducted in which mice received four weekly courses of MTX_{300}. The toxic effect of this high dose of MTX was mitigated with leucovorin (LV) given at 4.5 and 21 hr after MTX. After four weekly treatments the average tumor size was significantly smaller; 919 mg for treated tumors (N = 7) compared to 3760 mg (N = 6) for the saline-treated control. At this point the tumors of all mice were excised and separately analyzed for TKase activity. The activities were essentially the same for the control group and the MTX/LV group; 14.74 ± 7.51 and 14.90 ± 7.12 pmol/min/mg protein, respectively.

Table 1. Comparative ornithine decarboxylase activities in $CD8F_1$ tumor and various host tissues 24 hr following i.p. treatment with $FUra_{100}$ or MTX_{300}

	ODCase activity (nmol/hr/mg protein)			
	Tumor	Spleen	Kidney	Intestine
Saline	0.321	0.140	3.369	0.032
$FUra_{100}$	0.032 (10.0)*	0.011 (7.6)	0.614 (18.2)	0.025 (76.2)
MTX_{300}	0.123 (38.2)	0.037 (26.3)	5.234 (155.4)	<0.01†
Saline/ DFMO	<0.01	<0.01	<0.01	<0.01

Tissues from three mice were pooled for each determination. Saline-treated preparations were assayed with or without DFMO (2 mM) to determine nonspecific decarboxylation.

* Percent of saline value.

† The limit of detection was 10 pmol/hr/mg protein.

Ornithine decarboxylase activity. Ornithine decarboxylase, a short half-life enzyme encoded by a short half-life message [23], was also assayed in the $CD8F_1$ breast tumor and selected normal host tissues and was found to be inhibited in all tissues examined 24 hr after FUra treatment (Table 1). FU_{100} produced 90.0% inhibition of tumor ODCase activity. Addition of the irreversible ODCase inhibitor α-difluoromethylornithine (DFMO, 2 mM) to control cytosol provided > 98% inhibition compared to the uninhibited activity (321 pmol/hr/mg protein).

MTX_{300} had varying effects on ODCase activity. ODCase was inhibited moderately in spleen and tumor and strongly in gut but the kidney activity was increased markedly (Table 1).

Reversal of FUra-mediated inhibition of TKase by uridine "rescue". Depending on the dose of FUra administered, a delayed uridine "rescue" schedule beginning 2 hr after FUra can control FUra-induced murine host toxicity without reversing the antitumor activity of FUra [24]. Although the precise mechanism of this selective rescue by Urd is not understood, the excess Urd may compete with FUra for incorporation into the RNA of normal tissue to a greater extent than in tumor tissue. Following Urd "rescue" of FUra in tumor-bearing mice, TKase activity was measured in both tumor and spleen (Fig. 5) to determine whether there may be a relationship between restoration of TKase activity and Urd "rescue". At doses of $FUra_{100}$ and $FUra_{225}$, uridine reversed the inhibition of TKase activity. The extent of rescue at $FUra_{100}$ was complete in the spleen (125% of control) but only 63% in the tumor tissue. At $FUra_{225}$ the spleen value was 69% of the control value while the tumor level was 43%.

Effect of FUra treatment on thymidine kinase activity from C26 and C38 colon carcinoma. Treatment of C57BL mice bearing C38 tumors with $FUra_{100}$ inhibited tumor TKase activity by 91.2% at 24 hr. The control value was 15.6 pmol/min/mg protein. However, similar treatment of BALB/c mice bearing C26 colon tumors inhibited tumor TKase only 47.5%. The saline-control activity was 47.8 pmol/min/mg protein. Although this partial inhibition of C26 tumor TKase activity was significant 24 hr after $FUra_{100}$ treatment, the incorporation of

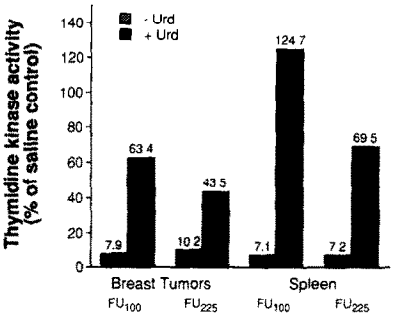

Fig. 5. *In vivo* reversal of FUra-mediated depletion of TKase activity by delayed uridine "rescue". Thymidine kinase activity was measured 24 hr after treatment with FUra. Saline-treated control activities were 37.4 and 334.3 pmol/min/mg protein for tumor and spleen, respectively. Uridine schedule (mg/kg) FUra – – 2 hr-> Urd_{1500} – – 2.5 hr-> Urd_{3500} – – 17 hr-> Urd_{3500} – – 2.5-> sacrifice.

[^{3}H]thymidine into DNA was not inhibited (saline control, 120.6 ± 13.9 cpm/μg DNA, N = 4; $FUra_{100}$, 190.1 ± 52.7 cpm/μg DNA, N = 4).

