The Effect of Chemotherapy on the Kinetics and Proliferative Capacity of Normal and Tumorous Tissues In Vivo

By S. H. Rosenoff, J. M. Bull, and R. C. Young

The proliferative state of a given tissue is a major determinant of its sensitivity to both phase-specific and cycle-specific chemotherapeutic agents. To study the extent of injury induced by antitumor agents to normal and tumorous tissues, a technique for following DNA synthesis as reflected in the incorporation of tritiated thymidine ($^3$H-TdR) into DNA was compared to the conventional radioautographic technique of the labeling index (LI) and to the functional kinetic technique of granulocyte colony formation in vitro. Alterations in DNA synthesis induced by a single dose of cyclophosphamide in normal and tumorous tissues in vivo paralleled in many respects the changes seen when the more time-consuming techniques of the LI or granulocyte colony formation were employed. However, the recovery of granulocyte colony formation after cyclophosphamide therapy lagged behind the recovery of DNA synthesis in the bone marrow, obscuring a kinetic event of potential therapeutic significance. The determination of DNA synthesis simultaneously in normal and tumorous tissues in vivo was easy to perform and supplied therapeutically pertinent results comparatively quickly.

Both toxic and therapeutic effects of antineoplastic agents are produced in a patient receiving chemotherapy, and the kinetics of both normal and tumorous tissues exposed to chemotherapeutic agents are altered by such therapy. Knowledge of the alterations in normal target tissues, such as the bone marrow, could be useful in the design of drug schedules.¹

In an effort to estimate the proliferative state of the bone marrow and to study its ability to recover from cytotoxic chemotherapy, several techniques have been utilized and each provides some estimate of recovery capacity. These techniques are: (1) the conventional cellular kinetic technique of radioautography utilizing tritiated thymidine, (2) the functional kinetic technique of bone marrow colony formation in culture, and (3) a biochemical technique in which the uptake of tritiated thymidine ($^3$H-TdR) into DNA is quantitated and expressed as the counts per minute per microgram of DNA (specific activity of DNA) and followed over time. However, no attempt has been previously made to compare three of the different techniques currently employed in the study of cell and tissue kinetics as to their ability to supply information about the recovery capacity of the bone marrow and tumor which might be useful to the clinician in the design of proper sequences of therapy.

In the present study, the three techniques mentioned above were utilized to study alterations in cell and tissue kinetics induced by a single dose of a classic...
alkylating agent, cyclophosphamide (CTX), in an attempt to elucidate any differences inherent in these three techniques.

MATERIALS AND METHODS

The L1210 murine leukemia was selected as a model system, as it already has been subjected to detailed, conventional, kinetic analysis, and it is a common tumor system for new antineoplastic agents. Cyclophosphamide was chosen as a representative of chemotherapeutic agents, as it is a widely used and clinically important alkylating agent with activity against the L1210 murine leukemia tumor system, and the dose of 200 mg/kg was selected, as it produces maximal antitumor effect with a single dose at acceptable toxicity (LD<sub>50</sub>).<sup>3,6</sup> BDF<sup>1</sup> female mice of an average weight of 20 g were obtained from Hazleton Laboratories (Falls Church, Va.) and used throughout these experiments. They were maintained in a constant temperature environment, in plastic cages, and fed laboratory chow and water ad libitum. L1210 murine leukemia carried intraperitoneally was used as the tumor source and transplanted to recipient mice by the injection of 1 x 10<sup>7</sup> L1210 cells intraperitoneally. BDF<sup>1</sup> female mice of comparable age and weight were utilized as normal controls. Experiments were carried out during the same time period each day, and all mice were sacrificed using cervical dislocation.

