Synchronization and Recruitment in Acute Leukemia

BEATRICE C. LAMPKIN, TAKESHI NAGAO, and ALVIN M. MAUER

From the Department of Pediatrics, University of Cincinnati, The Children's Hospital, and The Children's Hospital Research Foundation, Cincinnati, Ohio 45229

ABSTRACT The in vivo effects of several chemotherapeutic agents on the mitotic cycle of leukemic blasts in the bone marrow were evaluated by serial measurements of cells in mitosis and in deoxyribonucleic acid (DNA) synthesis as indicated by ability to incorporate tritiated thymidine or tritiated deoxyuridine. 28 studies were done in 23 children and 1 adult. The changes in the marrow after a single injection of L-asparaginase, hydrocortisone, cyclophosphamide, cytosine arabinoside, methotrexate, and an exchange transfusion (62% of the total blood volume) were evaluated. L-asparaginase and hydrocortisone were found to arrest the entry of cells into the S period. Cyclophosphamide appeared to inhibit DNA synthesis, arrest cells in mitosis, and inhibit the entry of cells into the S period. Cytosine arabinoside, and methotrexate inhibited DNA synthesis. During the period of time the cells were inhibited in the S phase by these two drugs, cells continued to enter the S period. Thus partial synchronization was achieved after these two drugs. An exchange transfusion had no consistent effect on the mitotic cycle, but partial synchronization in the S period was seen in one patient.

To take advantage of the ability of cytosine arabinoside, to synchronize leukemic cells in the S phase, a second cycle-dependent drug was given at the time the leukemic blasts were synchronized. The second cycle-dependent drugs evaluated were vincristine, methotrexate, and cytosine arabinoside given by intravenous drip over a 12 hr period. Recruitment was found after cytosine arabinoside alone, and after prior synchronization with cytosine arabinoside and then the administration of either of these drugs.

The results of these studies indicate that a greater therapeutic advantage can be achieved by a second cycle-dependent drug after synchronization than after the second drug alone.

This work was presented in preliminary form at the meeting of the American Society for Clinical Investigation, 4 May 1970, Atlantic City, N. J.

Dr. Lampkin is a recipient of Special Fellowship Award 5F03CA42380, from the National Cancer Institute.

Received for publication 1 March 1971.

INTRODUCTION

Several investigators have shown that in patients with acute leukemia only a portion of the leukemic cells are within the mitotic cycle, the remainder of the cell population being in a resting state with regard to proliferative activity (1–3). Most currently available chemotherapeutic agents for this disease are maximally effective on cells within the mitotic cycle, especially the deoxyribonucleic acid (DNA) synthesis phase (4, 5). They probably are minimally effective on resting cells (6). Recently, Saunders and Mauer (7), and Gabutti, Pileri, Tarocco, Gavosto, and Cooper (8), have demonstrated that resting leukemic cells reenter the active proliferative phase both during a steady state and upon chemotherapeutic perturbation, respectively. Thus the resting cells can achieve a greater degree of drug sensitivity in this manner.

The following study was done to determine if increased drug sensitivity of the leukemic cell population could be induced by partial synchronization of cells in DNA synthesis or by recruitment of cells from the resting state into the mitotic cycle. Synchronization may be defined as a significant increase in the number of cells in one particular phase of the mitotic cycle. Recruitment may be defined as an increase in the total number of leukemic cells within the mitotic cycle.

The results indicate that more leukemic cells can be killed by a second mitotic cycle-dependent drug after synchronization than after the same drug alone and that in some instances recruitment of resting cells into the mitotic cycle was possible.

METHODS

28 studies were done in 23 children and in 1 adult. Permission for the studies was obtained after explaining in detail to the parents of the children and to the one adult, the procedures to be used (multiple bone marrow aspira-

tions, etc.). The procedures used in this study were in accordance with standards established for clinical research in human subjects at our institution.

By morphological and histochemical criteria (9, 10), 13 children had acute lymphoblastic leukemia (ALL),[1] 3 had acute myeloblastic leukemia (AML), and 3 had acute myelomonoblastic leukemia (AMML). Two children and one adult had leukemic transformation of lymphosarcoma. Marrow samples in all patients at the time of study contained greater than 90% abnormal blast cells. Studies were done at the time of diagnosis except in four patients with ALL and one patient with AML. In patients not studied at diagnosis, all drug therapy was stopped at least 24 hr before the new drug evaluation.

The drugs studied were cytosine arabinoside, methotrexate, hydrocortisone, cyclophosphamide, and L-asparaginase.[2] In some studies the evaluation of the sequential administration of cytosine arabinoside followed by either vincristine, methotrexate, or cystosine arabinoside itself was done. In four patients the effect of an exchange transfusion with 62% of the calculated blood volume was determined. The effect of cystosine arabinoside on normal marrow cells was studied in two patients with advanced medulloblastoma. These children had received all radiotherapy possible, and had become refractory to treatment with vincristine. Cytosine arabinoside was being given for its possible antitumor effects.

The changes in leukemic cell–proliferative activity, caused by the drugs or procedures, were followed by means of serial marrow aspirates. The marrow samples were obtained before the study and at intervals thereafter for the duration of the study. In patients in whom two drugs were given sequentially, marrow samples were obtained before the study and immediately before administration of the second drug as well.

The mitotic index of the marrow samples was determined by a modification of the method of Japa (11). About 0.25 ml of marrow was aspirated into a plastic syringe and discharged onto a watch glass where the sample was immediately covered with acetocarmine solution. After 10 min, marrow particles were transferred to glass slides and covered with cover slips which were then sealed at the edges with silicone grease. The number of mitotic figures per 1000 nucleated cells was determined for 10 cover slip preparations for each marrow sample obtained during the study. The mitotic index was recorded as the number of cells in mitosis per 1000 nucleated cells.

In order to determine the per cent of cells in DNA synthesis, the bone marrow needle was redirected after the initial aspiration and about 1.5 ml of marrow was drawn into a plastic syringe wetted with a saline solution of heparin (200 U/ml). A 1 ml portion of the sample was incubated with 1 μCi of tritiated thymidine (^{3}HT) with a specific activity of 1.9 Ci/mmole for 50 min at 37°C with constant gentle shaking. The sample was then transferred to a disposable sedimentation tube and centrifuged for 10 min at 1200 *g*.

