

Repopulation in Murine Breast Tumors during and after Sequential Treatments with Cyclophosphamide and 5-Fluorouracil¹

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ABSTRACT

The rate of repopulation of surviving cells increases during fractionated radiotherapy and limits the ability to control tumors. Repopulation is likely to be more important during the longer intervals between courses of chemotherapy and is a potential cause of clinical resistance to chemotherapy. To evaluate the rate of repopulation of surviving cells in MXT and EMT-6 mouse breast tumors between successive cycles of chemotherapy, tumor-bearing mice were treated with up to three weekly cycles of cyclophosphamide or 5-fluorouracil (5FU). Animals were killed at different intervals during and after chemotherapy, and uptake of 5'-bromodeoxyuridine and immunohistochemical staining were selected to study repopulation. When tumors regrew after a single treatment, the proliferative rate of tumor cells returned to control values. During successive courses of chemotherapy, the proliferative rate of surviving cells increased in both tumors, *e.g.*, in 5FU-treated MXT tumors, the percentages of 5'-bromodeoxyuridine-labeled proliferating cells at 7 days after the first, second, and third treatment were $25 \pm 12\%$, $29 \pm 9\%$, and $27 \pm 11\%$; it was $32 \pm 6\%$ at 14 days after the third treatment. The corresponding value in control tumors was $19 \pm 2\%$ ($P < 0.05$ for each comparison). Accelerating repopulation after sequential treatments with cyclophosphamide or 5FU can lead to effective drug resistance in the absence of changes in the sensitivity of constituent cells.

INTRODUCTION

During and after treatment of experimental tumors with radiation, there is substantial evidence that the rate of proliferation of surviving clonogenic tumor cells increases with time (1, 2). This repopulation requires a marked increase in the total dose of radiation to obtain tumor control, when radiation therapy is given over prolonged periods. There is also strong, albeit less direct evidence for rapid proliferation of clonogenic cells during fractionated irradiation of human tumors. The total dose required to achieve local tumor control increases rapidly with duration of treatment, and interruptions in treatment have been shown to be associated with treatment failure in radiotherapy (3–6). Concurrent treatment with radiation and chemotherapy has been shown to improve therapeutic index in several sites, and this effect may be caused by inhibition of tumor cell repopulation by chemotherapy (7–9).

Repopulation is likely to be an even more important process during the longer intervals between courses of chemotherapy, but less is known about this process. We were able to identify only three studies of tumor cell repopulation after treatment of murine tumors with chemotherapy, and these studies were restricted to single treatments. Each of them showed evidence of increased proliferation of surviving tumor cells compared with the proliferative rate in untreated tumors, although the timing of such increased proliferation depended on the drug used and tumor model (10–12). Rapid proliferation of tumor

cells was also reported after induction chemotherapy in patients with oropharyngeal cancer (13). Davis and Tannock (14) have presented a theoretical model that showed increasing repopulation after sequential courses of chemotherapy could lead to regrowth of tumors after initial shrinkage without any change in the intrinsic sensitivity of the cells to the drugs used. Thus, accelerating repopulation might be an important cause of clinical drug resistance.

Quantitative analysis of tumor cell repopulation between cycles of chemotherapy is difficult, because it is important to distinguish the surviving clonogenic cells that can regenerate the tumor from lethally damaged cells that may remain morphologically intact. One method is to evaluate sequentially the number of surviving colony-forming cells by excision and plating, but the plating efficiency in tissue culture may not reflect conditions *in vivo*. Another method is to evaluate the average dose of radiation to control 50% of tumors under hypoxic conditions (TCD-50) at different times after drug treatment (12), but this is cumbersome and requires assumptions about unchanging radiosensitivity of surviving cells. Immunohistochemical staining of proliferation markers can be used to determine the changes of proliferation of tumor cells and repopulation between cycles of chemotherapy. Although this technique does not differentiate between surviving clonogenic cells and lethally damaged cells before their lysis, it is likely to give useful information when sufficient time after drug treatment has elapsed for lysis of drug-damaged cells.

