[CANCER RESEARCH 33, 882–887, April 1973]

Bleomycin as a Possible Synchronizing Agent for Human Tumor Cells *in Vivo*

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SUMMARY

At low doses bleomycin (BLM) reversibly inhibits cell progression at the S-G_2 boundary. Cells located in other stages of the cell cycle are essentially unaffected; therefore, when present for a complete cell cycle, BLM becomes a prospective *in vivo* cell-synchronizing agent. In the five trials reported here, BLM was used to synchronize human malignant melanoma cells *in vivo*. Tumor biopsies were pulse-labeled with tritiated thymidine and assayed by liquid scintillation and autoradiographic techniques. Following the synchrony block, cells at the S-G_2 boundary progressed to S phase in a partially synchronized wave. The labeling indices indicated about 1.5 to 4 times the normal number of cells in S phase at the peak times following the first BLM treatment. Therefore, BLM partially synchronizes cells *in vivo*, and this technique provides a rapid and accurate means of locating the synchronized cells and for scheduling of a second drug for a maximum effect on tumor cell killing.

INTRODUCTION

In the last 3 years data have been presented on the use of HU[1] as an *in vivo* synchrony agent in 2 animal tumor systems, (10, 13) and in mouse small intestine (7). Synchronized fractions of S-phase cells, ranging from 0.5 to 0.7, were obtained following treatment with HU.

In order for a drug to qualify as a synchronizing agent (whether *in vivo* or *in vitro*) the following criteria must be satisfied. The drug must produce cell progression delay at only 1 phase of the cell cycle.[2] The delay must be reversible, preferably within a short time after the end of the treatment for synchrony. In addition, if the procedure is to be used *in vivo* to synchronize tumor cells in humans, the tumor should conform to certain criteria. The tumor must be accessible for biopsy, and it must be large enough so that the process of multiple biopsies will not alter the kinetics of the tumor cells (14). The cell cycle time (T_C) of the tumor in question should also be known since a synchrony treatment can be better scheduled if T_C can be considered.

The purpose of this paper is to describe some preliminary data on attempts to produce partially synchronized populations of cells *in vivo* by BLM in patients with malignant melanoma.

MATERIALS AND METHODS

Five patients with biopsy-proven malignant melanoma who were not suitable for curative surgery or radiotherapy were considered in this synchrony study. Three patients had 5 s.c. melanoma nodules each and 2 patients had between 20 and 25 nodules. The latter 2 patients were the subjects of the studies reported in Charts 1, 2, and 3. All patients included in this study had a life expectancy of at least 3 months and had not been treated previously.

***In Vivo* Synchrony Procedure.** A single dose of BLM is cleared very quickly (4 hr) from the tissue and serum after injection (16). Since BLM causes cell progression delay only at the S-G_2 boundary (2, 15), then the cells must be in contact continuously with BLM before a significant degree of synchrony is induced. The greatest degree of synchrony might be achieved when the BLM is present for a time approximately equal to the T_C of the tumor cells. It follows, therefore, that the cells in the other compartments of the division cycle during the BLM treatment will progress until they reach the S-G_2 boundary where they will be delayed.

It has been reported (14) that the median cell cycle time (T_C) of human malignant melanoma cells *in vivo* is about 3 days (G_1 = 48 hr, S = 21 hr, G_2 = 5 hr). The lengths of S phase and G_2 phase are very consistent with data reported by Young and De Vita for human melanomas (17). Since the T_C of melanomas was also shown to vary considerably (14), it was decided to treat with BLM for a period slightly longer than the median T_C. In the clinical trials reported here, BLM dissolved in 0.9% NaCl solution was infused (i.v.) for 4 days into 5 melanoma patients (25 mg total dose per 24 hr or 15 mg total dose per 24 hr) with excisional biopsies of s.c. nodules performed at 12- to 24-hr intervals. With the exception of 2 samples taken at 3 and 6 hr after the end of the synchrony treatment, all 0-hr and daily samples were taken at 7 a.m. to eliminate the possible influence of diurnal variations within the tumors. After synchronizing with BLM, tumor cells blocked at the S-G_2 boundary should progress around the cell

[1] The abbreviations used are: HU, hydroxyurea; BLM, bleomycin; TdR-^{3}H, tritiated thymidine; ara-C, cytosine arabinoside; LI, labeling index.

