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Evaluation of Cytotoxic Agents in Human Colonic Tumor Xenografts and Mouse Gastrointestinal Tissues Using a ^{3}H-Thymidine Fractional Incorporation Assay

J. A. HOUGHTON and P. J. HOUGHTON

Department of Radiopharmacology, Division of Biophysics, Institute of Cancer Research, Sutton, Surrey SM2 5PX United Kingdom

Abstract—*Drug induced changes in ^{3}H-thymidine fractional incorporation (TFI) in 4 human colonic xenograft lines and mouse gastrointestinal tissues have been related to the growth inhibition and host toxicity respectively. Using the TFI assay, the combinations of actinomycin D with cyclophosphamide and methyl CCNU with pentamethyl-melamine have been examined. Both combinations showed greater than additive activity against only 1 of the 3 xenograft lines in which they were tested. The TFI assay allows more rapid evaluation of drug activity and is more sensitive than growth delay studies. It also allows quantitative measurement of cytotoxic activity in gastrointestinal tissues after sublethal dose levels.*

INTRODUCTION

THE DEVELOPMENT of xenografting techniques for the growth of human tumors in congenitally athymic mice [1–3] and immune-deprived mice [4–6] have yielded potentially more relevant models of particular human neoplasms. However, it has become apparent that for xenografts to be of significant value in selecting clinically useful therapy in any particular cancer type, a sufficient number of xenograft lines must be examined in order to encompass at least some of the heterogeneity observed in the clinical disease. Obviously to generate some 20 xenograft lines from colorectal cancers, and use these for screening new and existing single agents, in addition to the evaluation of drug combinations, and schedules, would be extremely time consuming. In previous studies we have characterized 5 human colonic tumors and 1 rectal adenocarcinoma which were maintained in immune deprived mice (T–B$^+$) for up to 10 passages [7–9]. It was shown that these 6 xenograft lines remain characteristic of the original human specimens during maintenance in the murine host. The chemosensitivity of these tumors to a spectrum of clinically used agents has been reported [10]. Each tumor line responds in an individual and unpredictable manner, but the agents most effective against clinical colorectal cancer are the most effective against these xenografts.

A major practical limitation of this colorectal xenograft system is that tumors are relatively slow growing, and within a passage of any line there is a wide variation in growth rates between tumors. The tumor growth rate appears to be at least partially host dictated [8]. Growth delay studies thus tend to be prolonged, and due to the variation in tumor growth rates lack sensitivity in detecting slight antitumor activity. In earlier studies we have suggested that changes in the incorporation of [6-^{3}H]thymidine into tumor DNA may be related to the response to chemotherapy [11, 12] both in murine and human (xenografted) neoplasms. More recently Tew and Taylor [13] have shown that the recovery of [6-^{3}H]thymidine fractional incorporation (TFI) in normal tissues of the rat, following

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Present address: Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, Tennessee 38101, U.S.A.

methotrexate administration, could be correlated with tissue regeneration. In the present paper we have related the drug induced TFI changes in both tumors and mouse gastrointestinal tissues to the antitumor and "antihost" activities in order to develop a more rapid method for evaluating single agents and drug combinations.

MATERIALS AND METHODS

CBA/lac male and female mice were immune-deprived by thymectomy, lethal whole body irradiation and syngeneic bone-marrow transplant, as previously described [12].

Tumor lines

The four human colonic tumor xenografts, used in the study have been described elsewhere [7]. Here, they are listed in decreasing order of differentiation: (1) $HxHC_1$, a moderately well differentiated adenocarcinoma of the ascending colon, maintained only in female mice (passages 7–13 inclusive); (2) $HxGC_3$, a poorly differentiated adenocarcinoma of the transverse colon maintained in male mice (passages 3, 4, 5, 8, 9 and 10); (3) $HxVRC_5$, a poorly differentiated adenocarcinoma of the caecum, maintained in male mice (passages 4 and 5) and (4) $HxELC_2$, a poorly differentiated carcinoma of the caecum, maintained in male mice (passages 4 and 5).

Tumor pieces were implanted subcutaneously (s.c.) in the dorsal flanks so that 4 discrete tumors (of the same line) were maintained in each animal. Caliper measurements of tumors were initiated approximately 3 weeks after tumor implantation. Tumors were treated at approximately 8 mm diameter and growth delay subsequent to cytotoxic agent administration was assessed as the increase in the time required for treated tumors to grow to 4 times the pretreatment volume compared to untreated tumors.

