JCI The Journal of Clinical Investigation

LEUKEMIC CELL PROLIFERATION AS DETERMINED BY *IN VITRO* DEOXYRIBONUCLEIC ACID SYNTHESIS

C. G. Craddock, G. S. Nakai

J Clin Invest. 1962;41(2):360-369. https://doi.org/10.1172/JCI104490.

Research Article

Journal of Clinical Investigation
Vol. 41, No. 2, 1962

LEUKEMIC CELL PROLIFERATION AS DETERMINED BY *IN VITRO* DEOXYRIBONUCLEIC ACID SYNTHESIS *

By C. G. CRADDOCK and G. S. NAKAI

(From the Department of Medicine, School of Medicine, University of California at Los Angeles, and the Hematology Research Laboratory, Wadsworth Hospital, Veterans Administration Medical Center, Los Angeles, Calif.)

(Submitted for publication July 31, 1961; accepted October 5, 1961)

The immature cells circulating in the blood of leukemic patients are believed to reflect the proliferative activity of the malignant tissue. The rate of DNA synthesis by these cells may provide information as to the rate of growth of leukemic cells, since the DNA synthetic period is believed to occupy a specific interval in the generation cycle of dividing cells. This report gives our findings concerning this parameter of leukemic proliferation, as reflected by the uptake of isotopes into leukemic cell DNA *in vitro*. The results indicate that the conceptualization of the acute leukemic process, as one involving rapid cell growth, must be viewed in the perspective of the total bulk of divisible cells, since the amount of DNA synthesized by acute leukemic cells is often much less than that synthesized by normal immature cells in the same time period. This implies that the generation time of acute leukemic cells is often prolonged.

METHODS

Patients were studied in the untreated state, except in those instances indicated. Blood was withdrawn under aseptic conditions, in the fasting state, into siliconized tubes containing dextran and heparin (without perservative), and the leukocytes separated in the usual way (1). The leukocytes were resuspended in the patients' plasma, and an equal volume of Hank's solution containing the isotopes, was added. Radiophosphorus (P^{32}) was employed at a concentration of 15 mc per 10^9 cells and tritiated thymidine (H^3TDR) (Schwartz BioResearch Laboratories; specific activity, 0.36 c per mmole) at a concentration of 20 μc per 10^9 cells. The mixtures were incubated at 37° C with very gentle, constant shaking. Aliquots were removed at intervals of 1, 5, and 18 to 20 hours.

The cells exposed to H^3TDR were divided into samples for radioautographic analysis and for DNA extraction. P^{32}-labeled DNA was determined as previously described (1, 2). DNA extraction from the cell populations was performed by a modification of the method of Kirby (3). After separation, the leukocytes were washed with 1 per cent citric acid to remove the bulk of hemoglobin and cytoplasm. They were then homogenized in the cold in 6 per cent para-aminosalicylate (PAS) and extracted with an equal volume of phenol (88 per cent) at room temperature for 1 hour. The material was centrifuged in the cold for 1 hour and the top layer, containing DNA, removed. The DNA was precipitated with equal volumes of absolute isopropyl alcohol, resuspended in water, and reprecipitated after the addition of PAS to 6 per cent with 0.54 vol of isopropyl alcohol. The final precipitation was carried out with isopropyl alcohol (0.54 vol) from a 4 per cent sodium acetate solution of the DNA. The reprecipitations provided a final yield of about 60 per cent of the original DNA, with negligible contamination with RNA phosphorus and other non-DNA phosphorus. This procedure also effectively removed any tritium not in DNA and reduced the variables of quenching to a minimum. It was our experience that attempts to quantitate H^3TDR uptake into DNA by counting whole cells or cell fractions (e.g., nuclei) resulted in such highly variable quenching, self-absorption, and non-DNA H^3 contamination that results were difficult to interpret. The isolated DNA, on the other hand, did not exert more quenching than the water solvent at a concentration range up to four times that employed in the experiments.