In order to evaluate changes in the cellularity of the marrow, the marrow buffy coat of each sample was measured and recorded as per cent of the total volume (12). The buffy coat was then carefully removed with a Pasteur pipette, transferred to a watch glass, and mixed well with the same pipette. Cover slip smears of the buffy coat were made and radioautographs were prepared with Kodak AR10 stripping film. The radioautographs for each study were prepared simultaneously and stored at 4°C. After an exposure period from 7 to 13 days, the films were developed and the cells stained with Wright's stain. 3000 leukemic cells from six cover slip preparations were counted for each marrow sample and the per cent of labeled cells recorded.

Since methotrexate inhibits the formation of deoxythymidine-5-monophosphate from deoxyuridine-5-monophosphate, in order to evaluate the blocking effect of methotrexate it was necessary to use tritiated deoxyuridine (^{3}HdU). Tritiated thymidine (^{3}HT) was used to determine what would happen if thymidine were supplied after giving methotrexate. In these studies 2.5 ml of marrow was aspirated after the initial sample was obtained for mitotic index. 1 ml of the sample was incubated with ^{3}HT as described before. Another milliliter was incubated with 1 μCi of ^{3}HdU with the ^{3}H in 6 position and a specific activity of 3.1 Ci/mmole. After 1 hr incubation, each sample was divided into 2 portions. One portion from each sample was processed for radioautography as described before. The other portion was processed for determination of radioactivity by means of liquid scintillation counting. The buffy coat was removed and resuspended in 5 ml of Hanks' balanced salt–solution. This suspension was centrifuged at 300 *g* for 10 min. The resulting cell button was washed twice with Hanks solution and then resuspended in 5 ml of the solution. 1×10^7 cells were removed and centrifuged at 300 *g* for 10 min. This cell button was dissolved in 1 ml of 2 N NaOH with heating to 80°C for ½ hr. If a clear but colored solution resulted after cooling, two to four drops of 30% H_2O_2 were added to decrease the quenching effect.

To the clear solution, Scintisol TD™ (Isolab)[3] and two drops of saturated aqueous ascorbic acid were added. These ingredients were mixed well and 10 ml of Scintolute™ (Isolab)[3] was added. This mixture was transferred to a counting vial and the radioactivity determined in a Packard Tricarb Scintillation Counter (Packard Instrument Co., Downers Grove, Ill.) The results were expressed as cpm per 1×10^7 cells.

RESULTS

Drugs were evaluated singly and some in combination with cytosine arabinoside. It had been found that cytosine arabinoside given intravenously in a dose of 5 mg/kg body weight was capable of inducing partial synchronization of cells in DNA synthesis in some patients (4). Therefore, studies were done to evaluate the effect of a second drug given at the time synchronization had been achieved.

Single drug effects

Cytosine arabinoside. Studies were done in seven patients with ALL, three with AML, two with AMML, three with leukemic transformation of lymphoma; and in two patients with advanced medulloblastoma but normal bone marrows. These patients were given a rapid

[1] *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; AMML, acute myelomonoblastic leukemia; ^{3}HdU, tritiated deoxyuridine; ^{3}HT, tritiated thymidine.

[2] Batch SK 30,000 CE, supplied by Sloan-Kettering Institute for Cancer Research, New York.

[3] Isolab, Inc., Elkhart, Ind.

intravenous injection of the drug in a dose of 5 mg/kg body weight. The effect of two injections of the drug given 1 wk apart was evaluated in three patients with ALL. Except for patients 6, 7, 8, and 14, all were studied at time of diagnosis (Table I).

The results are shown in Table I. The labeling index increased over pretreatment values in patients with ALL from 24 to 96 hr after injection, indicating partial synchronization of the cells in DNA synthesis. In the three patients in whom a second injection was also evaluated, the time of the peak labeling index was the same as or close to the original one in all three patients.

In patients with AML and AMML, partial synchronization of the cell cycle occurred 24–36 hr after drug administration. In the patients with lymphoma, this effect was achieved 24–60 hr after the injection. Also shown in Table I are the result in the two patients with normal marrows in whom the partial synchronization of the cell cycle was found at 24 hr only in erythroid precursors. No change in labeling indexes of myeloid precursors occurred. No indication of synchronization was found in two patients with ALL, one with AML, and one with AMML.

The changes in mitotic indexes were the expected changes reflecting those in DNA synthesis activity. Mitotic indexes decreased after slowing or cessation of DNA synthesis, and increases in mitotic indexes occurred after prior recovery of DNA synthesis.

A significant decrease in the volume of buffy coat was found in all patients with leukemia or lymphoma by 24 hr after drug administration. In two patients with AML and one with lymphoma, the decrease was already present by 4 hr after the injection. Increases in buffy coat volume over pretreatment levels were found at time of partial synchronization in one patient with AML and one with lymphoma. No changes in buffy coat volume were observed in the two patients with normal marrows.

Methotrexate. The effect of methotrexate given intravenously in a dose of 1 mg/kg body weight is shown

TABLE I

Sequential Changes in Labeling Index after Administration of Cytosine Arabinoside