[2] Throughout this paper G_1 is used as the preDNA synthesis gap period; S is the DNA synthesis period; G_2 is the postDNA synthesis or premitotic period; and M is mitosis.

Received May 8, 1972; accepted January 18, 1973.

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cycle in a partially synchronized manner, reaching S phase 2 to 3 days later (or the length of $G_2 + M + G_1$). This movement of cells into S phase was detected by *in vitro* labeling of the tumor biopsies with TdR-^{3}H.

Biopsy Sample Preparations. Excisional biopsies of s.c. melanoma nodules were performed before treatment, immediately after treatment and at 12- to 24-hr intervals for the next 3 to 7 days. Small (1-cu mm) pieces of tumor were placed immediately into warm McCoy's 5A medium containing TdR-^{3}H, 3 μCi/ml (6.0 Ci/mmole), and incubated for 30 min at 37°. After labeling, the tumor samples were centrifuged and washed 3 times with cold balanced salt solution. Several small pieces of the tumor sample were fixed in 10% formalin and were subsequently sectioned and prepared for autoradiography. The remaining portion of the tumor sample was weighed (wet weighed) and dissolved in a 10 X concentration of hydroxide of Hyamine solution (Packard Instrument Co., Inc., Downer's Grove, Ill.) at 100°. The quantity of Hyamine solution used in dissolving the tumor was always adjusted to yield a final concentration of 58 mg of dissolved tumor per ml of Hyamine solution. Once this adjustment was made, it was possible to add the same volume of dissolved tumor to the scintillation fluor each time. A volume of 0.1 ml dissolved tumor was added to 15 ml of Triton X fluor containing 1 ml of distilled water; the sample was then cooled and counted for 10 min on a Packard Tri-Carb liquid spectrophotometer. The scintillation fluor was prepared as follows: 1 part Triton X-100 (Rohm and Haas, Philadelphia, Pa.); 2 parts toluene; PPO, 4 g/liter; and POPOP, 50 mg/liter. The data are calculated and plotted as cpm/mg tumor and are, therefore, directly comparable between samples of the same patient and between patients. Samples of dissolved tumor were also precipitated with trichloroacetic acid in order to obtain a radioactivity assay in acid-insoluble material. It was found that the cpm/mg of dissolved tumor was equal to the activity found in nonprecipitated samples (soluble and insoluble fractions). Therefore, the precipitation step was eliminated in these studies. Thus the liquid scintillation data are qualitative measurements of the synchrony procedure obtainable within 1.5 hr after biopsy and are, therefore, most important in determining the exact time for administering the 2nd (S-phase) drug. The autoradiographs of the same sample are a quantitative analysis of the synchrony, and the movement of the partially synchronized cells into S phase is detected by increases in the numbers of TdR-^{3}H-labeled cells.

RESULTS AND DISCUSSION

General Information about the Patient. The 1st patient (MH) had 25 s.c. melanoma nodules and biopsies were taken before and at 24-hr intervals after the BLM synchrony for 7 days. The scintillation counts and labeling data for the presynchrony biopsy and for the biopsy obtained on the day on which the partially synchronized cells entered S phase (peak day) are shown in Table 1. This patient was not given an S-phase drug on the peak day during the 1st synchrony procedure in order that complete cell progression kinetics data could be obtained relative to the BLM synchrony. The patient was treated twice more (at 5-week intervals) with the BLM synchrony regimen, and ara-C (25 mg/24 hr for 2 days) was administered at the time that the partially synchronized cells entered S phase.

The next 3 patients (MY, MMC, and MC) each had only 5 s.c. melanoma nodules. During the 1st synchrony treatment, enough biopsies were processed to locate the wave of cells moving into S phase (see Table 2), and then the patients were treated with ara-C (same dose as for Patient 1). Five weeks later each patient was treated again according to the kinetics data obtained during his 1st treatment, with ara-C administered on the expected peak day.