The goals of the present study were to quantitate the proliferation of surviving MXT and EMT-6 mouse breast cancer cells after sequential cycles of chemotherapy with CY³ and 5FU. We have elected to use immunohistochemistry to evaluate proliferation markers and evaluated tumors at 1-week intervals after treatment to allow for the lysis of drug-damaged cells.

MATERIALS AND METHODS

Tumor Models. The MXT mouse breast tumor (15) was kindly provided by Dr. Kiss (Universite Libre de Bruxelles, Brussels, Belgium) and maintained by serial transplantation in the flanks of syngeneic BDF mice. EMT-6 tumor cells were maintained in culture with reestablishment from frozen stock at ~3-month intervals. Tumors were generated in syngeneic BALB/C mice by injection of $\sim 10^6$ cells into the right flank of each mouse. B₆D₂F₁/J and BALB/C male mice were provided and raised by the animal colony of the Ontario Cancer Institute, University of Toronto (Toronto, Canada). They were 6–8 weeks old when used for experiments. Mice were housed five per cage. Tap water and food were given *ad libitum*. All procedures were carried out following approval of the Institutional Animal Care Committee.

Drugs and Reagents. CY was purchased from Sigma-Aldrich Canada Ltd. (Oakville, Canada). A solution of CY was prepared before use and kept at 4°C until the time of the experiment. 5FU was provided by the hospital pharmacy. BrdUrd was provided by an affiliate of Merck KGaA (Darmstadt, Germany).

Treatment with Chemotherapy. All tumor-bearing mice were divided randomly into groups of 8–12, and treatment with chemotherapy was initiated when the diameter of tumors was ~8 mm (referred to as day 0).

³ The abbreviations used are: CY, cyclophosphamide; 5FU, 5-fluorouracil; BrdUrd, 5'-bromodeoxyuridine.

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One group was selected as the controls, and the others were treated with chemotherapy. In experiments, 100 mg/kg CY body weight or 50 mg/kg 5FU were injected i.p. weekly for a total of three doses. Control mice were given equal volume of PBS.

The two maximal perpendicular diameters of tumors were measured every 3 days to document tumor growth. Tumor measurements were converted to tumor volume (V) using the formula ($V = W^2 \times L/2$), where W and L are the perpendicular smaller and larger diameters, respectively, and plotted against time. Body weight of mice was measured twice weekly to monitor treatment-related toxicity.

Cell proliferation in treated and control tumors was compared as follows. In each of four replicate experiments, two of the control mice were killed on days 7 and 14. In the CY or 5FU-treated group, 2 mice were killed 7 days after the first and second dose and at 7 and 14 days after the third dose (i.e., on days 7, 14, 21, and 28). In all of the above mice, 100 mg/kg BrdUrd were injected i.p. 4 h before they were killed to label cells undergoing DNA synthesis (16).

Immunostaining of Tissues. We first evaluated the four proliferation markers proliferating cell nuclear antigen, Ki67, Histone 3, and BrdUrd uptake and selected BrdUrd uptake for use in experiments because of its superior resolution. Tumors were excised from the mice and fixed in 10% neutral buffered formalin for 12 h. Standard dehydration and paraffin wax-embedding procedures were used on fixed tissues. Serial sections (5 μ m in thickness) were mounted on 3-aminopropyl-triethoxysilane-treated slides, dewaxed in toluene, and rehydrated through descending concentrations of alcohol. After three washings in tap and then distilled water, slides were immersed in 3% hydrogen peroxide for 10 min to block the endogenous peroxidase activity. Sections were treated with 0.25% pepsin for 5 min at room temperature to enhance the sensitivity of BrdUrd to its antibody. The primary antibody, mouse anti-BrdUrd (Caltag, Burlingame, CA) 1:3000, was applied, and slides were incubated overnight. Sections were rinsed in PBS three times for 5 min. The secondary antibodies, biotinylated rabbit antimouse and antirabbit IgG mixture (ID Labs, London, Canada) 1:200 in 10% normal rabbit serum, were applied for 30 min. Sections were rinsed three times in PBS for 5 min. Immunoperoxidase staining was carried out using the NovaRed Substrate Kit for peroxidase (Vector, Burlingame, CA) following the instructions of the manufacturer. Horseradish peroxidase-conjugated streptavidin (Ultra HRP Detection system; ID Labs) 1:1000 was applied to all sections for 30 min (17). Slides were counterstained with hematoxylin.