The ^{3}H-thymidine fractional incorporation assay has been described previously [12]. Mice were given 25 μCi thymidine-6-^{3}H (Radiochemical Centre, Amersham, U.K., Specific Activity 27 Ci/mmole) at various times after administration of the cytotoxic agent, and were killed 1 hr later. Tumors and normal tissues of the gastrointestinal tract were rapidly excised and frozen (−26°C) until being extracted using a modified Schmidt–Thannhauser technique [12]. The TFI is given by the formula

$$\text{TFI} = \frac{F_2 \text{ (d.p.m.)}}{F_1 + F_2 \text{ (d.p.m.)}} \times 100$$

where F_1 is the non-incorporated acid soluble radioactivity and F_2 is the radioactivity in the DNA fraction. Tumor TFI results after treatment are expressed as a percent of the TFI in untreated tumors of equivalent weight within the same tumor line. Mouse gastrointestinal tissues were evacuated by gentle squeezing and were subsequently extracted.

Radioactivity was measured by liquid scintillation spectrometry (Intertechnique Ltd. Model SL40) using a mixture of Toluene (containing 0.6% butyl PBD) 7 parts and tergitol TP9 3 parts. Corrections were made for variable quench and efficiency.

RESULTS

TFI changes relative to tumor response

The relationship between the TFI recovery time (i.e., the time taken for TFI to return to the pretreatment level) and the mean growth delay for the same treatment is shown for 3 xenograft lines (Fig. 1). For each line there appears to be a direct relationship between the TFI recovery time and growth delay

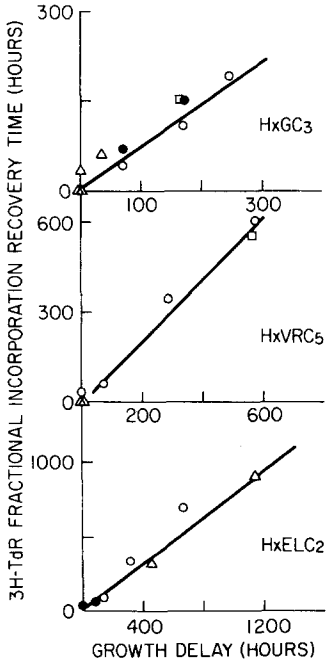

Fig. 1. The TFI recovery time is plotted against the mean growth delay induced in groups of tumors for the same treatment, in 3 xenograft lines. Each symbol type represents a different cytotoxic agent administered at various dose levels. △ 5-fluorouracil; ○ cyclophosphamide; □ methyl CCNU; ● actinomycin D.

induced, independent of the agent used. No growth delay has been measured after administration of agents which do not depress TFI. The changes in TFI with time after drug administration are shown for xenograft line $HxELC_2$ (Fig. 2). Both 5-fluorouracil and cyclophosphamide depress TFI considerably during the first few days after treatment, with a subsequent recovery of TFI to the pretreatment level. The slope of the latter part of the TFI recovery curve is similar to that of the tumor growth curve. Similar results have been obtained with lines $HxVRC_5$ and $HxGC_3$ after cyclophosphamide treatment, and $HxHC_1$ after methyl CCNU. It is worthy of note that even after the highest dose level of 5-fluorouracil (200 mg/kg) no reduction in tumor volume was measured in $HxELC_2$ tumors, although growth was inhibited for about 50 days.

Assessment of drug activity

An estimate of the proportion of cells involved in repopulating a tumor after treatment may be made by backward extrapolation of the regrowth curve to the time of treatment [14]. Alternatively, where it is assumed that regrowth proceeds at the pretreatment rate, the number of tumor volume doubling times that would be expected during the induced growth delay may be calculated. The "repopulating fraction" (P) has been calculated (Table 1) from either backward extrapolation of the regrowth curve, or by substituting into the formula

$$P = \frac{1}{2^{t/c}}$$

where, t, is either the growth delay or the TFI recovery time and, c, is the mean tumor volume doubling time, before treatment. The data in Table 1 represent the most effective single agent therapy examined, to date, against each tumor line.