The 5th patient to be entered into this study had 20 s.c. melanoma nodules. A complete BLM synchrony study was performed on this patient during the 1st treatment and no S-phase drug was administered. However, during the 2nd and 3rd treatments, ara-C was given when the partially synchronized cells entered S phase (see Table 1).

Although the purpose of this paper is to present data relative to the BLM synchrony procedure, certain general statements can be made about the response of the patients to this regimen. A complete report on these 5 patients as well as for 15 additional patients currently in this study will be the subject of another paper. Preliminary pathology reports on biopsy specimens obtained after treatment indicated an increased amount of necrosis and the presence of many cells with fragmented nuclei, as compared to biopsies obtained

Table 1
The TdR-^{3}H-labeling patterns in malignant melanoma cells from patients treated with bleomycin in vivo

Patient	Scintillation counts (cpm/mg tumor)			% labeled cells			BLM dose
	Treatment 1	Treatment 2	Treatment 3	Treatment 1	Treatment 2	Treatment 3	
1. MH							
A[a]	75.0	108		5.55	13.0		25 mg/24 hr for 4
B	153.5	205		20.50	28.6		days (total of 3
C	+2.05	+1.9		+3.7	+2.2		treatments)
5. MB							
A	149	205	264	7.95	21	29	15 mg/24 hr for 4
B	333	450	450	14.3	32.2		days (total of 3
C	+2.2	+2.19	+1.7	+1.8	+1.53		treatments)

[a] A, presynchrony biopsy; B, biopsy taken on peak day; C, fraction of cells in S phase on peak day following BLM synchrony treatment, as compared to presynchrony biopsy, *i.e.*, C = B/A.

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Table 2

The TdR-^{3}H-labeling patterns in malignant melanoma cells from patients treated with bleomycin in vivo

BLM dose was 25 mg/24 hr for 4 days (total of 2 treatments) in all cases.

Patient	Scintillation counts (cpm/mg tumor)
2. MY	
A[a]	73
B	146
C	+2.0
3. MMC	
A	236
B	460
C	+1.95
4. MC	
A	179
B	269
C	+1.5

[a] A, presynchrony biopsy; B, biopsy taken on peak day; C, fraction of cells in S phase on peak day following BLM synchrony treatment, as compared to presynchrony biopsy, *i.e.*, C = B/A.

before treatment. Because of the advanced stage of the disease in each of these 5 patients, no cures or regressions were obtained. However, during the course of 2 or 3 treatments with this BLM synchrony-S-phase drug regimen, no new s.c. nodules appeared, and the nodules present at the beginning of treatment either stopped growing or actually decreased in size.

Production of Partial Synchrony *in Vitro*. BLM has been shown to induce cell progression delay *in vitro* at the S-G_2 phase boundary (2, 15). At high doses (100 μg/ml for 1 hr), the progression block is irreversible (2, 15); however, at doses of 25 μg/ml or lower (for 12 hr), the block has been shown to be reversible immediately after treatment (S. C. Barranco and R. M. Humphrey, unpublished data) in Chinese hamster cells *in vitro*. Furthermore, Cohen *et al.* (4) have recently presented *in vivo* data on the antimitotic activity of BLM on mouse small intestine. At low doses (2 mg/kg body weight) BLM produced significant but transient inhibition of mitosis by blocking cells at the S-G_2 boundary. At higher doses (20 or 200 mg/kg), BLM exerted a direct effect on cells in the G_2 phase of the cell cycle. Therefore the G_2 progression effects of BLM have now been exhibited *in vitro* (2, 15) and *in vivo* (4).

For the synchrony procedure in 4 patients, a dose of 25 mg/day for 4 days was chosen for the initial studies since the total dose for the 4-day treatment (100 mg) was below the total dose found to be toxic to the patient (300 to 400 mg). Chart 1 contains the results for 1 such patient. The counts ranged from 3700 to 8900 cpm and, when adjusted to cpm/mg tumor, resulted in the liquid scintillation counts shown in Chart 1, *top*. The scintillation counts were 100 cpm/mg tumor in the sample obtained immediately before the start of the 1st treatment. By the end of the 4-day BLM infusion, the counts were decreased slightly and reached a minimum of 50 cpm/mg tumor 12 hr after the end of treatment. At Day 1, the counts were elevated above the pretreatment base line counts and ultimately reached a peak of 150 cpm/mg on Day 2.

