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TUMOR 5-FLUORODEOXYURIDYLATE CONCENTRATION AS A DETERMINANT OF 5-FLUOROURACIL RESPONSE*

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Abstract—The antitumor activity of 5-fluoruoracil (5-FU) for two murine colonic adenocarcinomas was correlated with the concentration and the clearance of the active antimetabolite, 5-fluorodeoxyuridylate (FdUMP). Mice inoculated with a cell suspension of murine colonic adenocarcinomas 38 and 51 were treated with 5-FU (100 mg/kg i.p.) on 3 day post-transplantation. For mice bearing adenocarconoma 38, treatment with 5-FU was associated with a 97 per cent reduction in mean tumor weight a day 30 and a 77 per cent reduction at day 37 of tumor growth. In contrast, mice bearing colonic adenocarcinoma 51, treated with the same dose schedule of 5-FU did not demonstrate a reduction in the rate of tumor growth *in vivo*. Two hr after i.p. injection of 5-FU (100 mg/kg) the intracellular concentration of free FdUMP in the sensitive tumor 38 was 560 fmoles/μg of DNA. The active antimetabolite was maintained at a concentration in excess of 100 fmoles/μg of DNA for 72 hr. In contrast, the 2-hr free FdUMP concentration in the resistant tumor line 51 was 240 fmoles μg of DNA ($P < 0.005$), and a concentration in excess of 100 fmoles/μg of DNA was maintained for only 24 hr. There was no difference in the rate of progressive accumulation of the competitive metabolite, deoxyuridine monophosphate (dUMP), during the first 24 hr of the study.
Two hr after i.p. injection of 5-FU (100 mh/kg), [^{3}H] deoxyuridine ([^{3}H]Udr) incorporation into DNA was reduced in both tumor lines to below 3 per cent of control. However, in the sensitive tumor, adenocarcinoma 38, DNA synthesis was maximally inhibited for 72 hr, compared to 24 hr in the resistant adenocarcinoma 51. The reinitiation of DNA synthesis corresponded to the reduction of free FdUMP concentration to less than 100 fmoles/μg of DNA. There was no linear relationship between the FdUMP/dUMP ratio and [^{3}H]UdR incorporation into DNA in either tumor line. These data demonstrate that the peak tumor FdUMP concentration and the kinetics of its clearance correlated with the responsiveness of the two specific murine tumors to 5-FU. The measure of peak FdUMP level should be tested for its potential clinical application as a means of selecting patients with gastrointestinal and breast cancer to be treated with this agent.

Since its synthesis in 1958 the antimetabolite 5-fluorouracil (5-FU)‡ has played a major role in the treatment of gastrointestinal cancer [1]. However, it is recognized that only 20 per cent of the patients with colonic cancer treated with this agent will achieve an objective response [2]. The selection of patients for therapy with 5-FU remains an exercise in clinical empiricism; there is no simple test that has been accepted as a predictive index for response. It has been demonstrated that FdUMP is the active metabolite of 5-FU, and forms a covalent complex with thymidylate synthetase in the presence of 5,10-CH_2H_4 folate [3]. Thymidylate synthetase catalyzes the methylation of the natural substrate, dUMP, to dTMP for DNA synthesis. The present study was conducted to correlate the kinetics of intracellular 5-FdUMP concentration with the extent and duration of inhibition of DNA synthesis, and with antitumor activity, for two chemically induced murine colonic tumors. The results demonstrate that FdUMP concentration serves as a useful predictive system for determining *in vivo* tumor sensitivity to 5-fluorouracil.

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‡Abbreviations used in the text are as follows: 5-FU, 5-fluorouracil; FdUMP, 5-fluorodeoxyridlyate; dUMP, deoxyuridine monophosphate; UdR, deoxyuridine; and 5,10-CH_2H_4 folate, 5,10-methylenetetrahydrofolate.