The extracted H^3-labeled DNA, dissolved in water, was suspended in a scintillator system composed of the following: Hyamine chloride (Rohm & Haas), recrystallized in toluene, 50 g; Thixin (Baker Castor Oil Co., Los Angeles), 30 g; PPO (2,5-diphenyloxazole), 4 g; POPOP [1,4-*bis*-2(5-phenyloxazolyl)-benzene], 100 mg; toluene, 1.54 L (19 ml of this system will hold 0.5 ml of the aqueous sample). Sample volumes of 0.1 or 0.2 ml were employed. Counting time was sufficiently long to give results reproducible within 1 per cent, and quenching was determined on sufficient samples to confirm the fact that it was constant. Counting efficiency with this technique was between 8 and 9 per cent. DNA-P^{32} specific activity was determined by windowless, gas-flow counting, as previously described (1). Specific activities for both P^{32}- and H^3-labeled DNA are expressed as counts per minute per microgram of DNA phosphorus. The H^3 counts are not corrected for loss due to inefficiency and are not expressed as counts per millimole of

* This work was supported in part by the U. S. Public Health Service, under Contract H-1069, and the California Institute of Cancer Research, and by a grant from Parke, Davis & Company.

DNA, and hence appear low in comparison to other reports employing similar techniques. The reason for not converting the results to this expression is that two additional variables, the phosphorus/DNA ratio, and the DNA content per cell, are imposed.

Smear preparations on glass slides coated with gelatin (0.05 g gelatin in a 6.5 per cent aqueous solution of chrome alum) were used for radioautography. The slides were initially fixed in methanol and subjected to acid hydrolysis before applying Kodak AR-10 stripping film (Kodak, Ltd., London). Subsequent experience showed that Carnoy's solution as a fixative eliminated the necessity of additional acid hydrolysis, removed red cells from the slide and provided better morphology. The slides were developed after 30 days' exposure and stained with Giemsa or Leishman-Giemsa. Accurate total nucleated cell counts and differential counts were recorded on all patients at the time of the study. The extent of labeling was recorded both in terms of the percentage of all cells which were labeled (counting 600 cells) and the number of labeled cells in each of four ranges of grain count above background. These were 2 to 4 grains, 5 to 10 grains, 11 to 20 grains, and 21 grains. The distribution of grains was also determined by counting 100 labeled cells for each point. The grains over the cell nucleus have been shown to represent label in DNA by

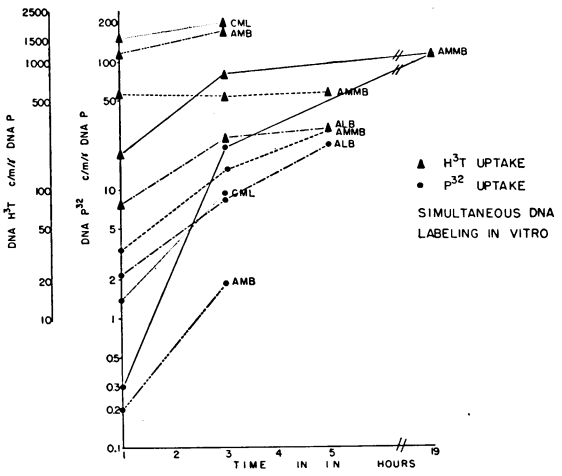

FIG. 1. COMPARATIVE RATES OF INCORPORATION OF H^3TDR (▲) AND P^{32} (●) INTO THE DNA OF LEUKEMIC BLOOD CELLS FROM FOUR CASES OF LEUKEMIA. The comparative uptake curves were obtained from aliquots of the same cell population, obtained at the same time and incubated under identical conditions. Paired samples are indicated by the type of line between points. For abbreviations, see text.