Combination studies

Methyl CCNU and pentamethylmelamine. The activities of methyl CCNU and pentamethylmelamine (PMM) given individually or in combination, against $HxHC_1$ xenografts are shown in Fig. 3. Methyl CCNU (35 mg/kg; LD_5 dose level) induced a growth delay of 4–5 days, but PMM did not inhibit the growth of $HxHC_1$ tumors at dose levels up to 100 mg/kg. The TFI changes with time after treatment using either agent alone, or for the combination of PMM given 1 hr before, or 1 hr after, methyl CCNU are also shown. Both combination sequences produced a marked depression in tumor TFI with a recovery time of between 12 and 13 days. During this period, $HxHC_1$ tumors showed no increase in volume. This combination has been examined against $HxGC_3$ and $HxELC_2$ xenograft tumors but in both cases only additive

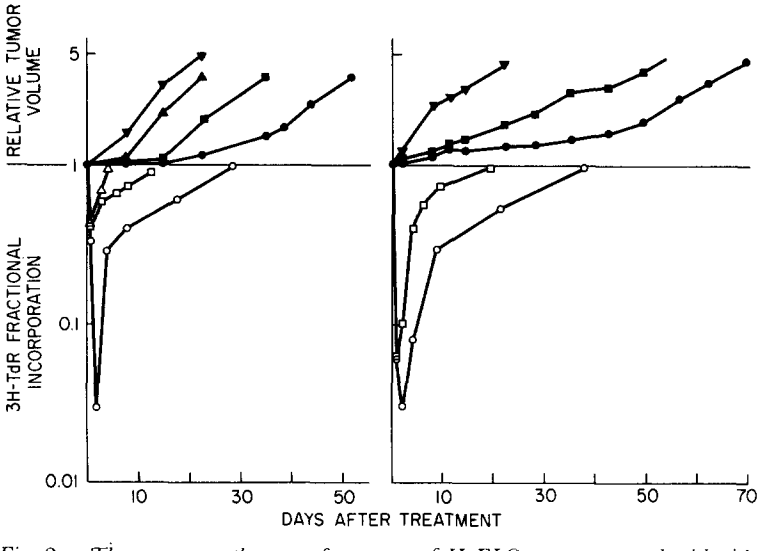

Fig. 2. The mean growth curves for groups of $HxELC_2$ tumors treated with either cyclophosphamide (left) ▲ 50; ■ 100; ● 200 mg/kg, *or 5-fluorouracil (right)* ■ 100; ● 200 mg/kg; ▼ *represents the mean growth curve for untreated tumours in each set of experiments. Below the TFI changes for the corresponding treatment (open symbols) are shown with time after drug administration.*

Table 1

Tumor line	Agent	Dose mg/kg	P^1	P^2	P^3
$HxHC_1$	Methyl CCNU	35	0.82	0.72	0.67
$HxGC_3$	Methyl CCNU	35	0.72	0.53	0.57
	Cyclophosphamide	00	0.87	0.77	0.83
		200	0.60	0.52	0.66
		300	0.65	0.39	0.48
$HxVRC_5$	Cyclophosphamide	50	0.95	0.80	0.82
		100	0.70	0.39	0.32
		200	0.35	0.15	0.13
$HxELC_2$	Cyclophosphamide	50	0.65	0.68	0.74
		100	0.47	0.39	0.37
		200	0.22	0.14	0.13
	5-Fluorouracil	100	0.66	0.27	0.41
		200	0.24	0.03	0.07

P^1 = repopulating fraction from a backward extrapolation of the regrowth curve.
$P^2 = 1/2\ t/c$ where t = mean tumor growth delay.
$P^3 = 1/2\ t/c$ where t = TFI recovery time; c is the tumor volume doubling time for untreated tumors.