The LI (fraction of cells in S phase) of the base line pretreatment biopsy was 5.55% and the LI of biopsies taken during the following week are expressed as a fraction of this base line and are shown in Chart 1, *bottom*. The labeled fraction was slightly elevated at the end of the treatment. The counts were reduced at 12 hr but reached a peak LI of 20.5% at Day 2 (Table 1). This represents a factor of about 3.7 times as many cells in S phase at 2 days after the BLM synchrony treatment. An S-phase drug administered at this time should have a 3.7 times greater effect than if the same drug were presented to the asynchronous population of cells having only 5.55% cells in S phase (see Table 1). The synchrony procedure was repeated resulting in 2.2 times as many cells reaching S phase on the peak day as were present in S phase before the 2nd treatment (see Table 1).

Of the 2 parameters, the data obtained from the autoradiographs (LI) are the most accurate and were required as definite proof that the scintillation counts were adequate measures of the synchrony procedure. In order to predict properly the location of the partially synchronized wave of S phase cells in time to administer the S-phase drug (while these cells were still in S phase) a rapid but adequate method to locate the cells

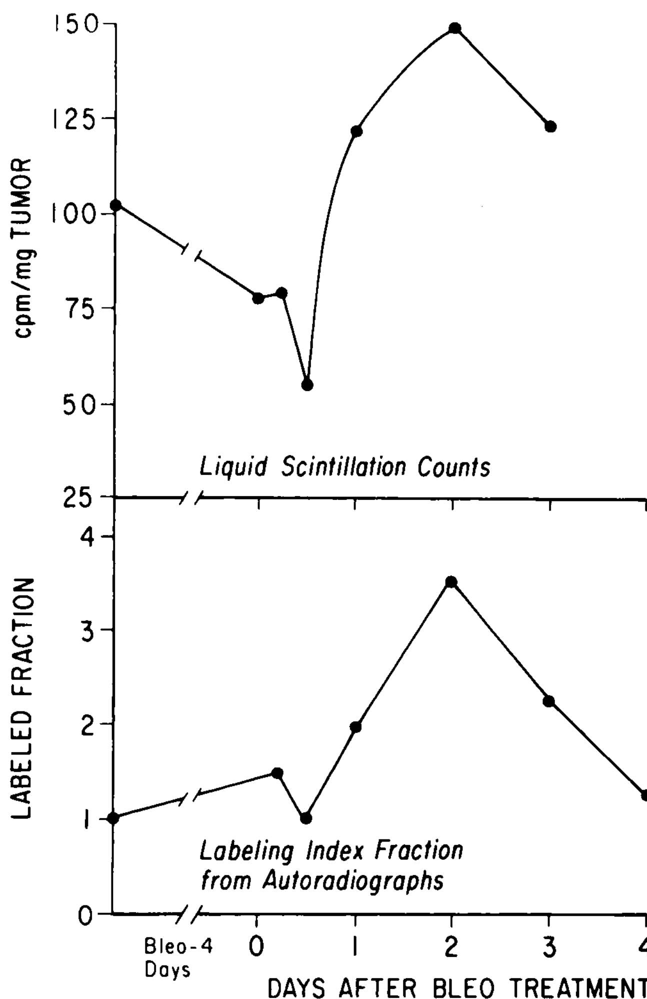

Chart 1. Changes in the TdR-^{3}H-labeling pattern in a patient receiving 25 mg of dose per 24 BLM (*Bleo*) total dose per 24 hr for 4 days. *Top*, liquid scintillation data expressed as cpm/mg tumor; *bottom*, autoradiography data of an aliquot of the same sample, expressed as the fraction of TdR-^{3}H-labeled cells relative to the 0 time pretreatment LI.

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must be used. The liquid scintillation counts serve nicely in this capacity and are obtainable within 1.5 hr after the biopsy, thereby allowing the clinician to administer the S-phase drug during the period when a partially synchronized wave of cells are moving into S phase. The data obtainable from autoradiographs, although more accurate, would take from 1 to 2 weeks to obtain and, of course, would no longer be of value to the clinician.