MATERIALS AND METHODS

Materials. 5-Fluorouracil, dUMP and tetrahydrofolic acid were obtained from the Sigma Chemical Co., St. Louis, MO. FdUMP (97 per cent pure as measured by u.v. absorption at 268 nm and paper chromatography) was obtained from Terra-Marine Bioresearch, LaJolla, CA. Thymidylate synthetase, prepared from dichloromethotrexate-resistant *Lactobacillus casei* by the method of Crusberg *et al.* [4], was a gift from Dr. Charles Myers, National Institutes of Health, Bethesda, MD. The enzyme preparation formed 5.5 mmoles dTMP/hr/mg of protein at pH 7.0 and 30°. [^{3}H] Deoxyuridine (23 Ci/mmole) and Aquasol counting scintillant, were obtained from the Amersham Searle Corp., Arlington Heights, IL. C57 BL/6 × DBA/2, hereafter called BDF_1, Balb/C and C57 BL/6 mice weighing between 18 and 25 g were obtained from the Hazelton Laboratories, Vienna VA. All mice were held in a controlled temperature environment and allowed water and Purina lab chow *ad lib.* The trans-

planted murine colonic adenocarcinomas 38 and 51 were the kind gift of Dr. D. P. Groswold, Jr., of the Kettering–Myer Laboratories, Southern Research Institute Birmingham, AL [5].

Preparation of stock tumor. Colonic tumor 51 was maintained in Balb/C mice, the host of origin, while chemotherapy trials were conducted in BDF_1 mice. Colonic tumor 38 was carried in C57 BL/6 mice, the host of origin, and chemotherapy trials were conducted in BDF_1 mice. The procedure for the inoculation of tumor was as follows: The tumor, 0.5 cm, was carefully dissected from mice bearing the specific line. The isolated tumor was minced with scissors into small fragments in Earl's solution. The brei was then passed through a No. 40 mesh wire seive in order to remove excess fibrous tissue. Earl's solution was added in quantity sufficient to result in a final concentration of 30 mg of tumor cell suspension in a volume of 0.5 ml. After confirmation of cell viability, a 0.5 ml tumor cell suspension was implanted subcutaneously into the abdominal wall of the mice.

Antitumor activity. Twenty BDF_1 mice each received a subcutaneous inoculation of a 30 mg cell suspension of adenocarcinoma 38. Three days later the mice were divided randomly and ten animals were given a single dose of 5-FU i.p. (100 mg/kg); the remaining 10 animals served as controls. Similarly, 20 BDF_1 mice were inoculated subcutaneously with 30 mg of cell suspension of the adenocarcinoma 51; 3 days after tumor implantation the mice were randomly divided, and ten were treated with a single dose of 5-FU i.p., using the dose schedule described above. Thereafter, all mice were examined twice weekly and the date at which a tumor first became palpable was noted. The perpendicular diameters of the tumor were then measured with the aid of a caliper and were converted to weight by the following formula:

$$\text{tumor weight (mg)} = \frac{a \times b^2}{2}, \; a \text{ and } b \text{ both in mm [6].}$$

Correlation of intracellular FdUMP and dUMP concentrations with [^{3}H] uridine incorporation into DNA. BDF_1 mice bearing 0.5 cm ($\pm$ 0.2) colonic adenocarcinomas 38 or 51 were used in this study. Each animal received 100 mg/kg of 5-FU, i.p., and at selected time intervals after drug administration the mice received 50 μCi [^{3}H]deoxyuridine i.p. After 1 hr of isotope incorporation the mice were killed and the tumor was carefully dissected from the peritoneum; one half of the tissue was placed in 0.5 ml NaF, 1 mM, in order to prevent enzymatic degradation of FdUMP [7]. Thereafter, the tissue was lightly homogenized at 4°, and FdUMP and dUMP were extracted by the method of Nazar *et al.* [8]. The homogenate was suspended in 10 vol. of 1.0 M acetic acid at 0°, freeze-thawed three times with liquid nitrogen, and then centrifuged at 500 *g* for 10 min. The supernatant fraction was saved and the pellet was washed two additional times with an identical volume of 1.0 M acetic acid. The combined supernatant fractions were lyophilized and then reconstituted in 0.5 ml of distilled water. The dUMP concentration was assayed, after incubation of this substrate with *L. casei* thymidylate synthetase, by monitoring the change in absorbance at 340 nm. One unit of enzyme activity was defined as the amount required to synthesize 1 μmole thymidylate/min at 40° in Tris–HCl buffer (pH 7.4.) The free FdUMP was determined spectrophotometrically by the method of Myers *et al.* [9].