TABLE I

Uptake of H^3TDR and P^{32} by blood cells of patients with chronic myelogenous (CML) and myeloblastic (AMB) leukemia

Disease		H^3T radioautographs				P^{32} uptake in DNA			H^3T uptake in DNA	
		Immature cells	Total population labeled	Immature cells labeled	Avg. grains per labeled cell*	1 hr	3 hrs	19 hrs	1 hr	3 hrs
		%	%	%		*cpm/μg DNA-P*			*cpm/μg DNA-P*	
CML	1	25					12.6	44.7		
	2	5	2.5	50	18.2		2.4	12.3		
	3	8					15.6			
	4	25	5.8	23	20.2	1.4	9.5		1,508	2,094
		22	6.0	27	17.5		7.1			2,675
	5	15	3.4	24	18.0		8.6			
	6	8	4.5	18	16.0	7.6	16.1			
	7	23	12.0	52	20.0		16.8	17.1		
	8	8	6.0	75	18.5	1.5	6.3	55.5		
	9	8	7.0	88	17.0	2.0	12.5			
	10	22	7.4	34	14.8		7.2			1,098
Mean		15	6.1	42	17.8	3.1	10.5	32.4	1,508	1,956
AMB	1	66	8.0	12	14.2	0.2	1.9		414	2,290
		70	7.0	10	9.9	0.2	1.5		1,170	1,880
	2	70	8.1	12	14.3		2.3			634
	3	90	3.0	3	18.4	0.0	10.9			959
	4	98	15.0	15	7.5					
	5	40	12.0	30	18.0					
	6	71	7.0	10	15.0		5.4	23.1		
	7	68	12.0	18	12.0	1.9	9.5	42.0		
Mean		72	9.0	14	13.7	0.58	5.2	32.6	792	1,441
	8†	98	50–100?	50–100?	1–3?	54.7	96.5			

* Cells with grain counts in excess of 21 were counted as 21 grains per cell.
† Patient in terminal blastic crisis.

TABLE II

Uptake of H^3TDR and P^{32} by blood cells of patients with chronic lymphocytic (CLL) and lymphoblastic leukemia (ALB)

Disease	H^3T radioautographs				P^{32} uptake in DNA				H^3T uptake in DNA		
	Immature cells (approx.)	Total population labeled	Immature cells labeled	Avg. grains per labeled cell*	1 hr	3 hrs	5 hrs	19 hrs	1 hr	3 hrs	5 hrs
	%	%	%			*cpm/μg DNA-P*				*cpm/μg DNA-P*	
CLL 1	4	0.05	1.25	18.2		0.9		1.7			
2	3	1.80	60.0	12.0	0.3	1.1					
3	5	1.10	25.0	10.8	0.3	1.5		1.5			
4	2	0.06	3.0	15.5		0.9				12.0	
5	1	0.00	0.0			0.2		0.9			
6	4	1.00	25.0	19.8	1.0	1.8			8.5	18.5	
7						0.6					
8						2.1		1.8			
9						0.6					
10						1.8		0.9			
11						0.6		0.6			
Mean	3	0.67	20.9	15.3	0.5	1.1		1.2	8.5	15.2	
ALB 1	86	4.0	4.6	8.2		6.8		6.8			
2	96	2.0	2.08	4.1	0.1	3.1			95	611	
	95	2.2	2.3	3.0		4.5				387	
3†	40	0.3	0.75	11.5	0.2	3.6	10.0				
	80				2.2	8.5	23.0		78	252	306
4	92	5.0	5.4	6.6							
5	85	1.2	1.4	6.5		1.2					
Mean	82	2.5	2.8	6.7	0.8	4.6	16.5		86.5	416.7	
6‡	99	18.0?	18.1?	6.5		10.9					

* Cells with grain counts in excess of 21 were counted as 21 grains per cell.
† Patient ran chronic course.
‡ Patient in terminal blastic crisis.