Quantitative Image Analysis. Sections were imaged using a Sony DX-CMD color charged coupled device (CCD) video camera (Sony Corp., Tokyo, Japan) mounted on a Zeiss Axioskop transmitted light microscope (Carl Zeiss, Oberkochen, Germany). Two methods were used to select areas of slides for quantitation of proliferative activity.

The Random Field Method. Using a $\times 25$ objective with a field size of $343 \times 343 \mu$ m squares representative fields were selected on the basis of two criteria: (a) the predominance of tumor cells and (b) the presence of some positive staining. At least 10 fields were randomly captured from the section. In each section, the tumor area was delineated using drawing tools to exclude necrosis and areas occupied by stroma and other nontumor cells. Thresholding according to color, hue, and intensity was performed on selected areas to identify all nuclei within the field and subsequently identify only the positively stained nuclei. For each section, the threshold was inspected visually, and settings were adapted if necessary. The proportion of nuclear staining (referred to as BrdUrd-labeling index) was computed as a ratio of the area occupied by all nuclei of tumor cells in the selected sections.

The "Hot Spot" Method. At least 10 fields demonstrating areas of positive staining at $\times 10$ magnification were captured and analyzed using M2 software for a MicroComputer Imaging Device (Imaging Research, Inc., St. Catharines Ontario, Canada). Within each section, ≥ 10 $100 \times 100 \mu$ m squares with maximally positive staining cellular areas, which represent areas of most intense proliferation, were selected (18). The proportional area was calculated as the ratio of the area occupied by the positively staining nuclei to that of the total scanned area.

Analysis of immunohistochemistry was performed by individuals without knowledge of the treatments that were received by the animals. As a test of the reproducibility of the method randomly chosen, tumor sections were also evaluated on two separate occasions without knowledge of the previous result.

Statistical Analysis. Data are presented as means \pm SE. The paired *t* test for independent samples of unequal variances was performed to compare

sample means. Statistical significance was based on two-sided *P*s < 0.05 . To investigate whether the proliferative rate increases significantly with time, an ANOVA for the regression of labeling index and time (days) from starting treatment was used to determine whether the slope of the regression line is significantly different from zero.

RESULTS

The growth of transplanted MXT and EMT6 mouse breast tumors after sequential treatments with CY and 5FU is shown in Fig. 1. Growth delay caused by these treatments is expressed as the increase in time for tumors to grow from 130 to 160 mm³ (median treatment volume) to a median volume of 1 cm³ and summarized in Table 1. These doses of CY and 5FU caused no obvious toxicity to the animals nor changes of body weight. Sequential doses of 100 mg/kg CY and 50 mg/kg 5FU body weight were chosen in the following experiments.

Estimation of the BrdUrd-labeling index by immunohistochemistry was quite reproducible, e.g., in control MXT tumors excised on day 7 that were evaluated on two occasions, the independent estimates of labeling indices were $19.2 \pm 0.4\%$ (based on a total of 83 fields) and $18.1 \pm 0.4\%$ (based on 57 fields; *P* = 0.31).