DNA was extracted from the remainder of the tumor by a modification of the method of Schneider [10]. To determine the incorporation of [^{3}H]deoxyuridine into DNA, a 0.5 ml aliquot of each final supernatant fraction was added to 10 ml Aquasol and counted in a Searle Mark III liquid scintillation spectrometer, with automatic quench correction and counting efficiency for tritium of 35 per cent. [^{3}H] UdR incorporation was corrected for dUMP pool size by the method of Myers *et al.* [11]. An additional 0.5 ml aliquot of the supernatant fraction was used for measurement of DNA by the method of Burton [12]. The concentration of dUMP was expressed as pmoles/μg of DNA; the FdUMP concentration was specified as fmoles/μg of DNA. The values for FdUMP and for deoxyuridine incorporation at each time point represent the average of three separate experiments, and each experimental determination was performed on pooled tissues from two animals. The individual values from separate experiments were in close agreement; the standard error of the mean was less than 10 per cent during this study.

RESULTS

Antitumor activity. Colonic adenocarcinoma 38 was palpable in nine of ten untreated mice at day 20, but remained undetectable in 5-FU-treated mice at the same period post-implantation (Table 1). By day 30, nine untreated animals demonstrated a measurable mass, with a mean tumor weight of 775 mg with a range of 322–1064 mg. In contrast, only two of ten mice treated with 5-FU demonstrated a measurable mass at day 30. By day 37, the average weight of nine tumors in the untreated control group was 2431 with a range of 956–4220 mg. The mean tumor weight of treated animals at the same time point was 580 mg, with a range of 120–1120 mg ($P < 0.001$). A similar study was conducted using colonic adenocarcinoma 51. The rate of tumor growth was substantially more rapid than that observed with an equal inoculation of adenocarcinoma 38. Tumors were palpable in all animals, treated and control, by day 10. There was no significant difference in tumor weights when control and 5-FU-treated groups were compared at day 20 ($P = 0.26$) (Table 1).

Kinetics of tumor dUMP and FdUMP concentrations and DNA synthesis in colonic adenocarcinoma 38. Two hr after the i.p. administration of 100 mg/kg of 5-FU, the mean *in vivo* tumor FdUMP concentration of mice bearing adenocarcinoma 38 was a maximum of 560 ($\pm$ 110) fmoles/μg of DNA (Fig. 1) The disappearance of the free FdUMP continued and at 72 hr was 120 ($\pm$ 30) fmoles/μg of DNA. The tumor dUMP concentration was increased 60 per cent over the control by 24 hr after treatment; the dUMP concentration then plateaued at 310 ($\pm$ 55) pmoles/μg of DNA during the following 24 hr. This was followed by a further increase by 96 hr to 790 ($\pm$ 90) pmoles/μg of DNA, or 380 per cent of the pretreatment concentration.