Specific Activity of DNA (cpm/μg DNA)

Details of the procedure for the extraction of DNA from mouse bone marrow (BM), GI mucosa, and ascites tumor following the administration of tritiated thymidine (3H-TdR) in vivo have been described elsewhere.<sup>1,7</sup> In the present study, on day 6 of tumor growth, 72 mice received 200 mg/kg of cyclophosphamide intraperitoneally. Six mice were left untreated and served as the zero-hour control group. At time 0, 1, 6, 12, 24, 36, 48, 72 hr and daily thereafter through day 8, six mice at each time point received 100 μCi 3H-thymidine (specific activity, 6.7 Ci/m mole; New England Nuclear, Boston, Mass.) intraperitoneally and 1 hr later were sacrificed. Ascites tumor was aspirated from the peritoneal cavity with a Pasteur pipette. Additional ascites was obtained by washing the peritoneal cavity with iced phosphate-buffered 0.85% NaCl, pH 7.4 (PBS). A 3-cm length of proximal duodenum was next removed from each mouse, gently agitated in PBS, and blotted on absorbent paper to remove adherent ascites tumor cells. The duodenum was split lengthwise on a glass slide, and a second glass slide was used to strip the mucosa from the muscularis and serosal layers. The mucosa was then removed from the second slide with wooden applicator sticks and dispersed in PBS. The bone marrow was harvested from both tibias of each mouse by stripping the muscle from the bone, sectioning the bone at each end, and expressing the marrow using a 25-gauge hypodermic needle and a syringe of iced PBS. Separate samples of ascites tumor and bone marrow from each of six mice were pooled into two groups of three. These pooled samples were spun at 800 g for 5 min in a refrigerated centrifuge (4°C), the supernatant was discarded, and the button of tissue was frozen for further processing. The DNA content of each of the pooled specimens was extracted by a modification of the Schneider method.<sup>8</sup> An 0.5-ml aliquot of the final supernatant was added to 15 ml Aquasol (New England Nuclear, Boston, Mass.) and counted in a Packard Tri-Carb liquid scintillation spectrometer. An 0.5-ml aliquot of the final supernatant was processed by the Burton method<sup>9</sup> for DNA determination. The results were then expressed as the counts per minute (cpm) per microgram of DNA and graphed as per cent of control over time.

Intravenous 3H-thymidine

On day 6 of tumor development 42 mice received 200 mg/kg of cyclophosphamide intraperitoneally. Six mice were left untreated and served as the zero-hour control group. At time 0, 1, 6, 12, 24, 36, 48, and 72 hr after cyclophosphamide therapy six mice at each time point received 100 μCi 3H-thymidine (specific activity, 6.7 Ci/m mole; New England Nuclear, Boston, Mass.) intravenously. All intravenous injections were performed in the tail veins of the mice using a 27-gauge hypodermic needle. The volume of injection was 0.1 cc. The dissection and processing technique was identical to that described above.

Labeling Index (LI)

On day 4 of tumor development 26 mice received 200 mg/kg of cyclophosphamide intraperitoneally. Three mice remained untreated and served as the zero-hour control group. Three
animals were sacrificed at various intervals in time for 8 days following cyclophosphamide therapy. At these times ascites tumor and BM were collected from each animal for radioautography. Each animal received 25 µCi 3H-TdR (6.7 Ci/mmole; New England Nuclear, Boston, Mass.) intraperitoneally, 1 hr prior to sacrifice. Alcohol-cleaned, gelatinized slides were utilized for radioautography. LI studies were performed on two separate tissues: bone marrow (BM) and ascites tumor. The collection and preparation of these two tissues for radioautography will be discussed separately in this section.

**L1210 ascites tumor.** Ascites fluid was collected from the peritoneal cavity of each mouse and suspended in 5 ml of 1% sodium citrate solution for 5 min and then centrifuged at 800 g for 5 min. The supernatant was discarded and the pellet resuspended in 5 ml of a 9:1 solution of methyl alcohol and glacial acetic acid for 10 min for fixation. Three drops of the cell suspension were placed on a gelatinized slide, allowed to spread, and then blown upon to produce an even spread of cells and to rupture cell membranes. After drying, slides were exposed for 7 days in Kodak NTB2 emulsion at 4°C, developed, fixed, and stained with a modified Giemsa technique.

**Bone marrow.** Bone marrow was collected from each animal at the time of sacrifice by the technique described earlier in this section. One per cent sodium citrate solution was used in place of the PBS. The BM obtained was then processed, fixed, developed, and stained as described in the preceding paragraph. The exposure time was 14 days. The LI was determined by examining at least two slides at each time point and counting 1000 consecutive mononuclear cells in the case of the BM.