Patient	Hours											Hour of greatest synchronization
	0	<4	24	36	48	60	72	84	96	108	120	
	labeling index											
Acute lymphoblastic leukemia												
1, 1st study	16.4	.4	1.2		20.0		21.0	34				84
1, 2nd study	10.0	0	1.0		11.5		13.0		21.0			96
2, 1st study	8.2	0	6.5		24.8		39.7					72
2, 2nd study	10.0	0	1.5		12.0		26.0					72
3, 1st study	9.7	4.5	15.3		13.4							24
3, 2nd study	2.0	3.0	4.0	16.5								36
4	7.4	0.08	6.8		12.5							48
5	1.8	0.2	0.3		1.9							No synchronization
6	4.2	1.5	4.9		5.0		4.7		3.9			No synchronization
7	11.9	0.1	—		10.7		16.9		13.2		11.1	72
Lymphosarcoma												
8	23.7	2.4	10.2		23.1	36						60
9	17.5	1.2	23.8		15.2							24
10	28.3	4.6	37.3									24
Acute myeloblastic leukemia												
11	6.4	0.1	5.4	13.7								36
12	5.7	0.5	9.0									24
13	12.0	1.0	12.0									No synchronization
Acute myelomonoblastic leukemia												
14	9.8	6.2	8.6	10.8								No synchronization
15	6.0	1.8	10.0									24
Red cell precursors of normal marrow												
16	40	10	60									24
17	46	16	65									24

in Fig. 1. This patient was studied in his first relapse and had not been given methotrexate before. The rate of incorporation of both ^{3}HdU and ^{3}HT increased after methotrexate when measured by determination of the radioactivity of the whole cell suspension. In contrast, after the injection of the drug ^{3}HdU incorporation by the cells as indicated by radioautography was promptly blocked, indicating cessation of DNA synthesis. The effect persisted for at least 48 hr, but recovery of incorporation was found by 72 hr. Only the labeled precursor incorporated into DNA remains in the cell after processing the sample for radioautography; therefore absence of labeled cells in the radioautograph is indication that these cells were incapable of DNA synthesis with ^{3}HdU as the precursor substance. The decrease in mitotic indexes, as shown in Table II (patient 18), was confirmation that methotrexate had effectively blocked DNA synthesis by these cells. The increase in the radioactivity of ^{3}HdU and ^{3}HT in whole cell suspensions, indicates that these nucleosides are readily taken up by the cells, but determining radioactivity of whole cell preparations does not indicate DNA synthesis activity.

After the injection of methotrexate, the incorporation of ^{3}HT as measured by radioautography increased progressively up to 72 hr. Thus, there was indication that the flow of cells into the DNA synthesis phase of the cycle was not impeded. The cells subsequently accumulated until the block was alleviated by 72 hr and a decrease of cells in DNA synthesis was found by 96 hr. Thus by supplying thymidine, the compound needed for DNA synthesis beyond the block introduced by methotrexate, partial synchronization of the cells in the DNA synthesis phase could be achieved.

Cyclophosphamide. Two patients with ALL were given the drug intravenously in a dose of 15 mg/kg body weight. Both patients had relapsed while receiving methotrexate and had not had any drug therapy for 2 wk. Neither patient had received cyclophosphamide before.

The results are shown in Table III. In both patients, a decrease in labeling index was found 4 hr after the drug was given. In patient 19, the per cent of cells in

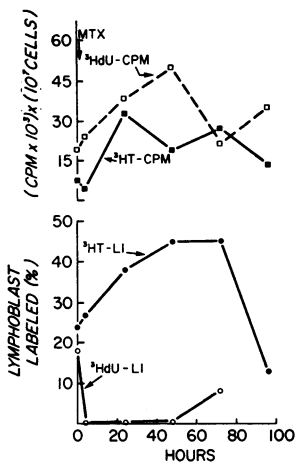

FIGURE 1 Effect of methotrexate on DNA synthesis of marrow lymphoblasts. Upper portion, scintillation counts of whole cell preparations. Lower portion, labeling index (LI) obtained from radioautographs. Note the decreased uptake of ^{3}HdU as measured by radioautograph indicating inhibition of DNA synthesis. The increase in the ^{3}HT labeling index (LI) indicates that synchronization could be achieved if thymidine were supplied.

DNA synthesis continued to decrease with some recovery apparent at 72 hr. In patient 1, however, fluctuation in the per cent of cells in DNA synthesis was observed, with increased values being found at 24 and 72 hr. The intensity of labeling in the labeled cells was not altered, suggesting that changes in labeling index reflected changes in the per cent of cells in DNA synthesis rather than a significant alteration of the rate of DNA synthesis. In both patients, a decrease in the volume of buffy coat was found after 24 hr. Thus, in both of these patients, the total number of cells in DNA synthesis was probably markedly reduced by the cyclophosphamide injection.

In both patients, increases in mitotic indexes were found at 4 hr, more pronounced in patient 19. In this patient, an increased mitotic index persisted for 48 hr. Thus, especially in patient 19, there is indication of a delay in mitosis introduced by the drug.

TABLE II

Sequential Changes in Mitotic Index after Administration of Cytosine Arabinoside, and Methotrexate

	Hours after cytosine arabinoside				Hours after methotrexate						
Patient	0	4	24	48	0	4	24	48	72	96	120
	labeling index				*labeling index*						
4	4.2	0.4	1.1	5.7	5.7	1.8	0.8	0.3	1.0	2.0	1.3
5	0.5	0	0.1	1.4	1.4	5.9	1.1	1.9	2.5	1.0	1.1
10	8.1	2.1	3.6		3.6	3.0	1.7	9.8	3.8		
13	7.1	1.2	13.4		13.4	5.6	9.7				
18					6.8	2.7	1.1	2.6	1.9	3.2	

L-*Asparaginase.* One patient with ALL (patient 20, Table III) was given this drug in a dose of 1000 U/kg body weight by an intravenous injection over a period of 1 hr. This patient had received and was resistant to all other standard chemotherapeutic agents at this time. The results are shown in Table III. 1 hr after completion of the injection, there was a small decrease in mitotic index but no change in labeling index. By 24 hr, however, both mitotic and labeling indexes had decreased markedly and remained decreased for the period of observation. There was also a decrease in the volume of buffy coat.

Hydrocortisone. In two untreated patients with ALL (patients 21 and 22, Table III), hydrocortisone was given intravenously in a dose of 35 mg/kg body weight. The results are shown in Table III. A second injection of hydrocortisone (35 mg/kg) was given to patient 21, 24 hr after the first. In neither patient was a change in the volume of buffy coat observed within the first 24 hr after the injection. No change was found during the period of observation in patient 21, even though a second injection of the drug was given. In patient 22, a decrease in the volume of buffy coat was found at 36 hr.