The BrdUrd-labeling index in control MXT tumors and at various times after sequential treatments with CY or 5FU is shown in Fig. 2. The mean labeling index for control tumors on days 7 and 14 was $19 \pm 2\%$. The labeling indices of the CY-treated tumors were significantly higher than that of controls except for evaluation on day 7 after only the first dose of CY ($20 \pm 3\%$; Fig. 2A). The BrdUrd-labeling index of CY-treated tumors on day 14 (after two doses of CY) was $27 \pm 2\%$ and on days 21 and 28 (after three

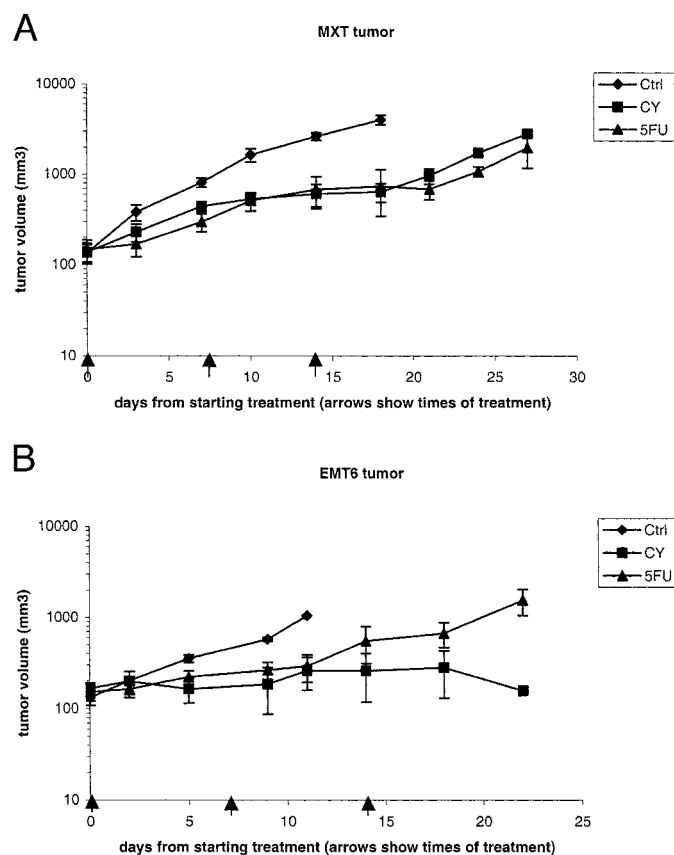


Fig. 1. Growth curves for MXT (A) and EMT-6 (B) tumors during and after three courses of CY (100 mg/kg) and 5FU (50 mg/kg). Points are the means of 8–12 animals; bars, SE. Arrows, days when animals received chemotherapy.

Table 1 Summary of growth characteristics of MXT and EMT-6 tumors during and after three courses of chemotherapy^a

Tumor	Drug	Doubling time between and after courses of chemotherapy				Time to grow to 1 cm ³ (days)
		d0-d7	d7-d14	d14-d21	d21-d28	
MXT	Control	2.3	4.3	/ ^b	/	18
	5FU	4.4	10.2	8.8	4.8	29.5
	CY	6.9	6.2	13.9	4.9	36
EMT-6	Control	5.2	4.7	/	/	20.5
	5FU	9.6	10.6	6.1	/	29.5
	CY	14.2	8.8	13.1	/	>32 ^c

^a Chemotherapy 100 mg/kg CY or 50 mg/kg 5FU was given i.p. once weekly on days 0, 7, and 14.

^b Not available.

^c When experiments were ceased on day 32 after tumor inoculation, the average tumor volume of CY-treated mice was <1 cm³.