**In Vitro Bone Marrow Culture**

Control mice were injected with 5 cc saline intraperitoneally. Test mice were injected with cyclophosphamide 200 mg/kg intraperitoneally. The animals were sacrificed 1, 6, 12, 30, and 48 hr, and subsequently at 24-hr intervals for 9 days. Immediately after sacrifice the BM from one femur from each of three test and control mice was aspirated into 3 cc of McCoy's SA medium. Nucleated cell counts were then performed. One cubic centimeter of the marrow sample was then diluted to a final concentration of 7.5 × 10^4 nucleated cells per cc in McCoy's 5A medium.

**Culture Medium and Plating Technique**

One-half cubic centimeter of the diluted marrow sample previously prepared was mixed in a Falcon plastic test tube with 5.0 cc of a suspension of 2.5 cc of 5% methylcellulose, 1.25 cc McCoy's medium, 0.5 cc fetal calf serum, 25 cc of bovine serum albumin, and 0.5 cc of a colony-stimulating factor made of a supernate prepared from a L611 tissue culture suspension. Four 1.1-cc aliquots were syringed from the test tube into 35 × 10-mm Falcon plastic Petri dish and incubated in a humidified chamber with 10% CO₂ at 37°C. After 7 days of incubation the total number of colonies containing ≥50 cells (range 50-1000) were counted on each plate using an inverted microscope at x 35 magnification. The colony counts obtained were expressed as per cent of control and graphed over time.

**Toxicity Studies**

BDF₁ female mice were treated at time zero with CTX (200 mg/kg intraperitoneally). Treated mice were subdivided into groups of 30 each which varied only in the timing of a second (200 mg/kg intraperitoneally) dose of CTX. These groups were zero hour only (one dose), zero hour only (two doses, 400 mg/kg intraperitoneally at time zero), zero hour and 24 hr, and zero hour and other times as indicated in Table 1.

**RESULTS**

**The Incorporation of 3H-TdR into the BM, Gl Mucosa, and Ascites Tumor (Fig. 1)**

The optimal time to sacrifice mice after the administration of 3H-TdR in order to determine the uptake of the isotope into DNA (cpm/µg DNA) would be a time at which the uptake was both maximal and stable. The uptake of 3H-TdR into the DNA of the ascites tumor is nearly complete by 5 min and
Table 1. Toxicity of Cyclophosphamide in BDF, Mice: Dose Schedules Variations

<table>
<thead>
<tr>
<th>Treatment Groups*</th>
<th>Lethality†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 1 dose: 200 mg/kg CTX</td>
<td>10</td>
</tr>
<tr>
<td>B. 2 doses: 200 mg/kg CTX each (0 hr only)</td>
<td>80</td>
</tr>
<tr>
<td>C. 2 doses: 200 mg/kg CTX each (0 hr and 12 hr)</td>
<td>25</td>
</tr>
<tr>
<td>D. 2 doses: 200 mg/kg CTX each (0 hr and 24 hr)</td>
<td>45</td>
</tr>
<tr>
<td>E. 2 doses: 200 mg/kg CTX each (0 hr and 36 hr)</td>
<td>40</td>
</tr>
<tr>
<td>F. 2 doses: 200 mg/kg CTX each (0 hr and 48 hr)</td>
<td>50</td>
</tr>
</tbody>
</table>

*Thirty mice in each treatment group treated intraperitoneally
†Per cent of animals dead 30 days after therapy.
¶Group C differs significantly from treatment Groups D and E combined (p < 0.05) and from Group F (p < 0.05).§
§§Significance obtained using single tail analysis.²²

![Graph showing time vs. CPM/μg DNA for L1210 ascites, normal bone marrow, and gastrointestinal mucosal cells.](image)

**Fig. 1.** The in vivo incorporation of ³H-thymidine into DNA in L1210 ascites tumor, normal bone marrow, and gastrointestinal mucosal cells. Each point represents the mean of two pooled groups of three animals each. Values are expressed as the cpm/μg of DNA for each of the three separate tissues over time.

reach a maximum at 1 hr. In the BM and GI mucosa, the uptake is more gradual and is nearly complete at 30 min and maximal at 1 hr. In all three tissues the uptake of ³H-TdR into DNA remains essentially unchanged for another 2 hr. Therefore, in all further studies, mice were sacrificed 1 hr after the intraperitoneal injection of ³H-TdR.