The pretreatment DNA synthesis rate, measured as the mean rate of incorporation of [^{3}H]UdR into DNA

Table 1. effect of 5-fluorouracil on the *in vivo* growth of Murine colonic carcinomas 38 and 51*

Tumor	Treatment	Tumor weight† (mg)			
		Day 10	Day 20	Day 30	Day 37
38	Control		210 (110–301)	755 (332–1064)	2431 (956–4220)
	5-FU (100 mg/kg i.p.)				580 (120–1120)
51	Control	129 (96–180)	844 (340–1300)		
	5-FU (100 mg/kg i.p.)	102 (95–110)	708 (150–1150)		

*Groups of ten BDF_1 mice were treated with 5-FU, 100 mg/kg i.p., 3 days post-transplantation of a 30 mg cell suspension of murine colon adenocarcinoma 38 or 51. Two perpendicular diameters of each tumor were measured at the designated days post-implant; the measurements were converted to weight in mg. The effect of 5-FU treatment on tumor growth was compared to untreated mice bearing the comparable colon tumor. With adenocarcinoma 51, 50 per cent of treated and control mice died by days 30–35.

†Numbers in parentheses represent ranges of values.

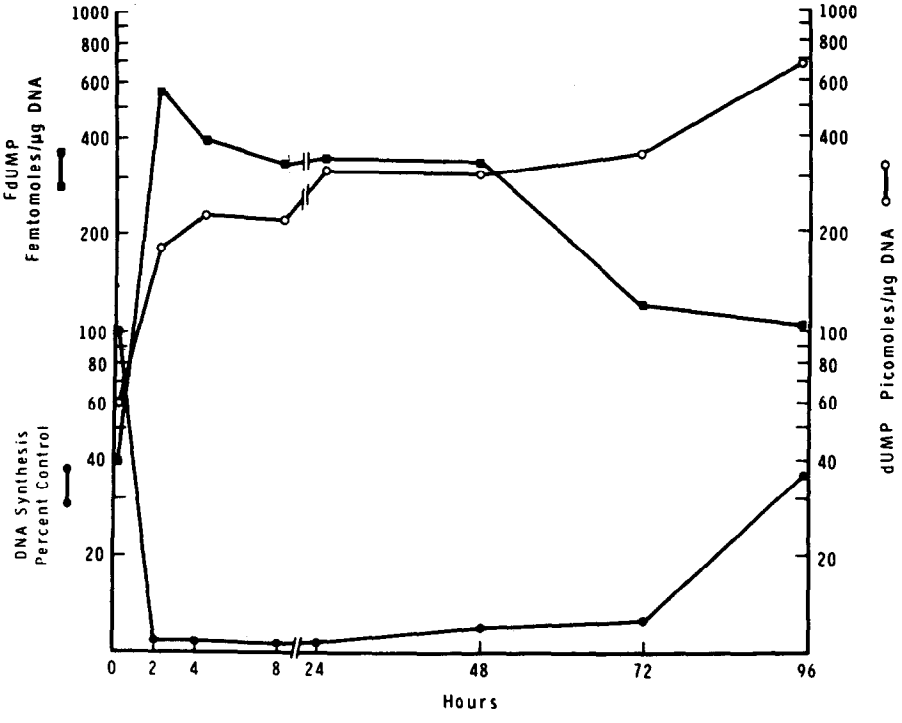

Fig. 1. Correlation between the concentration of FdUMP (■, fmoles/μg of DNA, dUMP (○, pmoles/μg of DNA) and the rate of UdR incorporation (●, per cent control into DNA of the sensitive murine colonic tumor 38, after 100 mg/kg of 5-FU i.p. The values of FdUMP, dUMP and deoxyuridine incorporation for each time point represent the average of three separate experiments, and each experimental determination was performed on pooled tissues from two animals.

in untreated mice, was 20 dis/min/mg of DNA. Within 2 hr after 5-FU treatment, there was 96 per cent reduction in [^{3}H]deoxyuridine uptake into DNA. The incorporation of this precursor into DNA remained below 5 per cent of control for 72 hr, and thereafter rose to 34 per cent of control by 96 hr of study. A dUMP concentration of 310 pmoles/μg of DNA and an FdUMP concentration of 120 fmoles/μg of DNA coincided with the resumption of DNA synthesis, measured as the incorporation of [^{3}H]UdR into DNA.