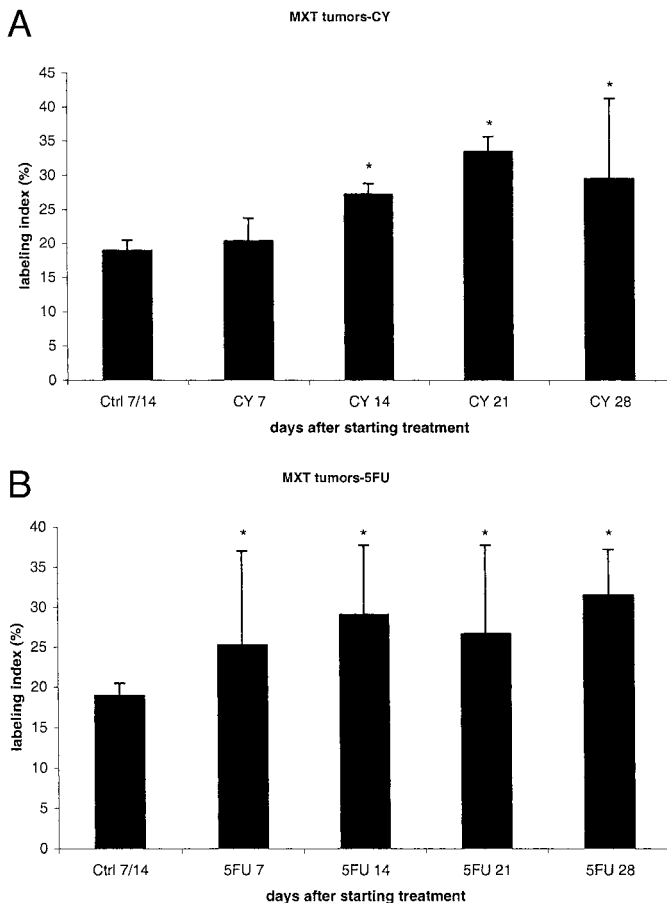


Fig. 2. BrdUrd-labeling index estimated by the random field method in MXT tumors after sequential weekly treatments with CY (A) or 5FU (B). Values are means \pm SE based on ≥ 10 fields/tumor and a total of eight tumors in four separate experiments. Except for day 7 in A, values of the BrdUrd-labeling index after chemotherapy were significantly higher than that of controls. * $P < 0.05$.

doses of CY) were $33 \pm 2\%$ and $30 \pm 11\%$, respectively ($P < 0.05$ for each comparison with controls). Similarly, compared with controls ($19 \pm 2\%$), the proportional areas of the 5FU-treated tumors on days 7, 14, 21, and 28 were $25 \pm 12\%$, $29 \pm 9\%$, $27 \pm 11\%$, and $32 \pm 6\%$, respectively. These rates of cell proliferation were significantly higher than that of controls ($P < 0.05$ for each comparison; Fig. 2B).

Results for EMT6 tumors are shown in Fig. 3; there is also evidence of increased cell proliferation after treatment with CY and 5FU. For the CY-treated tumors, the BrdUrd-labeling index on days 7, 14, and 21 was $22 \pm 10\%$, $28 \pm 8\%$, and $21 \pm 2\%$, respectively, higher than

the mean value for controls ($12 \pm 2\%$; Fig. 3A). The BrdUrd-labeling index of the 5FU-treated tumors on days 7, 14, and 21 was $27 \pm 1\%$, $22 \pm 11\%$, and $45 \pm 8\%$, respectively. Each of these values is significantly different from that of the control tumors (Fig. 3B).

There is a trend for the rate of repopulation in both tumors to increase with time during and after sequential treatments with CY or 5FU. However, the slopes of the regression lines relating BrdUrd-labeling index with time from starting treatment were not significantly different from zero ($P > 0.05$ for each regression line).

Results obtained by using the hot spot method, where heavily labeled fields were chosen for analysis, are shown in Table 2. Note that this gives a ratio of areas, and not the relative number of stained cells, so absolute values are lower than those reported above. In general, these results are parallel to those using the random field method and show higher rates of proliferation in both tumors after sequential treatments with CY or 5FU. Thus, there is evidence for increased rates of repopulation after sequential courses of chemotherapy, when assessed either in randomly selected areas of viable tumor tissue or in areas of maximal proliferation.