**Alterations in DNA Synthesis Induced by Cyclophosphamide in Normal and Tumorous Tissues**

The simultaneous changes in DNA synthesis in the tumor (ascites) and normal host target tissues (BM and GI mucosa) following a single dose of cyclophosphamide 200 mg/kg are shown in Fig. 2.
Fig. 2. Effect of a single dose of 200 mg/kg intraperitoneally of CTX on the incorporation in vivo of 
$^3$H-thymidine into DNA in L1210 ascites tumor, normal bone marrow, and gastrointestinal 
mucosal cells. Each point represents the mean of two pooled groups of three animals each. Values are 
expressed as per cent of control of the cpm/$\mu$g of DNA for each of the three separate 
tissues over time.

Suppression of DNA synthesis in the BM and GI mucosa began between hours 1 and 6 after therapy, was maximal by hour 12, initiated its recovery between hour 12 and 24 after therapy, markedly overshot control levels of 
$^3$H-TdR incorporation from day 2 to day 4, and then gradually returned toward control levels.

Cyclophosphamide therapy was followed by a pattern of suppression of DNA synthesis in ascites tumor which was quite different from that seen in the BM or GI mucosa. Suppression of DNA synthesis was noted in the ascites tumor by the first hour following therapy, reached a maximum by 24 hr, re-
mained markedly depressed through day 5, and only initiated its recovery by day 6 following cyclophosphamide therapy.

To explore the possibility that the route of $^3$H-TdR administration might result in a different distribution of isotope and subsequently different results, a similar experiment was carried out in which the $^3$H-TdR was given intravenously (Fig. 3). When the results obtained with the intravenous route (Fig. 3) are compared with the intraperitoneal route (Fig. 2), it can be seen that although the exact percentages at each time point were not identical, the timing of suppression and recovery of DNA synthesis in the BM, GI mucosa, and ascites tumor was similar. As the data were comparable, for purposes of simplicity of technique, the intraperitoneal route for injection of $^3$H-TdR was used in future experiments.

Alterations in the Labeling Index (LI) Induced by Cyclophosphamide

Bone marrow (Fig. 4). The LI of control animals in this study was 12.2%. One hour after cyclophosphamide treatment the LI rose to 123% of control. The LI then fell below control levels by 6 hr, reached a nadir in 12–24 hr, and
recovered to control levels by approximately 48 hr. The LI then rose above base-line levels and remained greater than 200% of control from day 3 to day 5, and returned to control levels by day 7.

*L1210 ascites tumor.* The control LI in the ascites tumor was 61%. The LI then fell to 12.7% of control over the first 24 hr and did not rise above 20% of control for the remaining 7 days of the study.

Granulocyte Colony Formation

Following a single dose of cyclophosphamide, 200 mg/kg intraperitoneally, granulocyte colony production became markedly depressed by 1 hr and remained essentially unmeasurable for the next 30 hr (Fig. 5). Recovery of granulocyte colony production commenced between 30 and 48 hr after cyclophosphamide therapy, overshot control from day 2 to day 5, and returned to control levels between day 5 and day 6.

The Effect of Dose Schedule Upon Lethal Toxicity

The single-dose studies with CTX showed a pattern of suppression and recovery of DNA synthesis in the BM (cpm/μg DNA and LI) (Figs. 2 and 3) which was different during the initial 48-hr period from the changes seen using the technique of bone marrow colony formation in vitro. To explore the
possibility that this time period would have therapeutic importance in the selection of the timing of a second dose of CTX which would minimize toxicity, the study as shown in Table 1 was conducted. CTX, 200 mg/kg, was an LD$_{10}$, and two doses of CTX given simultaneously at time zero (0 hr) (400 mg/kg) was an LD$_{80}$. When a single dose of 200 mg/kg of CTX was given at 0 hr and a second dose 12 hr later, the LD$_{80}$ effect of the 400 mg/kg of CTX was reduced to an LD$_{25}$. When the second dose of CTX (200 mg/kg) was administered 24, 36, or 48 hr after the first dose, toxicity was seen to increase from the LD$_{25}$ observed with the 0 + 12 hr schedule to approximately an LD$_{45}$.