In both patients, however, an effect of the drug on the mitotic cycle was found. After the second injection in patient 21, labeling indexes decreased followed by decreases in the mitotic indexes. Recovery of the labeling indexes were evident in the 120- and 148-hr specimens. Return of the mitotic index to pretreatment levels was found at 148 hr. In patient 22, decreases in the mitotic index and labeling index were found at 12 and 36 hr, respectively. Samples were not adequate for complete evaluation of all three measurements at these times in this patient.

Combined drug effects

Cytosine arabinoside and vincristine. The effect of partial synchronization of the cells in DNA synthesis after administration of vincristine at this time was studied in three patients with ALL, two with AML, one with AMML, and one with leukemic transformation of lymphoma. It had been shown previously that when vincristine was given alone, in a dose of 0.075 mg/kg body weight, the maximal increase in number of mitotic figures occurred 12–24 hr after injection. The mitotic index then returned to pretreatment levels within 24–48 hr.

The effect of prior partial synchronization of leukemic cells in DNA synthesis with cytosine arabinoside and subsequent administration of vincristine in a dose of

TABLE III

Sequential Changes in Labeling Index, Mitotic Index, and Buffy Coat Volume in Patients with Acute Lymphoblastic Leukemia Given Cyclophosphamide, L-Asparaginase, or Hydrocortisone

		Hours										
Patient	Study	0	1	4	12	24	36	48	72	96	120	148
Cyclophosphamide												
1	Labeling index	16.9		9.9		18.8		9.2	24.1		15.7	
	Mitotic index	6.5		7.8		3.2		3.6	5.7		9.6	
	Buffy coat, *vol*	33.3		46.9		2.0		1.5	3.5		9.4	
19	Labeling index	45.2	31.1	30.4	23.0	16.0		18.4	23.0			
	Mitotic index	4.4	7.6	17.5	9.7	15.4		7.3	4.8			
	Buffy coat, *vol*	5.1	3.8	4.4	5.1	4.2		1.5	1.6			
L-Asparaginase												
20	Labeling index	4.1	4.6			1.2		1.2	1.5	0.5	0.5	
	Mitotic index	4.8	3.2			1.7		0.5	0.4	0	0.1	
	Buffy coat, *vol*	2.4				1.0		2.8	1.0	1.5	0.5	
Hydrocortisone												
21	Labeling index	14.1	15.3			13.5*		6.8	4.4	5.1	10.5	10.1
	Mitotic index	6.5	6.7			4.5		4.0	4.5	2.7	2.2	6.0
	Buffy coat, *vol*	5.2	5.2			6.9		6.1	4.2	5.8	5.6	6.6
22	Labeling index	12.3					5.4					
	Mitotic index	9.2			4.6							
	Buffy coat, *vol*	4.7			4.7		0.7					

* Second dose hydrocortisone given.

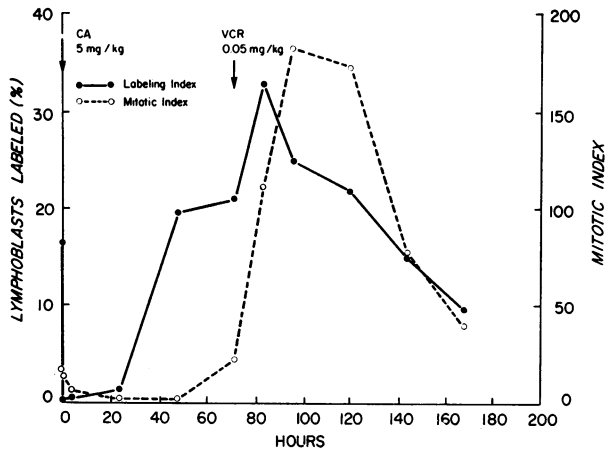

FIGURE 2 Changes in labeling and mitotic indexes of marrow lymphoblasts after cytosine arabinoside and vincristine (patient 1, first cycle). 72 and 84 hr after cytosine arabinoside there are more cells in the DNA synthesis period than before the drug was given, indicating synchronization in the S phase. The prolonged increase in the labeling and mitotic indexes after these drugs indicates recruitment into the mitotic cycle.

0.05 mg/kg body weight is shown in Figs. 2 and 3. These patients with ALL are numbers 1 and 2 in Table I. A preliminary report of the study in patient 1 has appeared (13). The number of cells in DNA synthesis was increased in both patients after the block produced by cytosine arabinoside—a result indicating partial synchronization in the S phase. In the second patient, of special note is the fivefold increase in the number of cells in DNA synthesis found 72 hr after cytosine arabinoside (8–40%). This increase is too great to account for by entrance of cells from G_1 into S. Instead, this marked accumulation of cells in the S period indicates that in this patient not only did synchronization occur but recruitment into the mitotic cycle also occurred.

In both patients, vincristine was given when some recovery in the mitotic index occurred after the initial depression by cytosine arabinoside. The mitotic index after vincristine remained significantly increased over pretreatment values as long as 96 hr after drug administration—two to four times longer than after vincristine alone. Thus in both patients even though a smaller dose of vincristine was given than when the drug was evaluated by itself, its effect as indicated by the number of accumulated cells in mitosis was greater when vincristine was given at the time leukemic cells were synchronized in the S period.

Increases in the number of cells in DNA synthesis over pretreatment values were found until 72 and 96 hr after vincristine in patients 1 and 2, respectively. The prolonged increase in the labeling and mitotic indexes in both patients indicates that recruitment of resting cells into the mitotic cycle occurred, and that more cells were arrested in mitosis by prior synchronization with cytosine arabinoside, than would have been with vincristine alone. Similar results for the combined therapy were also found in an additional patient with ALL and one with lymphoma.

A second cycle of therapy was begun 1 wk after the 1st. The results of a representative study (patient 1) are shown in Fig. 4. These responses were similar to those in the first cycle except that not as striking increases in labeling or mitotic indexes were achieved.