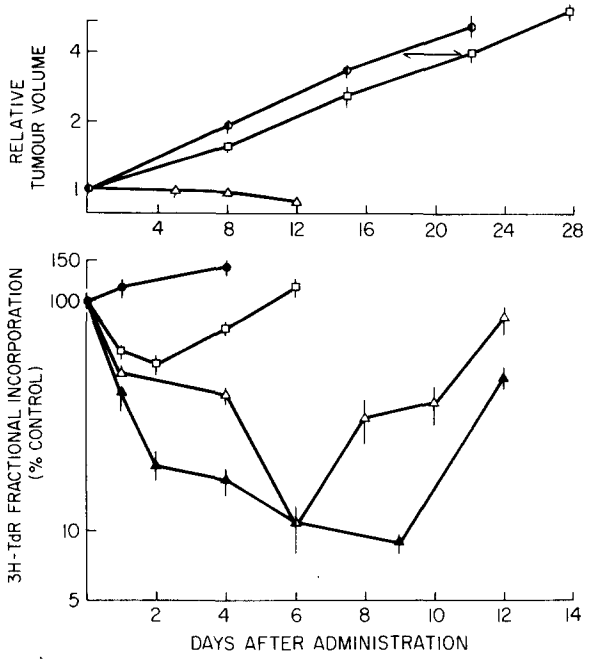

Fig. 3. *Top: Growth curves for groups of* $HxHC_1$ *tumors after treatment;* ● *PMM* 100; □ *CCNU* 35 mg/kg; △ *PMM administered* 1 hr *before methyl CCNU;* ○ *control. Each point is the mean* ±1 S.E. *for* 4 *animals. Bottom: Corresponding TFI changes in* $HxHC_1$ *tumors with time.* ● *PMM* 100; □ *methyl CCNU* 35 mg/kg; △ *PMM* 1 hr *before methyl CCNU;* ▲ *PMM* 1 hr *after methyl CCNU. Each point is the mean* ±1 S.E. *for* 8 *tumors.*

activity of methyl CCNU and PMM has been measured.

Actinomycin D and cyclophosphamide. The combination of actinomycin D with cyclophosphamide was suggested because of the differential response between $HxELC_2$ tumors and mouse small intestine after low doses of actinomycin D. The TFI changes with time are shown for $HxELC_2$ tumors and mouse small intestine after the administration of various dose levels of actinomycin D (Fig. 4). This antibiotic has little effect upon the growth of $HxELC_2$ tumors [10] but does induce a significant overshoot in the TFI recovery at low dose levels in these tumors but not in the mouse small intestine. The effect of administering cyclophosphamide alone (an active agent in this line) or at the time of maximum TFI overshoot following actinomycin D treatment (0.075 mg/kg) is shown (Fig. 4). Administration of cyclophosphamide after actinomycin D increased the TFI recovery time from 340 to 540 hr (cyclophosphamide 100 mg/kg). Administered simultaneously the TFI recovered at the same time as cyclophosphamide administered alone (data not shown). Actinomycin D did not induce a similar overshoot in TFI in tumor lines $HxHC_1$ or $HxGC_3$.

Repeated administration of methyl CCNU. Methyl CCNU has been found to be the most effective single agent against $HxHC_1$ tumors. After an LD_5 dose, growth is inhibited in these tumors by only 5 days, at which time the TFI had recovered to the pretreatment level. Consequently, in order to reduce the tumor burden it is necessary to retreat the host within 5 days. The data in Fig. 5 show the changes in TFI for $HxHC_1$ tumors and mouse gastrointestinal tissues, treated with methyl CCNU (35 mg/kg) at intervals of 4 days. The results indicate that it is possible to reduce the tumor TFI sequentially, whilst

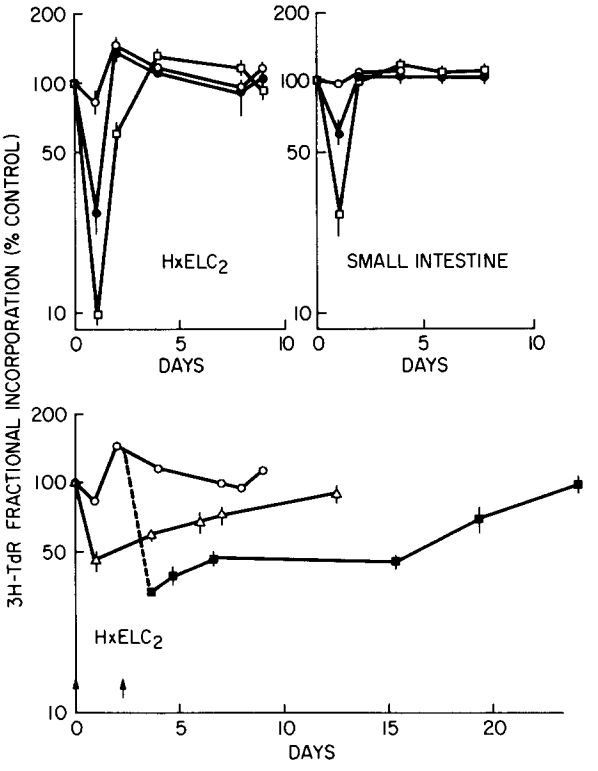

Fig. 4. Top: TFI changes with time in $HxELC_2$ tumors (left) and small intestine (right) after actinomycin D administration; ○ 0.075; ● 0.15; □ 0.3 mg/kg. *Mean* ±1 S.E. *Bottom: TFI changes in $HxELC_2$ tumors after treatment.* ○ *act.* D 0.075 mg/kg; △ *cyclophosphamide* 100 mg/kg. ■ *act.* D 0.075 mg/kg *at time* 0 *then cyclophosphamide* 100 mg/kg *administered at maximum TFI overshoot (arrow). Mean* ±1 S.E.