**DISCUSSION**

Since the introduction of $^3$H-TdR into biologic science in 1957 by Taylor, Woods, and Hughes,$^{11}$ and the definition of the specificity of $^3$H-TdR as a DNA precursor in radioautography$^{12}$ in 1958, $^3$H-TdR has been used to study the cell kinetics of tumors and normal tissues. Pelc and others have expressed the incorporation of $^3$H-TdR into DNA as the specific activity of the DNA of tissues studied$^{13}$ and found it to be reflective of the state of activity of DNA synthesis at the time of injection of the isotope. Skipper,$^{14}$ utilizing the technique of conventional radioautography, and Bruce and his co-workers,$^{15,16}$ employing the technique of hematopoietic and lymphoma colony formation, and Young et al.,$^1$ using the technique of the specific activity of DNA, have stressed that the selection and schedule of chemotherapeutic agents may be optimized by exploiting any inherent difference in the cell kinetics of tumorous tissues as compared to the cell kinetics of normal host target tissues. If a technique used in
the study of cellular and tissue kinetics is to be clinically useful, however, it should supply information simply, quickly, and reproducibly.

In this study, the technique for the determination of the specific activity of DNA (cpm/μg DNA) after the administration of ^3^H-TdR, has been utilized, which allows one to follow simultaneously the alterations in DNA synthesis in tumor and normal host target tissues in vivo. This system has been compared to conventional radioautography as represented by the LI and to the functional technique of BM granulocyte colony formation in culture.

The optimal time to harvest tissue from in vivo sources after the administration of ^3^H-TdR was determined to be 1 hr, a point where the ^3^H-TdR incorporation into DNA was both maximal and stable (Fig. 1). The importance of the stability of uptake of ^3^H-TdR into DNA arises from the fact that between 5 and 20 min are required to process material from multiple tissues. If ^3^H-TdR was being actively incorporated into DNA during the time of sacrifice, tissues removed first would have a lower specific activity than tissues removed from in vivo sources later. This difference would be artificial, due solely to the fact that the dissection occurred during the rising phase of the DNA-incorporation curve, and not due to the actual state of activity of DNA synthesis at that particular point in time. This potential problem is obviated if one selects the 1-hr postinjection point.

Injury and recovery of tumor and normal tissues induced by single dose of
KINETICS AND PROLIFERATION OF TISSUES

Fig. 6. Comparison of the effect of 200 mg/kg intraperitoneally of CTX on the incorporation in vivo of 3H-thymidine into DNA in normal bone marrow cells and L1210 ascites tumor cells (each point represents the mean of two pooled groups of three animals each; values are expressed as per cent of control of the cpm/µg of DNA for each of the three separate tissues over time) to the effect of the same dose of CTX on the labeling index of normal bone marrow cells and L1210 ascites tumor cells expressed as per cent of control over time.

cyclophosphamide were studied by the conventional kinetic techniques of the LI and compared to the results obtained using inhibition and recovery of DNA synthesis (Fig. 6). The two techniques gave slightly discordant results only during the first few hours of the study. That is, DNA synthesis as measured by the cpm/µg DNA techniques showed a more rapid fall from control levels than did the LI. In fact, the LI of the BM rose above control levels during the first few hours after therapy. If cyclophosphamide was causing a reduction in the de novo thymidine pool, thus favoring temporarily the uptake of exogenous thymidine, one would expect an increase in the specific activity of DNA in parallel with the rise seen in the LI. If, however, the early rise in the LI reflects DNA repair of sublethally injured cells which have had only one strand of DNA alkylated at the guanine moiety, but have not been cross-linked, this injury would perhaps result in many cells synthesizing small amounts of DNA, causing a temporary increase in the LI. Under the same conditions, the specific activity of DNA, when compared to control levels, would not be expected to rise, but would actually fall, reflecting more accurately the actual state of DNA synthesis of the tissue under study. After this initial 24-hr period, the results obtained with the two techniques were comparable. As radioautography of the BM requires approximately 1 mo from the start of the study until the data is complete, and the determination of DNA synthesis by the cpm/µg DNA requires only 24-48 hr, the latter technique would be preferable for clinical purposes.