DISCUSSION

The peak of FUra incorporation into total RNA *in vivo* in $CD8F_1$ mouse mammary tumors occurred at 4–5 hr after FUra administration. This result is similar to that obtained *in vivo* in L1210 leukemia cells after i.p. FUra treatment [25] where a peak of 3 hr was observed. In the present study, the percent replacement of uracil residues in RNA was estimated to be less than 1% after a single bolus dose of $FUra_{100}$ and is in good agreement with other results [7] obtained with mammalian cells both *in vitro* [1] and *in vivo* [26]. However, these measurements are for total RNA and do not distinguish between RNA species. Various RNA species possess differential stability [27] and may incorporate FUra depending on their rate of turn-over. For example, mRNA represents a few percent of total cellular RNA and

the half-lives among different mRNA species vary from several minutes to several days [27], whereas rRNA, the RNA species of greatest abundance, has a half-life of 5 days [7].

It was reasoned that the RNA species most likely to be affected by FUra incorporation would be those in which a substantial proportion of the population would be synthesized during the peak of intracellular FUTP concentration. Although a number of immediate-early gene products related to proliferation have been characterized and their mRNAs can be quantitated by hybridization assays, we chose to investigate certain soluble enzymes. Specifically we investigated thymidine kinase and ornithine decarboxylase activities which are included in a class of short-lived proliferation-related enzyme activities encoded by short half-life mRNAs [28].

Thymidine kinase activity is increased intracellularly in preparation for DNA synthesis and cellular division and has gained much attention due to its role in proliferating tissues and in cell-cycle progression [22]. Recently, Robertson and coworkers [29] measured serum levels of TKase from patients with breast cancer and reported that serum levels reflected the stage of disease, and for patients with systemic breast cancer the TKase level reflected response to therapy.

In the present report, two doses of FUra were investigated and their effects on TSase and TKase were measured. $FUra_{25}$ inhibited TSase activity but had little effect on TKase activity as measured by either enzyme activity or by [^{3}H]thymidine incorporation into DNA. After $FUra_{100}$ treatment TSase was inhibited due to FdUMP formation and, in addition, the activity of TKase was gradually reduced over a period of 24 hr. The latter inhibition lasted for a period of several days.

Since the nadir of TKase activity did not correspond to the time when peak concentrations of FUra metabolites would be expected [6], this loss of TKase activity was considered to be a result of the loss of enzyme activity rather than FUra-metabolite inhibition (e.g. FUdR). This was confirmed when separation of FUra-treated cytosolic protein from endogenous small molecules failed to restore TKase activity. The addition of heat-inactivated cytosol to untreated cytosol also failed to provide inhibition of control TKase activity.

Since thymidine kinase is a cell cycle-dependent enzyme [22], the loss in enzymatic activity might be simply due to the concomitant inhibition of cell proliferation by FUra as an S-phase agent and, therefore, not be a specific effect of (FU)RNA. However, we and others [30] have shown that methotrexate, another inhibitor of DNA synthesis, did not inhibit TKase activity. Further, hydroxyurea at concentrations which inhibited DNA synthesis $> 98\%$ *in vitro* has also been reported to have no adverse effect on TKase activity [31]. Indeed, the accumulation of TKase mRNA and the increase of TKase activity are not inhibited *in vitro* in synchronized cells that are blocked at the G_1-S border by hydroxyurea [31, 32]. Thus, on this correlative basis the FUra-induced inhibition of TKase appears to be a specific effect of (FU)RNA.

Of great significance to the relationship between the loss of TKase and the presence of (FU)RNA (Fig. 2) is the coupling of the reversal of the loss of TKase by uridine "rescue" (Fig. 5) and the known clearance of FUra from RNA by uridine "rescue" [24, 33, 34]. It has long been generally agreed that the DNA-directed effect of FUra could be reversed completely by an exogenous supply of thymidine, unless (a) there was also a FUra-induced RNA-directed effect or (b) the cells were selected for thymidine kinase deficiency [35, 36]. Similarly, it is reasonable that the RNA-directed effects of FUra could be averted by supplementation with Urd at doses sufficient to compete successfully with fluoropyrimidine metabolites for activation, incorporation and maintenance of FUra levels in RNA [35, 36]. Over 14 years ago it was indicated [37, 38] that under certain conditions interference with RNA function by FUra could be as important as inhibition of thymidylate synthase. In these studies both thymidine and uridine were required to completely overcome toxic FUra effects.