Colonic adenocarcinoma 51. After the i.p. administration of 100 mg/kg of 5-FU, the mean intracellular free FdUMP concentration in adenocarcinoma 51 reached a maximum of 240 (± 100) fmoles/μg DNA within 2 hr (Fig. 2). This was significantly lower than

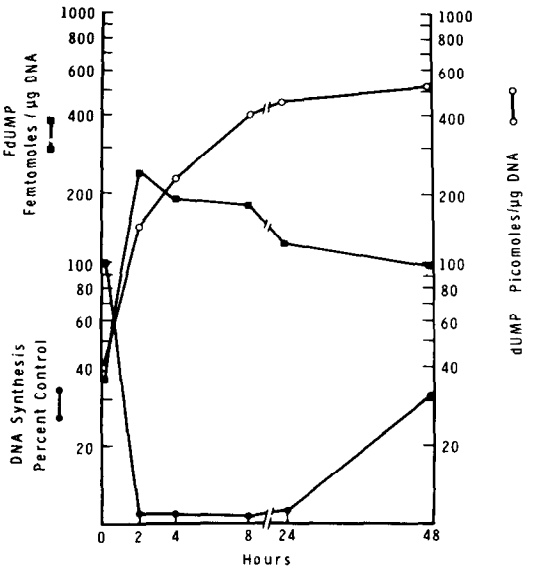

Fig. 2. Correlation between the concentration of FdUMP (■, fmoles/µg of DNA, dUMP (○, pmoles/µg of DNA) and the rate of UdR incorporation (●, per cent control) into DNA of the resistant murine colonic tumor 51, after 100 mg/kg of 5-FU, i.p. The values of FdUMP, dUMP and deoxyuridine incorporation for each time represent the average of three separate experiments, and each experimental determination was performed on pooled tissues from two animals.

that observed in adenocarcinoma 38 ($P < 0.005$), a consistent and reproducible finding in three separate experiments.

The incorporation of [^{3}H]UdR into DNA was reduced to below 5 per cent of control by 2 hr from the pretreatment DNA synthesis rate of 20 dis/min/mg of DNA, and remained depressed for only 24 hr after 5-FU administration. DNA synthesis then increased to 31 per cent of control for 48 hr. Tumor FdUMP and dUMP concentrations at the time of recovery of DNA synthesis were 120 fmoles/µg of DNA and 400 pmoles/µg of DNA respectively.

DISCUSSION

Several investigators have attempted to correlate the intracellular activity of one or more of the essential enzymes for 5-FU activation with objective tumor response to this agent. Kessel *et al.* [13] have reported an *in vivo* correlation between tumor levels of a pyrimidine: phosphoribosyltransferase and its subsequent response to 5-FU, and a similar correlation has been described for human colonic tumors [14]. In the present study we have directly measured the end product of this process of enzymatic activation, FdUMP, and correlated its free intracellular concentration with response.

There was a significant difference between the mean intracellular concentration of FdUMP at 2 hr for 5-FU-sensitive tumor 38 compared with a 2-hr FdUMP concentration for 5-FU-resistant tumor 51 ($P < 0.005$). The rate of clearance of FdUMP within the first 24 hr in the two tumors was not significantly different ($P = 0.30$). However, the total time for which FdUMP remained at concentrations in excess of 100 fmoles/µg of DNA differed significantly. The tumors had a comparable pretreatment DNA synthesis rate (20 dis/min/mg of DNA for tumor 51, vs 22 dis/min/mg of DNA for tumor 38), and in both tumors DNA synthesis was maximally suppressed within 2 hr after 5-FU administration. However, in the sensitive tumor 38 this inhibition persisted for 72 hr, compared to 24 hr for the resistant line. The resumption of DNA synthesis in both tumors was correlated with a reduction of free intracellular FdUMP concentration to less than 100 fmoles/µg of DNA.