allowing normal ileum and colon to recover between drug administrations. The data show that stomach TFI fails to recover following the second administration of methyl CCNU.

In order to prevent bone-marrow aplasia during the course of this experiment 3×10^6 syngeneic bone-marrow cells from thymectomized donors were injected intravenously (i.v.) 4–6 hr after each dose of methyl CCNU.

In mouse ileum and colon there appears to be a dose-related depression in TFI 1 day after treatment (Fig. 6) with actinomycin D, *cis*-dichlorodiammino platinum II, methyl CCNU and cyclophosphamide, although the relationship in stomach is poor. It appears that a treatment depressing TFI in the ileum by about 60% corresponds to a toxicity level at which the occasional death is observed (LD_5–LD_{10}) due to gastrointestinal toxicity.

DISCUSSION

In xenograft lines $HxGC_3$, $HxVRC_5$ and $HxELC_2$ there is good agreement between the TFI recovery time and the growth delay achieved, independent of the agent administered. At present methyl CCNU is the only agent to inhibit the growth of $HxHC_1$ tumors, thus no relationship can yet be attained. It would appear that the tumors start to regrow at about the time that the thymidine incorporation into DNA recovers to the pretreatment level. After a treatment which induces a long TFI recovery time there is some indication that the rate of recovery, from about 1 week after treatment, is similar to the tumor growth rate prior to treatment. We have shown that in the Lewis lung carcinoma growing as small colonies in the lungs, that changes in TFI parallel the changes in %LI

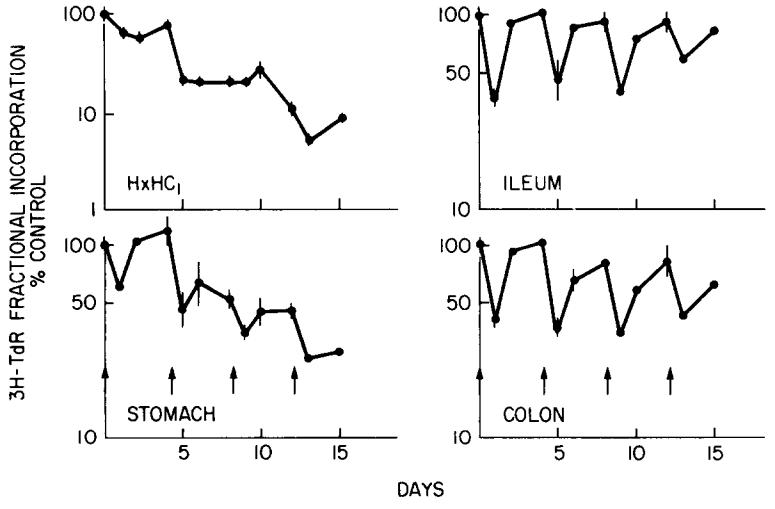

Fig. 5. The effect of repetitive administration of methyl CCNU on $HxHC_1$ xenografts and mouse gastrointestinal tissues. TFI changes are shown following administration of methyl CCNU (35 mg/kg) *every* 4 days (*arrows*). *Mean* ±1 S.E.

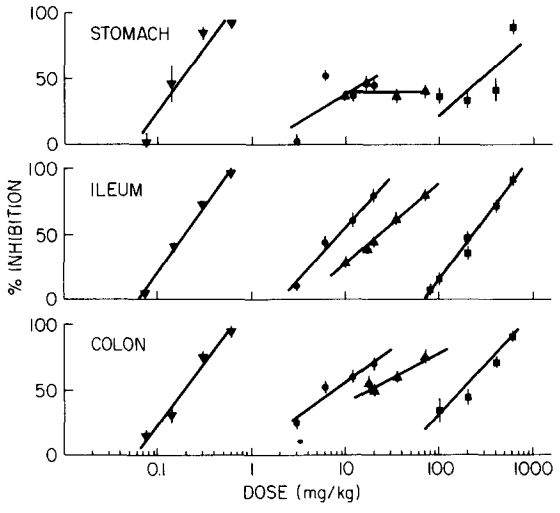

Fig. 6. The inhibition of TFI (100-TFI) plotted against dose 24 hr after drug administration for mouse gastrointestinal tissues. ▼ actinomycin D; ● cis-dichlorodiammino platinum; ▲ methyl CCNU; ■ cyclophosphamide. Mean ± S.E.