Partial synchronization of the mitotic cycle was achieved in the two patients with AML (patients 11 and 12, Table I). However, vincristine had little effect on the cells even though the drug was given in a dose of 0.075 mg/kg body weight. The results of a representative study in patient 12 are shown in Fig. 5. Partial synchronization after cytosine arabinoside was not found in patient 14, Table I, who had AMML, and vincristine likewise had little effect.

The drug cycles were given to all of the patients studied according to the schedule determined on the first study. A complete remission was obtained in the three patients with ALL. Three cycles were needed for patient 1, and four and seven cycles, respectively, for patients 2 and 3. Then 6-mercaptopurine was used for maintenance therapy. The first patient remained in remission for 12 months and the second and third for 4 and 3½ months, respectively.

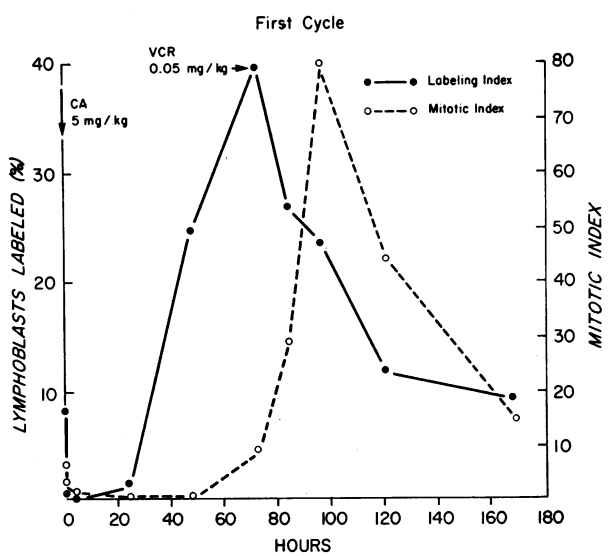

FIGURE 3 Changes in the labeling and mitotic indexes of marrow lymphoblasts after cytosine arabinoside and vincristine (patient 2, first cycle). Synchronization and recruitment are evident after cytosine arabinoside alone. Recruitment is still evident after vincristine.

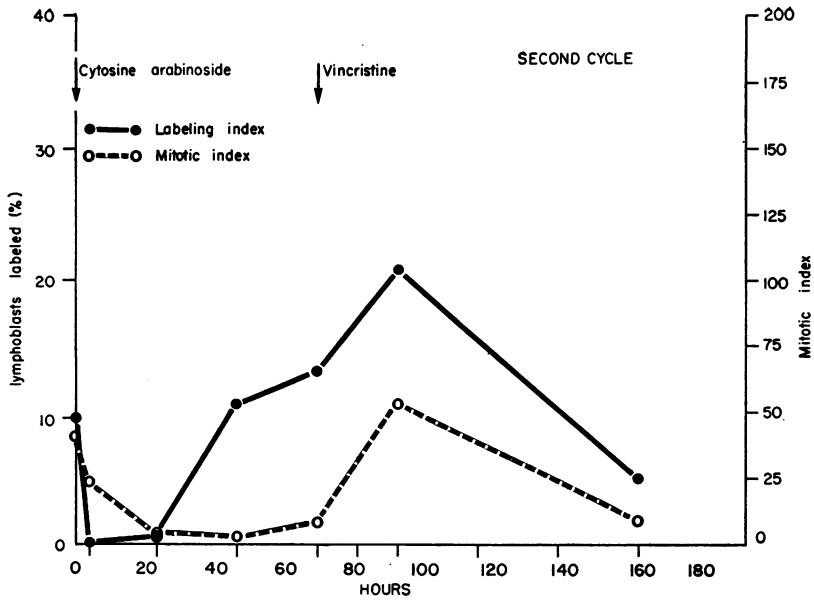

FIGURE 4 Changes in labeling and mitotic indexes of marrow lymphoblasts after cytosine arabinoside and vincristine (patient 1, second cycle). Note that the time of response after cytosine arabinoside and vincristine is the same as the first cycle, but the degree of response is less.

A good partial remission was achieved in the patient with lymphoma and in one with AMML after four and six cycles, respectively. This result in the patient with AMML was somewhat surprising in view of the lack of evidence for drug effect on the cell cycle.

Cytosine arabinoside, and methotrexate. The effect of partial synchronization of cells in DNA synthesis with cytosine arabinoside and subsequent administration of methotrexate was studied in two patients with ALL (patients 4 and 5, Table I), one with AML (patient 13, Table I), and one with leukemic transformation of lymphoma (patient 10, Table I). All of these studies were done at time of diagnosis.

A representative result in patient 4, Table I, is shown in Fig. 6. After the cytosine arabinoside injection, a decreased incorporation of ^{3}HT and ^{3}HdU was found at 4 hr. Some return in DNA synthesis activity had occurred by 24 hr, and partial synchronization was evi-

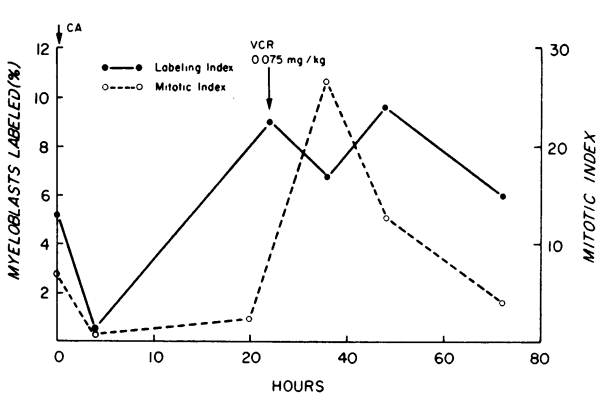

FIGURE 5 Changes in labeling and mitotic indexes of marrow myeloblasts after cytosine arabinoside and vincristine. Note that synchronization occurred after cytosine arabinoside, but vincristine had little effect on the mitotic cycle.