TABLE III

Uptake of H³TDR and P³² by blood cells of patients with myelomonoblastic (AMMB) leukemia

Disease	H³T radioautographs				P³² uptake in DNA			H³T uptake in DNA			
	Immature cells (approx.)	Total population labeled (approx.)	Immature cells labeled (approx.)	Avg. grains per labeled cell*	1 hr	3 hrs	5 hrs	19 hrs	1 hr	3 hrs	5 hrs	19 hrs
	%	%	%		*cpm/μg DNA-P*				*cpm/μg DNA-P*			
AMMB 1	48	19.3	40.2	3.4		24.3		75.1				
	60	5–6?	11–12?	2.0		2.8		14.7				
2	95	7?	7–8?	11.6	0.3	21.6		116.9	191	814		1,191
	99	7?	7–8?	7.4		32.8				1,081		
3	93	20?	22?	3.0	1.4	6.6						
4	81	15?	20?	5.0		8.9			558	548	584	
5	54	3.0	5.9	19.7	3.4	14.8	29.1					
6	91	8?	9?	7.0		23.5						
7	83	17?	20?	3.5	4.0	24.5						
Mean	78	3 – 20	5.9 – 40.2	7	2.3	17.8	29.1	68.9	374.5	814.3	584	1,191
8†	90	30?	33?	1 – 4		90.1						

* Cells with grain counts in excess of 21 were counted as 21 grains per cell.

† Patient in terminal blastic crisis on 6-MP and steroids.

acid hydrolysis and treatment of slides with ribonuclease and deoxyribonuclease.

In the results below, the radioautographic data were determined after 3 hours' incubation with H³TDR. Results after 1 hour and 19 hours were essentially the same.

RESULTS

The time course of H³TDR and P³² uptake into DNA by the same cell population, under identical conditions, differs in every cell system yet studied. Some examples of this are shown in Figure 1. The initial uptake of H³TDR is more rapid, the curve tending to plateau after the first hour. P³² uptake follows a more exponential curve, as would be expected if the endogenous rate of DNA synthesis in the continued presence of the label is not being altered by the experiment.

The results of *in vitro* labeling of DNA by H³TDR and P³² are shown in Tables I, II, and III. The radioautographic data provide, in some instances, an indication of the fraction of each cell population studied that is participating in DNA synthesis. However, it will be noted that, in some cases of myeloblastic leukemia (AMB), and many of lymphoblastic (ALB) and myelomonoblastic (AMMB) leukemia, the grain count per labeled cell was so low that grain counts could not be accurately expressed (i.e., many cells with labeling only questionably above background). Nevertheless, it is probable that this pattern of low labeling by many cells is the true pattern of DNA labeling in many acute leukemias, in view of the significant and occasionally marked uptake of the labels into DNA by the population as a whole. It may be argued that the failure to see highly labeled cells under these circumstances is due to some peculiar physical property of the leukemic cells that prevents the weak beta emission of tritium from reaching the photographic film. However, the frequency with which this pattern was observed in different types of leukemic populations, as well as correlation with the data obtained from extracted DNA, suggests that it is not the result of such technical factors.

In those instances where possible, the extent of labeling, as estimated by grain count distribution, is expressed as the average number of grains per cell. A maximum of 21 grains per cell was scored; cells with more than 21 grains were counted as containing 21 grains. This cutoff point

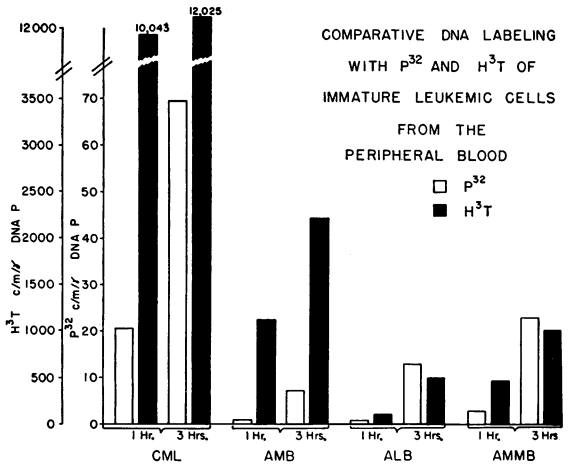

FIGURE 2. COMPARATIVE INCORPORATION OF H^3TDR AND P^{32} INTO LEUKEMIC BLOOD CELLS, EXPRESSING THE MEAN VALUES GIVEN IN TABLES I, II, AND III IN TERMS OF THOSE CELLS THEORETICALLY CAPABLE OF PROLIFERATION, AS DERIVED FROM THE PERCENTAGE OF IMMATURE CELLS IN THE POPULATION SAMPLE.