The counts for all 4 patients receiving BLM synchrony treatment of 25 mg/day for 4 days peaked at 2 days after the end of treatment in all of the treatment courses to each patient. For all 5 patients, the fraction of cells in S phase on the peak day was greater than the base line by a factor of 1.5 to 3.7 (see Tables 1 and 2). Because of the temporal spacing of the biopsies after synchrony, it has not been possible to obtain data on fluctuations in the mitotic index.

The minimum length of time required for the progression of the partially synchronized cells from G_2 to early S phase would be about 2 days if the G_1 phase in all melanomas *in vivo* were 48 hr long and G_2 were 5 hr (14). If the cells required a recovery period to overcome the BLM block, the peak would occur later than 2 days. Therefore, the data in Chart 1 may be interpreted in several ways. It is possible that the liquid scintillation and autoradiography data peaked at Day 2 after synchrony because the cells recovered immediately and were not inhibited in progression into S phase after the synchrony treatment. BLM is apparently cleared from the tissues and serum within 4 hr of injection (16); therefore a peak in LI could occur in 2 days. On the other hand, the 25-mg/day dose of BLM for 4 days may be sufficiently high to kill all of the cells in the division cycle and the peak in LI at 2 days might be due to the synchronous movement of cells from the G_0 compartment (viable but nondividing cells) into the division cycle. If the cells in the growth fraction are killed by the BLM synchrony treatment, and if the G_0 cells move immediately into the division cycle, they could also reach S phase in 2 days, since it is likely that they reenter the cycle through the G_1 phase (12).

To reduce the possibility of extensive cell killing by the synchronizing BLM dose, we lowered the amount of BLM administered to the 5th patient to 15 mg/day for 4 days. The data presented in Chart 2 indicate that the overall fluctuation in the scintillation counts and LI are similar to those in Chart 1, with the exception that the peak occurred on the 3rd day after the end of the synchrony treatment. The base line pretreatment LI was 7.95% and it rose to 14.3% at Day 3, representing a 1.8-fold increase in the number of cells in S phase at that time (Table 1) and treatment at that precise time with an S-phase agent should have had 1.8 times greater effect than on unsynchronized cells. That the partially synchronized population of cells reached S phase in 3 days suggests either that the cells required 24 hr for recovery from the BLM block or that G_1 phase might be longer in the tumor in this patient. The synchrony procedure was repeated, and 1.53 times as many cells were in S phase on the peak day (Day 3 after the end of BLM treatment) as were present at the start of the 2nd BLM treatment.

It can be seen in Table 1 that this fraction was actually lower than that obtained in the 1st treatment (1.53 *versus* 1.8). The same phenomenon occurred in Patient 1. These data