DISCUSSION

When patients with cancer receive chemotherapy, they are usually treated with sequential courses that are typically 3–4 weeks in duration. Chemotherapy is given at or near the start of each course, and the intervening time is necessary to allow recovery of normal tissue, particularly the bone marrow. This process of recovery is attributable to repopulation of the bone marrow as stem cells enter cycle, and their

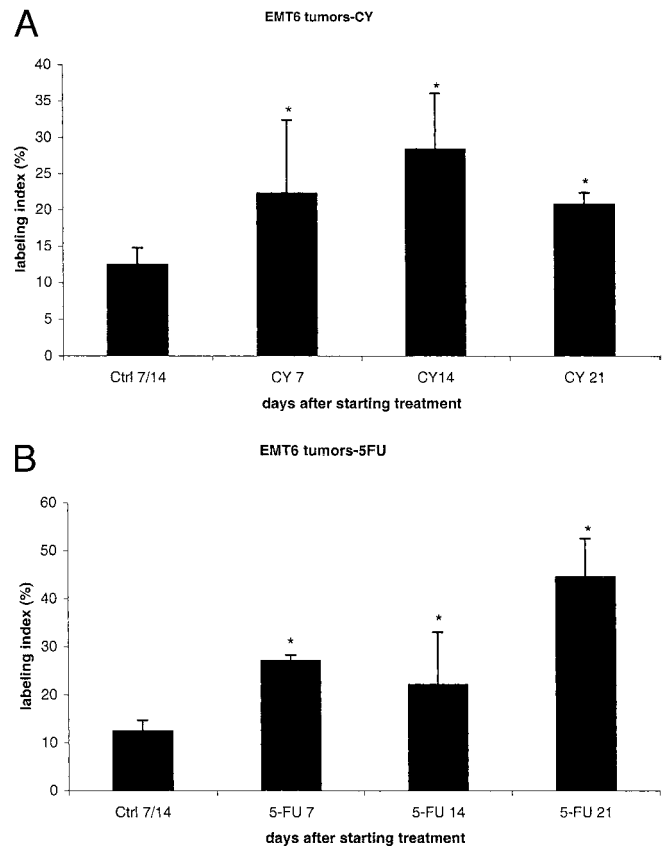


Fig. 3. BrdUrd-labeling index estimated by the random field method in EMT-6 tumors after sequential weekly treatments with CY (A) or 5FU (B). Values are means \pm SE based on ≥ 10 fields/tumor and a total of eight tumors in four separate experiments. The labeling index at all time points after chemotherapy was higher than in the control tumors. * $P < 0.05$.

Table 2 Proportional areas occupied by BrdUrd-labeled tumor cell nuclei after three weekly courses of chemotherapy^a

Groups	No. of hot spots	Proportional areas of positive cells (% , means \pm SE)	
		MXT tumor	EMT-6 tumor
Ctrl d7	49	10.7 \pm 0.1	3.9 \pm 0.9
CY d7	44	11.9 \pm 0.7	5.0 \pm 0.3 ^b
CY d14	34	14.7 \pm 2.9 ^b	13.3 \pm 2.8 ^b
CY d21	44	14.9 \pm 2.0 ^b	10.9 \pm 3.9 ^b
CY d28	44	20.7 \pm 0.4 ^b	N/A ^c
Ctrl d7	44	9.0 \pm 0.9	3.9 \pm 0.9
5FU d7	34	11.0 \pm 0.3 ^b	11.0 \pm 0.3 ^b
5FU d14	34	9.1 \pm 2.2	8.1 \pm 3.1 ^b
5FU d21	44	15.8 \pm 0.5 ^b	14.0 \pm 0.9 ^b
5FU d28	44	14.2 \pm 3.6 ^b	N/A ^c

^a Data were based on the hot spot method (note that this estimates labeled nuclei per unit area and not the proportion of labeled nuclei as in the random field method).