was arbitrarily made to simplify counting, since it was apparent that those populations with predominantly low-labeled cells rarely showed cells that exceeded 20 grains. It will be noted that cells from the acute leukemias generally show a lower average grain count, per labeled cell, than do cells from chronic myelogenous leukemia (CML) or chronic lymphocytic leukemia (CLL). The difference between the means (plus 1 standard deviation) of CML and CLL on the one hand, and between ALB and AMMB on the other, are highly significant ($p < 0.001$). The difference between AMB and CML is significant at $p < 0.01$.

In the case of CLL, it is very difficult to determine the percentage of potentially proliferative cells morphologically. The percentage of labeled cells is always extremely low, usually less than 1 per cent; however, it is apparent that the few cells which incorporate the label become highly labeled in comparison with the cells of acute leukemias.

The percentage of immature cells (myeloblasts, promyelocytes and myelocytes) which incorporate the H^3TDR in CML is about 40 per cent. This percentage labeling is comparable to that of normal marrow myeloid precursors in our laboratory. Others have reported a higher labeling index for normal marrow (4). In any event, the contrast between CML blood and normal marrow on the one hand, and acute leukemic blood cells on the other, is very obvious. This is especially noted in ALB, where only one patient showed more than 5 per cent of the blast cells labeled. This patient (ALB no. 6; Table II) was in terminal blastic crisis, with a steadily increasing leukemic cell mass at the time of study.

The results with radioautographic quantitation of DNA labeling with H^3TDR, have been correlated, where possible, with P^{32} and H^3 labeling of DNA, as determined by chemical extraction. If the specific activity of the DNA label, as determined by extraction of the DNA from the entire cell sample, is expressed in terms of those cells *theoretically* capable of division (i.e., immature cells which can synthesize DNA), the differences between CML cells and acute leukemic cells become quite marked (Figure 2).

Three patients with CML have been studied serially during the transition from the chronic to the acute form of the disease. Each has shown a change in the labeling pattern to that seen in

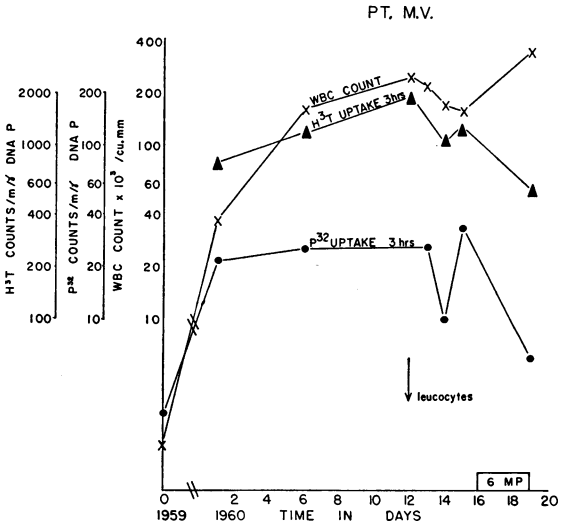

FIG. 3. SERIAL STUDIES OF THE *in vitro* INCORPORATION OF H^3TDR AND P^{32} INTO THE DNA OF THE LEUKEMIC CELLS OF A PATIENT WITH ACUTE MYELOMONOBLASTIC LEUKEMIA. The grain count score throughout did not change significantly, although the percentage of immature cells was less (25 per cent) in 1959, as compared with 1960 (85 per cent). During the period of progressive increase in cell numbers in 1960 (Days 2 to 12), the extent of DNA labeling did not change significantly. The sharp drop after Day 12 was related to the intravenous administration of normal leukocytes (indicated by the arrow).