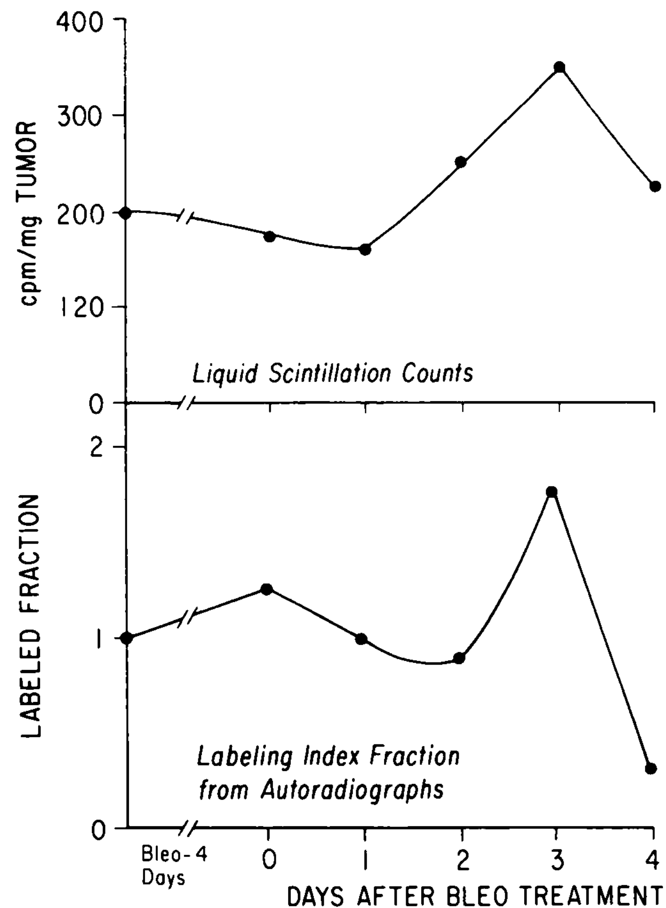

Chart 2. Changes in the TdR-^{3}H-labeling pattern in a patient receiving 15 mg BLM (*Bleo*) total dose per 24 hr for 4 days. *Top*, liquid scintillation data expressed as cpm/mg tumor; *bottom*, autoradiographic data of an aliquot of the same sample, expressed as the fraction of TdR-^{3}H-labeled cells relative to the 0 time pretreatment LI.

appear to suggest that the degree of synchrony was also decreased. This is not true, however, and if the LI (fraction of cells in S phase) obtained before any treatment is compared with the LI after the 2nd treatment (Table 1) one can readily see that the quality of synchrony is improving. For example, in the 1st patient (MH), the LI before any BLM synchrony treatment was 5.55% and it increased to 28.6% on the peak day of the 2nd treatment. This represents a 5.15-fold increase in the number of cells in S phase after 2 synchrony treatments. In the 5th patient (MB) the LI increased from 7.95% (before the 1st treatment) to 32.2% on the peak day of the 2nd treatment, a 4.05-fold increase in the number of cells in S phase at that time.

The data presented here have been considered for the construction of a kinetic model for chemotherapy (B. W. Brown and J. R. Thompson, in preparation). The model has been based primarily on the conclusion that it is possible to synchronize *in vivo* such solid human tumors as melanoma. Furthermore, their model predicts that the degree of synchrony should increase with the number of synchrony treatments. How this may occur can best be illustrated by the following. It is possible that treatment of tumors alters their growth kinetics and growth fractions. If altered tumor growth

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is the result of the killing of a large fraction of cells, then the inhibitory effects due to crowding would be diminished and a rapid regrowth of the surviving cells would occur. Many of these surviving cells would have been in a "G_0" nondividing compartment before and during drug treatment. Movement of these cells into the dividing compartment would substantially alter the growth fractions of the tumor. If this is the case, then subsequent drug schedules must also be altered. DeVita (5) has presented evidence that this does occur, since the LI of leukemia cells tends to increase after recovery from drug treatment. Reductions by 34% in the length of cell cycle time of rat bone marrow following irradiation (1) have also been reported. Changes in the LI in the human melanomas that we have studied *in vivo* have also been observed and are shown in Chart 3. The LI in untreated human melanomas in our study ranged from 5 to 8% (Patients 1 and 5) representing growth fractions of 20 to 30%. In Chart 3 it can be seen that prior to the 1st BLM treatment the LI for Patient 5 was 7.95%, representing a calculated growth fraction of 27%. The growth fractions (GF) are calculated according to Mendelsohn's equation (11) as follows: $GF = LI/(T_S/T_C)$ where T_S = length of S phase (21 hr) and T_C = length of the cell cycle (72 hr). Between the 1st and 2nd treatments the LI increased to 21% (Table 1) and then to 29% immediately before the 3rd treatment. This 29% LI represents a calculated or theoretical growth fraction of 100%.

By this time the number of nodules from this patient that were suitable for biopsy was exhausted and no additional labeling data could be obtained. It was estimated, however, that between 40 and 50% of the cells should have been in S phase on the peak day of the 3rd treatment and that subsequent treatments would also have enriched the fraction of cells in S phase.