^b $P < 0.05$ compared with control tumors.

^c Not available; because skin ulcerations were very common in EMT6 tumors after 21 days of treatment, the animals had to be killed.

progeny proliferate rapidly and produce mature functional cells. However, just as the bone marrow repopulates between repeated toxic insults of chemotherapy, so also do the target tumor cells. This process lessens the net effect of chemotherapy, because the killing of tumor cells is partially compensated by repopulation.

Repopulation has been recognized as an important process that decreases the overall effectiveness of fractionated irradiation, and there is evidence that the rate of repopulation increases with time during a course of such treatment (1–6). The mechanisms underlying this “accelerating” repopulation are unknown but might involve improved nutrition of surviving tumor cells as well-oxygenated tumor cells close to blood vessels are killed and undergo lysis and the stimulation of residual tumor cells by circulating growth factors. There is very limited evidence from experimental tumors that the rate of repopulation may increase after treatment with some anticancer drugs as compared with the rate of proliferation in untreated tumors (10–12). However, there is no available information about repopulation after sequential treatments that reflect those used clinically. Because the intervals between treatments with chemotherapy (typically \sim 3 weeks) are much longer than those between fractions of radiation therapy (typically 1 day), the potential for repopulation of surviving cells to overcome the cell death caused by each treatment is greater, even if one allows for an effect of chemotherapy to inhibit cell proliferation for a limited time after each treatment. Indeed, modeling of the number of viable cells during three weekly cycles of chemotherapy with assumptions of substantial cell kill per cycle, and rates of repopulation found during radiotherapy of human tumors, has shown that accelerating repopulation can lead to initial shrinkage (or “response”) of human tumors, followed by regrowth, as is observed frequently during clinical treatment (14). This modeling indicates that acquired resistance of human cancer to chemotherapy might occur without any change in the intrinsic chemosensitivity of the constituent cancer cells.

In the present study, we have obtained information about the rate of repopulation of tumor cells during sequential treatment of two experimental tumors with two commonly used anticancer drugs, CY and 5FU. Treatments were given at weekly intervals, because time-related events in the mouse occur at shorter intervals than in man. Our results indicate that the rate of cell proliferation was greater at intervals after treatment with chemotherapy, with a trend to increase further after sequential courses of treatment. Thus, we have obtained evidence that accelerating repopulation may occur after treatment with chemotherapy.

Our study might be criticized because our method for assessment of

proliferation is not restricted to the important clonogenic cells that are the targets of cytotoxic treatment and which are ultimately responsible for tumor growth and death of the host. The method would not, *e.g.*, differentiate between viable cells that retain reproductive integrity and lethally damaged cells before their lysis. However, we were careful to assess proliferation \geq 7 days after previous treatment, and just before the next course, when lysis of damaged cells might be expected to be complete.

The observation of accelerating repopulation during sequential courses of chemotherapy has important implications for treatment. Antitumor effects and therapeutic index might be improved substantially if repopulation could be inhibited between treatments by agents that are relatively tumor specific and which would not inhibit repopulation in the bone marrow. Hormonal agents, which inhibit proliferation of hormone-responsive breast or prostate cancer cells, or inhibitors of growth factor receptors represent agents that are used clinically that might selectively inhibit tumor repopulation between cycles of chemotherapy. These agents are being investigated as inhibitors of repopulation during fractionated radiation therapy (19, 20). The use of such agents to inhibit repopulation between courses of chemotherapy will be more complex because anticancer drugs are more active against proliferating cells. Short-acting agents would be preferred, and they would need to be stopped just before the next cycle of chemotherapy to allow tumor cells to reenter cycle and regain chemosensitivity. Experiments of this type, designed to improve the therapeutic effectiveness of repeated cycles of chemotherapy, are ongoing in our laboratory.

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