[11]. It is not likely that the TFI changes represent a change in the rate of DNA synthesis in the tumor cells *per se*, but, rather reflect the level of proliferating cells in the tumor. The incorporation of DNA precursors must be interpreted with some caution in tumors after treatment with cytotoxic agents, although the TFI appears to be a useful parameter to measure. That TFI recovery is coincidental to the renewed tumor growth, suggests that TFI recovery corresponds to tumor repopulation. Consequently, in order to sequentially reduce the tumor burden it must be retreated before the TFI recovers completely.

Calculation of the "repopulating fraction" by backward extrapolation of the regrowth curve gives slightly higher values than by calculation using either the growth delay, or the TFI recovery time. This is probably due to the tumors increasing in volume after treatment, before a period of growth stasis. This is apparent in $HxVRC_5$ tumors which continue to enlarge for about 8 days following cyclophosphamide administration. The "repopulating fraction" data in Table 1 demonstrates the relative insensitivity of these colorectal xenograft lines to the present spectrum of cytotoxic agents. Calculating P, assuming that repopulation occurs at the pretreatment growth rate, may lead to some inaccuracy, as this need not be correct [15]. However, in each experiment the post treatment growth rate eventually returned to that of the control tumors, and the technique allows comparison of the sensitivity between different tumor lines.

In selected tumor lines it is possible to combine agents and induce a TFI recovery time greater than anticipated (i.e., > additive), but the same combination may have little activity against other tumor lines. This was demonstrated with the 2 combinations used. In the actinomycin D–cyclophosphamide experiments using $HxELC_2$ the increased activity of cyclophosphamide may have been due to an increased proliferative state after actinomycin D administration, but this combination failed to show a similar activity in 2 other xenograft lines. It would appear that in order to exploit cell kinetic perturbations in colorectal cancer the "priming" agent must induce its desired effect in a high proportion of tumor lines.

The combination of methyl CCNU with PMM initially looked promising against $HxHC_1$ tumors. The TFI was considerably depressed with a recovery in about 12 days. This compares with a TFI recovery of 5 days after methyl CCNU alone, which is the most effective single agent used against this tumor line. The TFI depression in ileum one day after administration of the combination (77%) is approximately additive (methyl CCNU 62%; PMM 13%). The scheduling of this combination was intended to avoid potential cell proliferation kinetic changes in the tumor. The results suggest that PMM is not influencing methyl CCNU uptake as the same result is obtained in either sequence. In $HxGC_3$ and $HxELC_2$ tumors the effect of this combination was additive only.

In the small intestine and colon of the mouse each agent examined produced a dose related depression in TFI 1 day after treatment. Presumably the gastrointestinal toxicity induced by these agents is due to reduction of the stem cell population in the crypts. When the small intestine TFI was $\geqq 50\%$ of the control value 1 day after drug administration complete TFI recovery was measured at 2 days. With increasing TFI depression the recovery takes longer and deaths from gastrointestinal toxicity are observed, as shown for small intestine after increasing doses of actinomycin D (Fig. 4). The repetitive administration of methyl CCNU results in a sequential depression of TFI in $HxHC_1$ tumors, with full recovery in both ileum and colon between administrations. The TFI in stomach failed to recover after the second dose and this may be a major contributing factor in the death of mice treated in this manner.

In this paper data have been presented which show that by measurement of the

changes in TFI in these tumor lines it is possible to evaluate the activity of an individual agent or combination quite rapidly. The technique is less time consuming and more sensitive than growth delay measurement, and has the advantage that it may be applied to tumors which are not accessible to physical measurement (i.e., pulmonary metastases). In addition it allows the measurement of cytotoxicity in gastrointestinal tissues which otherwise require laborious histological analysis [16, 17]. Using the TFI technique it is therefore possible to determine which agents have the greatest selective toxicity against each tumor line.

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