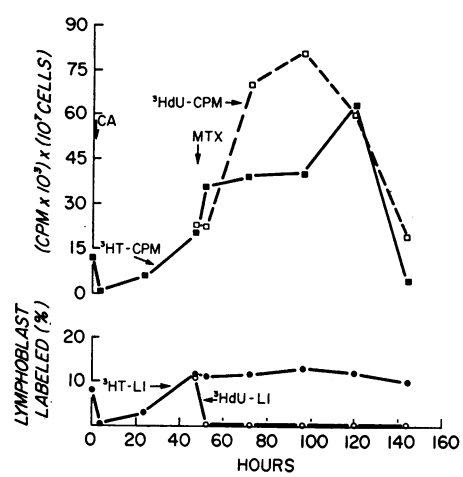

FIGURE 6 Changes in uptake of ^{3}HT and ^{3}HdU in patient with ALL who was given cytosine arabinoside and then methotrexate at the time the cells were synchronized in the S phase. Upper, scintillation counts of whole cell preparations; lower, labeling index (LI) obtained from radioautographs.

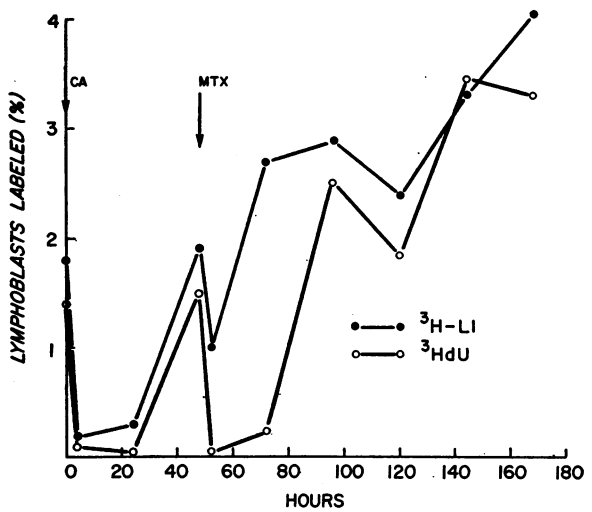

FIGURE 7 Example of lack of synchronization after cytosine arabinoside, but presence of synchronization after cytosine arabinoside plus methotrexate in a patient with ALL.

dent at 48 hr. At that time the methotrexate was given. DNA synthesis, as measured by ^{3}HdU incorporation determined by radioautography, was then promptly blocked and this effect persisted throughout the period of observation.

The per cent of cells in the DNA synthesis phase, as evidenced by their capacity to incorporate ^{3}HT determined by radioautography, increased slightly until 96 hr and then decreased somewhat. As in the study of methotrexate effect alone, cellular incorporation of both

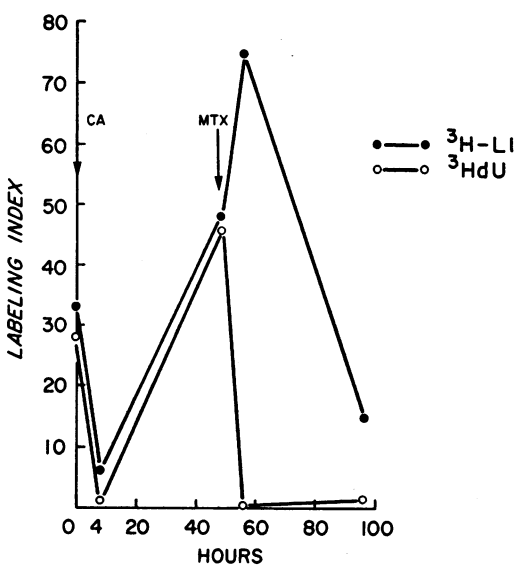

FIGURE 8 Example of synchronization after cytosine arabinoside and of recruitment when methotrexate was given at the time the cells were synchronized in the S phase.

^{3}HT and ^{3}HdU when determined by whole cell radioactivity measurement, was strikingly increased after the drug injection. Mitotic indexes decreased after methotrexate administration as shown in Table II and remained less than at preinjection levels throughout the period of study.

The cycles of drug therapy were repeated at weekly intervals with increase of methotrexate dose by 1 mg/kg body weight per wk. After three cycles, the patient was in complete remission. This patient was then given methotrexate in a dose of 5 mg/kg body weight every 2 wk as maintenance therapy and is still in remission at time of writing, 18 months later.

Results of another study in a patient with ALL are shown in Fig. 7 (patient 5, Table I). Initially, the proliferative activity of the leukemic cell population was quite low (labeling index, 1.8%; mitotic index, 0.5%). No synchronization was achieved by cytosine arabinoside, and the inhibitory effect of methotrexate was transitory. However, partial synchronization of cells in DNA synthesis was found 48 hr after methotrexate injection. Four cycles of this drug regimen were given but a remission was not obtained. Similar results occurred in a patient with AML (patient 13, Table I).

In Fig. 8 are shown the results of this drug regimen

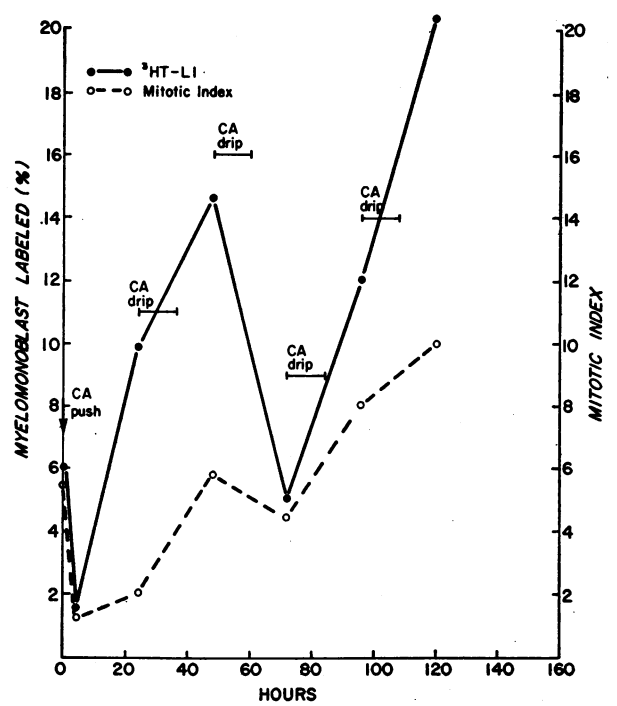

FIGURE 9 Changes in labeling and mitotic indexes after cytosine arabinoside given by push then by intravenous drip over a 12 hr period every 12 hr. Note synchronization 24 hr after the drug and recruitment, as evidenced by the marked increased labeling and mitotic indexes at 120 hr.