Figure 7 shows the changes in the kinetics of the BM induced by a single dose of cyclophosphamide. We have compared in this figure observations made when one studies DNA synthesis as opposed to those made when one studies
granulocyte colony formation in vitro. The timing of suppression and recovery of DNA synthesis in the BM roughly parallels the changes seen in the colony-forming ability of BM precursor cells with the notable exception that DNA synthesis in the BM can be seen to recover 24 hr sooner than does the colony-forming ability. Knowledge of this early recovery of DNA synthesis proves critical to the selection of a time of reduced toxicity of a second dose of a chemotherapeutic agent. For example (Table 1), when twice the LD_{10} dose of cyclophosphamide (400/kg) was administered, an LD_{90} was achieved. If an LD_{10} was given at time zero and a second LD_{10} at the nadir of DNA synthesis in the BM (12 hr after the first dose), the LD_{80} affect of the combined dose was reduced to an LD_{25}. This same study showed that if one delays the second LD_{10} dose to the recovery period of DNA synthesis in the BM (24, 36, or 48 hr after the first dose), one loses the protective advantage of the depressed state of DNA synthesis in the BM; and the toxicity rises from LD_{25} to LD_{45}. The determination of the specific activity of DNA allowed the prediction of the schedule-dependent reduced toxicity described above. As the granulocyte colony formation in vitro remained depressed until 48 hr after the first dose of cyclophosphamide, colony-forming ability would not have precisely defined the time for the markedly reduced toxicity of a second dose of CTX given at 12 hr, and additionally would not have predicted for the increasing toxicity of a second dose of CTX 24 or 36 hr after the first dose. Both of these observations might be predicted from the DNA-\textsuperscript{3H}-Tdr curve. As colony formation may reflect the more functional recovery of the granulocyte precursors, it is possible that during the initial phase of recovery of DNA synthesis in the BM (12–36 hr
after therapy), they are not yet functionally intact. However, these precursors may be quite sensitive to additional chemotherapy during this interval.

The system described herein for the analysis of in vivo alterations in DNA synthesis induced by chemotherapy as reflected in changes in the specific activity of DNA following the administration of $^3$H-TdR has the virtue of requiring only 24-48 hr for any single observation. Radioautography is time consuming in the sense that incubation periods are often lengthy, 2 wk in the case of the mouse BM$^2$ and much longer in the case of most human marrow and tumors.$^{18,19}$ and the analysis of slides requires an additional 1–2 wk. Such time delays make it impossible to design therapy for the individual patient involved in the study.

The technique of BM colony formation has permitted the evaluation of the proliferative capacity of BM stem cell populations$^{10,20}$ as well as leukemia and lymphoma cell lines.$^{15,16,21}$ Some of the difficulties we ascribed to radioautography are also present in this technique: (1) data are not available for 7–10 days awaiting colony growth, (2) the techniques are somewhat time consuming, (3) the necessity for sterile samples and environment leaves room for technical error, and of great importance, (4) at least as in the present study with cyclophosphamide, the recovery of granulocyte colony formation lagged behind the recovery of DNA synthesis in granulocyte precursors, beclouding a therapeutically important kinetic event. However, the in vitro culture technique does not require the use of radioisotopes and the potential hazards associated with these compounds.

It appears then, that the technique for following DNA synthesis simultaneously in tumorous and normal host tissues has the combined virtues of being comparatively easy to perform, of allowing results to be obtained rapidly, and of defining therapeutically important recovery intervals. These attributes may recommend this technique as a kinetic tool of potential clinical utility.

REFERENCES
5. Special Report to Pharmacology Committee, Acute Leukemia Task Force—Some Thoughts on Pharmacology: Cellular Kinetics and Experimental Therapeutics Associated with Cancer Chemotherapy (Used Alone or in Conjunction with Other Modalities). Southern Research Institute, June 19, 1968, p 25
14. Skipper, HE:: Kinetic behavior versus response to chemotherapy. Natl Cancer Inst Monogr 34:2, 1971
15. Bruce WR: The action of chemotherapeutic agents at the cellular level and the effects of these agents on hematopoietic and lymphomatous tissue. Can Cancer Conf 7:53, 1966
The effect of chemotherapy on the kinetics and proliferative capacity of normal and tumorous tissues in vivo

SH Rosenoff, JM Bull and RC Young