The accumulation of dUMP after treatment with 5-FU has been correlated with three independent factors: a decreased utilization as the result of direct inhibition of thymidylate synthetase by FdUMP; the derepression of deoxycytidylate deaminase activity due to decreased dTTP, with a resulting increased conversion of dCMP to dUMP [15]; and a reduced feedback inhibition of both *de novo* pyrimidine synthesis and ribonucleotide reductase activity [16]. Previous work has suggested a marked effect of increased dUMP concentration upon the rate at which FdUMP binds to thymidylate synthetase [11]. This led to the postulate that the recovery from inhibition of thymidylate synthetase was mediated in part by the increased pool of dUMP. Moreover, it was proposed that a linear relationship existed between the FdUMP/dUMP ratio and [^{3}H] UdR incorporation into DNA, supporting the concept that dUMP may have a major influence on the velocity of enzyme inactivation *in vivo*.

In our study of two colonic tumors, there is no linear relationship between FdUMP/dUMP ratio and [^{3}H]UdR incorporation in either tumor line. The present study also failed to demonstrate a significant difference in the course of dUMP accumulation for the two tumors in the first 24 hr of the experiment. Furthermore, there are no confirmatory data to support a role of increased dUMP pool size and its inhibitory effect on the rate of FdUMP binding to thymidylate synthetase. In addition, Klubes *et al.* [17] have reached a conclusion similar to ours in their studies employing the 5-FU-resistant Walker 246 sarcoma and the more sensitive murine leukemia L-1210. DNA synthesis ([^{3}H]UdR/µg of DNA) was maximally inhibited in both tumor lines. However, the duration of suppression differed; a prolonged inhibition of DNA synthesis in L1210 was correlated with the persistence of inhibitory concentrations of FdUMP, whereas the rapid recovery in Walker 256 could be related to the earlier disappearance of the active antimetabolite. Importantly, tumor dUMP concentration did not correlate with the recovery of DNA synthesis in either tumor line.

The difference in conclusions reached in our studies compared to those previously reported by Myers *et al.* [11] may be explained by our use of two histologically similar tumors rather than a comparison of normal and neoplastic tissues. Nevertheless, an expansion of dUMP pool as an independent variable for recovery from 5-FU-inhibited DNA synthesis could not be confirmed. In contrast, the concentration of FdUMP was demonstrated to be the principal determinant of response to 5-FU

It is of interest that the 5-FU-resistant tumor 51 demonstrated a significantly more rapid *in vivo* growth rate than the more sensitive tumor 38. If the

essential variable for response is the tumor cell kinetics, one would have anticipated an enhanced antitumor effect of the 'S' phase active antimetabolite 5-FU in the faster growing adenocarcinoma 51. Clearly, the drug resistance of this tumor must reflect a basic inability to activate 5-FU in sufficient concentration.

The maximum FdUMP concentration and the kinetics of its clearance correlated with tumor responsiveness to 5-FU for the murine models of colon cancer. The measurement of peak FdUMP concentration in tumors after intravenous administration of a therapeutic dose of 5-FU may have direct applicability for the future prospective selection of patients to be treated with this agent. We have, in our clinical studies, demonstrated the feasibility of measurine FdUMP concentration in small biopsy samples after the administration of 5-FU at therapeutic doses. We plan a prospective analysis of the predictive ability of FdUMP concentration for human tumors. Our animal studies suggest that the rate of tumor FdUMP clearance does not differ in the sensitive and the resistant adenocarcinomas, whereas the initial peak concentration of FdUMP is the principal variable. It is possible that a single biopsy will provide predictive information after the time point of maximum FdUMP concentration in human cancer has been determined, The development of such a system would save 80 per cent of patients with gastrointestinal cancer both the cost and toxicity of an ineffective therapy for their specific tumor.

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