TABLE IV

Effect of 62% Exchange Transfusion on Mitotic Cycle

Patient	Study	Hours 0	12	24	36	48
Acute myelomonoblastic leukemia						
15	Labeling index	9.7	9.0		6.0	
	Mitotic index	1.3	3.7		5.6	
	WBC, *total/mm³*	684,000		280,000		187,000
23	Labeling index	3.2		5.8		4.8
	Mitotic index	4.2		1.1		6.6
	WBC, *total/mm³*	107,000		39,200		35,300
Acute lymphoblastic leukemia						
22	Labeling index	7.6	9.6	14.6		
	Mitotic index	9.9	6.5	9.2		
	WBC, *total/mm³*	104,000		44,000		
24	Labeling index	4.8	5.6	6.2		
	Mitotic index	5.2	6.0	3.2		
	WBC, *total/mm³*	170,000		39,600		

in a patient with leukemic transformation of lymphoma (patient 10, Table I). Partial synchronization occurred after cytosine arabinoside, and methotrexate inhibited ^{3}HdU incorporation. A striking increase of cells in DNA synthesis was found 4 hr after methotrexate administration as determined by ^{3}HT incorporation measured by radioautography.

Cytosine arabinoside in sequential injections. The effect of partial synchronization with cytosine arabinoside after subsequent administration of the same drug given over a 12 hr period intravenously in a dose of 5 mg/kg body weight is shown in Fig. 9 (patient 15, Table I). After the initial dose of the drug, partial synchronization was found. 12 hr after the completion of the second injection of the drug, a further striking increase in cells in DNA synthesis was found. The injection of the drug over 12-hr periods was continued at intervals and a second wave of increased numbers of cells in DNA synthesis was found at 120 hr. A similar increase in the mitotic index was found at that time.

Exchange transfusion

It was apparent from these drug studies that recruitment of cells from the resting phase into proliferative activity had occurred in some patients. One possible explanation might be that reduction of the number of leukemic cells allowed for reentry of resting cells into the mitotic cycle in some manner. To test this hypothesis, leukemic cells were removed by a 62% exchange transfusion. Two patients with ALL and two with AMML were studied (Table IV). These patients seemed particularly suitable for the study because each had white blood counts greater than 100,000/mm³. A reduction of leukocyte concentration, proportional to the amount of blood exchanged, was found in each patient. There was no evident rapid entry of cells from the marrow and tissue stores to replace the removed cells. Recruitment did not occur in any patient 24–48 hr after an exchange transfusion. However, partial synchronization was evident in patient 22, 24 hr after the exchange. The changes in the mitotic cycle in the other patients were minimal and inconsistent.

DISCUSSION

An understanding of the mechanisms by which chemotherapeutic agents affect leukemic cells should make more rational therapeutic regimens possible. In addition, unsuspected in vivo effects might be uncovered to provide leads to new forms of treatment.

The in vivo effects of cytosine arabinoside, vincristine, and corticosteroids on marrow lymphoblasts have been reported (4, 5). Cytosine arabinoside was found to inhibit DNA synthesis, vincristine to arrest cells in mitosis, and corticosteroids to lyse lymphoblastic leukemic cells as well as inhibit their entry into DNA synthesis. In the studies reported here, the effects of methotrexate, cyclophosphamide, and L-asparaginase were determined, and further studies were done using the three other drugs.

The drugs were administered intravenously and bone marrow samples were obtained before and serially after the injection. Changes in the proliferative capacity of marrow were evaluated by serial measurement of cells in mitosis and in DNA synthesis as indicated by ability

to incorporate ^{3}HT or ^{3}HdU, specific indicators of DNA synthesis. The marrow sites sampled have been shown to be representative of the marrow leukemic cell population (2). An estimate of the marrow cellularity was obtained from measurement of the marrow buffy coat (12).

Methotrexate was found to inhibit DNA synthesis. Cyclophosphamide appeared to have several effects: inhibiting DNA synthesis, arresting cells in mitosis, and inhibiting cells from entering DNA synthesis. L-asparaginase inhibited entry of cells into DNA synthesis, as recently reported by Saunders (14).

It can be concluded that the major effect of most of the drugs used for treatment of acute leukemia is on some phase of the mitotic cycle. At time of diagnosis and during relapse, however, less than one-half of the marrow leukemic cells are in the mitotic cycle (15, 16). Two methods of increasing the number of leukemic cells in a drug-sensitive phase of the cell cycle were found possible in this study: partial synchronization of cells in the mitotic cycle; and recruitment of cells from the resting phase into the cell cycle.

Partial synchronization of cells in DNA synthesis by intravenous injection of cytosine arabinoside, in a dose of 5 mgm/kg body weight, has been reported in patients with ALL (4). In the studies reported here, partial synchronization by this method also occurred in patients with AML, AMML, and leukemic transformation of lymphoma. The timing of maximal synchronization varied from patient to patient, but was generally slowest in those patients with ALL. At time of maximal synchronization the number of cells in DNA synthesis varied from 1.5 to 5 times the pretreatment value. Methotrexate was also found to have a synchronizing effect on leukemic cells, similarly accumulating them in DNA synthesis. In the dose studied, however, methotrexate was a much less efficient synchronizing agent than cytosine arabinoside.

Of interest is the observation that in normal bone marrow, partial synchronization of erythroid precursors occurred 24 hr after injection of cytosine arabinoside, but no effect was found for myeloid precursors. The difference in drug effect on normal myeloid cells and leukemic myeloblasts may indicate biochemical alterations in the malignant cell line.

After the leukemic cells had been synchronized by cytosine arabinoside in the S phase vincristine, methotrexate, and cytosine arabinoside, given by an intravenous drip over a 12 hr period were administered. More cells were killed by these drugs administered after partial synchronization than without prior synchronization.