Thymidine supplementation is usually effective in restoring DNA synthesis after low dose FUra [1–7]. The administration of uridine can (a) reverse the cytotoxicity of high dose FUra, (b) prevent or reverse any alterations produced by fluoropyrimidines on RNA synthesis, and (c) reverse the effects of incorporation of FUra into RNA (via FUTP) without reversing the inhibition of DNA synthesis [1–7, 36, 37, 39–41]. For example, Wilkinson and Crumley [2] noted in Novikoff hepatoma cells that thymidine prevented death by low dose FUrd, but did not prevent growth inhibition by high dose FUrd. However, Urd had no effect on the inhibition of DNA synthesis but prevented growth inhibition by high dose FUrd. These authors concluded that the effects of FUrd on RNA metabolism contribute significantly to the cytotoxic activity. It must be emphasized that the extent of reversal depends on the relative utilization of pyrimidine salvage and *de novo* pathways by the tissue system, and also depends in particular on the dose of fluoropyrimidine employed, and the time of addition of the pyrimidine in relation to the fluoropyrimidine administration. Recently, the delayed administration of uridine was employed *in vivo* to selectively decrease the systemic toxicity of FUra and safely allow the use of higher doses of FUra with the result that superior antitumor activity was achieved [24, 42]. It is pertinent to the uridine reversal of the loss of TKase (Fig. 5) that this delayed Urd rescue (i.e. 2 hr after FUra administration) markedly reduces the level of FUra in RNA [24, 33, 34]. This rescue effect of Urd supports the concept that the FUra-induced depletion of TKase activity is specifically mediated by (FU)RNA.

The effect of FU_{100} on TKase activity from C38 colon tumor, an FUra-sensitive tumor [43], was similar to the effect on the breast tumor in that TKase activity was severely inhibited after 24 hr. However, at the same dose of FUra the TKase activity of C26 colon tumor, a tumor less sensitive to FUra [43], was inhibited 48% but the rate of [^{3}H]-dThd incorporation into DNA was not inhibited. These results suggest that circumvention of the *de novo* block on DNA synthesis by TKase activity

may be an important determinant in the response of various tumors to FUra.

Although the above data suggest that the loss of TKase (and ODCase) is a result of (FU)RNA, the mechanism by which this occurs is unknown. Inhibition may be a direct effect of incorporation of FUra into TKase mRNA *per se* causing inhibition of pre-mRNA splicing or leading to miscoded and inactive protein. However, inhibition of splicing of RNA requires a high level (> 84%) of uracil replacement by FUra [8]. This level is clearly unattainable in the total RNA of mammalian cells [7]. Whether certain mRNAs which are rapidly synthesized during the peak of FUTP can reach this level of replacement remains to be determined. As for a direct effect due to FUra incorporation into mRNA, when FUra was incorporated into dihydrofolate reductase mRNA *in vitro*, there was little effect on the rate of synthesis of the dihydrofolate reductase protein [9]. However, the protein product showed attenuated binding by polyclonal dihydrofolate reductase antibody and responded differently to binding of and inhibition by methotrexate [9].

There is also support for a secondary effect. Thus, the activity of transcription factors or regulatory proteins involved in the synthesis or stabilization of TKase mRNA could be attenuated by (FU)mRNA. For example, in studies with a T-lymphoma cell line, P1456, which is reversibly growth-inhibited by glucocorticoids, Thompson [44] has demonstrated the disappearance of TKase mRNA and other short half-life messages and has suggested the role of a specific transcription factor which regulates these activities. According to this model the transcription factor is transcribed poorly under optimal conditions and any small negative effect could reduce further the efficiency of transcription resulting in depletion of the gene products under its control. These types of labile regulatory proteins may be effectively attenuated by (FU)RNA and result in the secondary loss of mRNA and protein products.

In summary, the loss of TKase appears to be a specific effect due to (FU)RNA for the following reasons: (1) other DNA synthesis inhibitors (e.g. MTX) did not affect TKase activity; (2) uridine "rescue" restored TKase; (3) the time course of appearance and disappearance of (FU)RNA correlated with the loss and restoration of TKase; (4) the (FU)RNA-induced loss of TKase was found in several FUra-sensitive tumors (e.g. $CD8F_1$ breast, C26 colon and C38 colon); and (5) removal of small-molecular-weight inhibitors after FUra treatment did not restore TKase activity.

This effect of FUra on TKase activity may be one example of a more general effect of (FU)RNA on labile cellular proteins since ODCase, another labile enzyme, was also inhibited by FUra treatment. Although this report has focused on soluble enzymes, it is expected that other labile proteins such as certain transcription factors or regulatory proteins may also be affected by (FU)RNA. Therefore, although the loss of TKase induced by FUra appeared to contribute significantly to the antitumor effect of FUra, this TKase effect should be regarded as contributory rather than as the full effect of high-dose FUra-induced cytotoxicity. The identification of other activities which are affected by (FU)RNA should provide the possibility for designing strategies for the direct modulation of these activities or factors in combination with FUra.

Note added in proof. In collaboration with Drs. K. Keyomarsi and A. B. Pardee of the Dana-Farber Cancer Institute we have confirmed that the level of TKas mRNA from CD8F1 tumors decreased *in vivo* in parallel with the loss of enzyme activity. These results will be published elsewhere.

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