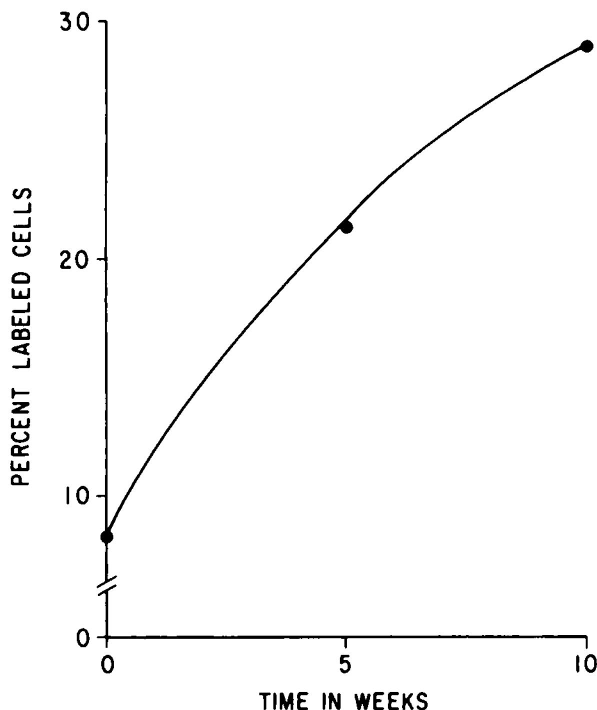

Chart 3. Increase in the LI in human malignant melanoma (*in vivo*) during a course of 3 BLM treatments. The samples were obtained immediately before each BLM treatment.

These changes in the LI and growth fractions bring up the question of the relative merits of synchrony followed by a dose of an S-phase agent (Schedule A) as opposed to a treatment schedule (Schedule B) in which several doses of an S-phase drug are administered to unsynchronized tumor cells at intervals separated by the T_S of the tumor. The preliminary data presented in Chart 3 suggest that, in tumors with small growth fractions and low LI, synchrony procedures may be effectively used in several initial treatments to enrich the fraction of cells in S phase. If after a synchrony treatment more tumor cells are synchronized into S phase as are there normally, then treatment at that time with a drug that kills cells specifically in S phase should have a greater killing effect on tumor cells. Since the S-phase agent would be more effective at that time, it should be possible to reduce the total treatment dose of this drug. By reducing the dose of the S-phase agent, its effect on proliferating populations of normal cells, such as intestinal epithelium and bone marrow should also be reduced, thereby providing a greater therapeutic advantage. Then, the subsequent use of S-phase drugs alone (as described in Schedule B) would be greatly enhanced. For example, the use of an S phase drug as the initial treatment on Patients 1 or 5 would have killed only 5 to 8% of the cells. However, if the S-phase drug is given after 1 BLM synchrony treatment, it is possible to kill 14 to 20% of the tumor cells. After the 2nd synchrony treatment, the S-phase drug may kill 28 to 32% of the tumor cells (Table 1).

Lampkin *et al.* (9) have achieved good synchrony in a patient with acute lymphoblastic leukemia. Seventy-two hr after a single injection of ara-C, they observed an increase from 18% to 33% in the LI of leukemia lymphoblasts. At that time, vincristine was administered. After this schedule was repeated 3 times, the patient was in remission and was put on a maintenance drug treatment. Synchronization of the mitotic cycle in HeLa cells has also been reported after treatment with ara-C *in vitro* (8). Ernst and Killman (6) have reported the partial synchrony of human leukemic blast cells *in vivo* with methylprednisolone. The drug caused the reduced progression of tumor cells from G_1 phase to S phase. It could not be determined whether the G_1 inhibition was the result of selective killing of G_1 cells or actual inhibition of cell progression. In addition, Gillette *et al.* (7), Mauro and Madoc-Jones (10), and Rajewsky (13) have reported a rather high degree of synchronized S-phase cells (50 to 70% of the total population) after treatment of experimental animal systems *in vivo* with HU. Their data on animal tumor synchrony *in vivo* show the highest degree of synchrony *in vivo* reported thus far.

Although the degree of synchrony reported to date for human tumor cells *in vivo* is not as good as that reported for animal tumor systems, we believe that this area of investigation should continue. We believe that important contributions (relative to synchrony) can be made especially for the treatment of melanoma and squamous cell carcinoma of the cervix. The *in vivo* cell cycle times for these tumors are known (3, 14) and most importantly they can be biopsied very easily. We submit that it is technically feasible to obtain partial synchrony in human solid tumors and in leukemia cells and that this technique may now be exploited in the treatment of

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these neoplastic diseases by well-designed chemotherapeutic regimens.

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