Three methods were used in an attempt to recruit resting leukemic cells into the mitotic cycle: synchronization of the cell cycle with cytosine arabinoside and subsequent administration of methotrexate, vincristine, or cytosine arabinoside; massive doses of hydrocortisone; and partial exchange transfusion.

Recruitment was indeed evident in three patients with ALL and one patient with leukemic transformation of lymphoma who were treated with cytosine arabinoside synchronization after vincristine injection. A representative result is shown in Fig. 2. Likewise, recruitment occurred after cytosine arabinoside synchronization, when methotrexate was the secondary agent in one patient with ALL and in one patient with leukemic transformation of lymphoma. Neither vincristine nor methotrexate had much effect in the patients with AML or AMML, even though partial synchronization had been obtained by cytosine arabinoside. In one patient with AMML in whom cytosine arabinoside was both the synchronizing and secondary drug, recruitment was seen.

Chan, Hayhoe, and Bullimore recently reported recruitment of resting cells into the mitotic cycle in patients with AML, when the absolute number of blood leukemic blast cells was decreased by extracorporeal irradiation (17). To determine if reducing leukemic cell numbers by other means might also be effective, we tried cell lysis by massive doses of hydrocortisone and partial exchange transfusion. Unfortunately, administration of the hydrocortisone did not result in significant reduction of leukemic cell concentration. Partial exchange transfusion did reduce the cell concentration to the expected level, but recruitment into the mitotic cycle did not occur during the period of observation. Additional factors, such as releasing cell products from the rapidly destroyed leukemic cells induced by extracorporeal irradiation, may be more important in the recruitment found in marrow cells by Chan and his coworkers than reduction of population density.

Further studies are certainly in order to find better means of recruitment of resting leukemic cells into the mitotic cycle. After recruitment of these drug-resistant cells into a sensitive phase, further potentiation of chemotherapeutic effect might be achieved by synchronization of those cells in DNA synthesis by a drug such as cytosine arabinoside for the administration of a secondary drug for maximal cell kill.

ACKNOWLEDGMENTS

The authors wish to thank Doctors Joseph H. Burchenal, and Dorris J. Hutchinson of Sloan-Kettering Institute for Cancer Research, for supplying the L-asparaginase. We also thank Misses Theresa Asbrock, Virginia Fisher, Ruth Rush, Kristen Schorr, and Mrs. Esther Ohlinger for technical assistance, and Doctors John Akabutu, Ann Lichtenberg, Judith Naylor, and K. Y. Wong for their help in obtaining bone marrow samples.

This investigation was supported by grants CA 04826, CA 05196, and R 00123, from the U. S. Public Health Service; and a grant from the American Cancer Society.

REFERENCES

1 Mauer, A. M. 1964. Characteristics of cell proliferation in a patient with acute leukemia. *Lancet.* **2:** 675.

2. Mauer, A. M., and V. Fisher. 1966. Characteristics of cell proliferation in four patients with untreated acute leukemia. *Blood.* **28:** 428.

3. Gavosto, F., A. Pileri, C. Bachi, and L. Pegoraro. 1964. Proliferation and maturation defect in acute leukaemia cells. *Nature (London)* **203:** 92.

4. Lampkin, B. C., T. Nagao, and A. M. Mauer. 1969. Drug effect in acute leukemia. *J. Clin. Invest.* **48:** 1124.

5. Ernst, P., and S.-A. Killmann. 1970. Perturbation of generation cycle of human leukemic blast cells by cytostatic therapy in vivo: effect of corticosteroids. *Blood.* **36:** 689.

6. Mauer, A. M., B. C. Lampkin, and T. Nagao. 1970. Prospects for new directions in therapy of acute lymphoblastic leukemia. *In* Hemopoietic Cellular Proliferation. F. Stohlman, Jr., editor. Grune & Stratton, Inc., New York. 260.

7. Saunders, E. F., and A. M. Mauer. 1969. Reentry of non-dividing leukemic cells into a proliferative phase of acute childhood leukemia. *J. Clin. Invest.* **48:** 1299.

8. Gabutti, V., A. Pileri, R. P. Tarocco, F. Gavosto, and E. H. Cooper. 1969. Proliferative potential of out-of-cycle leukaemia cells. *Nature (London).* **224:** 375.

9. Boggs, D. R., M. M. Wintrobe, and G. E. Cartwright. 1962. The acute leukemias. Analysis of 322 cases and review of the literature. *Medicine (Baltimore).* **41:** 163.

10. Hayhoe, F. G. J., D. Quaglino, and R. Doll. 1964. The cytology and cytochemistry of acute leukaemias; a study of 140 cases. *Med. Res. Counc. (G. Brit.) Spec. Rep. Ser.*

11. Japa, J. 1942. A study of the mitotic activity of normal human bone marrow. *Brit. J. Exp. Pathol.* **23:** 272.

12. Sturgeon, P. 1951. Volumetric and microscopic pattern of bone marrow in normal infants and children. I. Volumetric pattern. *Pediatrics.* **7:** 577.

13. Lampkin, B. C., T. Nagao, and A. M. Mauer. 1969. Synchronization of the mitotic cycle in acute leukaemia. *Nature (London).* **222:** 1274.

14. Saunders, E. F. 1970. Effect of L-asparaginase on nucleic acid metabolism of human leukemic cells. *Blood.* **36:** 859. (Abstr.)

15. Saunders, E. F., B. C. Lampkin, and A. M. Mauer. 1967. Variation of proliferative activity in leukemic cell population of patients with acute leukemia. *J. Clin. Invest.* **46:** 1356.

16. Foadi, M. D., E. H. Cooper, and R. M. Hardisty. 1968. Proliferative activity of leukaemic cells at various stages of acute leukaemia of childhood. *Brit. J. Haematol.* **15:** 269.

17. Chan, B. W. B., J. G. J. Hayhoe, and J. A. Bullimore. 1969. Effect of extracorporeal irradiation of the blood on bone marrow activity in acute leukaemia. *Nature (London).* **221:** 972.