AMB. Several patients with acute leukemia have been followed as the disease became more blastic. In those instances in which the pattern of *in vitro* DNA labeling could be established, the change observed was a progressive increase in the total number and percentage of immature cells which were labeled, rather than any obvious change in the extent of DNA labeling per cell. An example of this is depicted in Figure 3.

The effect of therapy on the DNA labeling process has been extremely variable in our hands. In the acute leukemias, our data are insufficient to be definitive, but 6-mercaptopurine (6-MP) has had no consistent effect on H^3TDR labeling, provided immature cells are still present. Myleran (busulfan) therapy in CML has, in most instances, suppressed DNA synthesis before any appreciable alteration in the blood picture. In one patient, who was followed serially during the favorable response to X-ray directed to the spleen only, the total white blood cells and the percentage of immature cells fell steadily, but those immature cells remaining showed the same pattern of DNA labeling observed before therapy (Figure 4).

Additional data concerning the effect of X-ray on H^3TDR uptake are shown in Table IV. In this experiment thoracic duct lymph was obtained from a normal dog and divided into 8 aliquots of 4.5 ml (3 control samples, 5 irradiated samples). The experimental samples were irradiated *in vitro* in 5-ml screw-cap bottles in continuous rotation.[1] After completion of the X-ray exposure, all samples were inoculated with H^3TDR simultaneously and examined radioautographically at the intervals shown. The percentage of the total cell population and the percentage of large

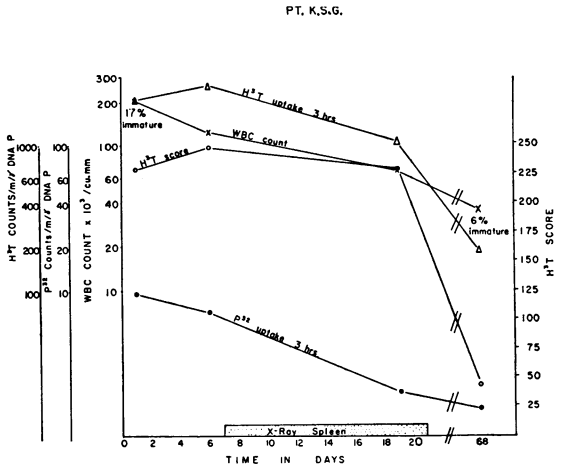

FIG. 4. SERIAL STUDIES OF H^3TDR AND P^{32} LABELING OF DNA *in vitro* IN A CASE OF CML UNDERGOING SPLENIC IRRADIATION. The fall in WBC count and percentage of blasts was accompanied by a fall in total DNA labeling, but those immature cells remaining showed no significant change in H^3TDR uptake, as judged by the radioautographs (H^3T score) and by correction of the specific activity curves for the reduced percentage of immature cells in the population sample.

[1] The irradiation procedure was directed by Dr. Benedict Cassen, Department of Nuclear Medicine and Radiation Biology, University of California at Los Angeles, whose help is gratefully acknowledged.

TABLE IV

DNA labeling with H^3TDR by dog thoracic duct lymphocytes after in vitro exposure to X-ray

Amount of X-ray exposure (in roentgens)*	Total population labeled†		Immature cells labeled		Immature cells degenerated		Immature cells degenerated and labeled	
	1 hr	24 hrs	1 hr	24 hrs	1 hr	24 hrs	1 hr	24 hrs
	%	%	%	%	%	%	%	%
0	7.4	8.8	80	92	6	52	5	50
0	10.0	6.3	81	80	12	49	10	30
0	11.8	11.3	90	90	10	36	4	26
100	7.9	7.4	98	66	2	50	6	20
200	8.3	3.7	70	52	26	61	13	21
400	7.6	6.9	72	72	21	45	5	26
1,000	9.7	7.1	73	60	20	46	11	32
2,000	10.9	6.9	70	69	16	70	6	40

* The control and irradiated samples are aliquots of the same lymph collection, hence the cells are of the same average age. H^3TDR was added at the same time to all aliquots; the isotope was added in equal amounts on the basis of cell number.

† Heavily labeled cells (>21 grains per nucleus).

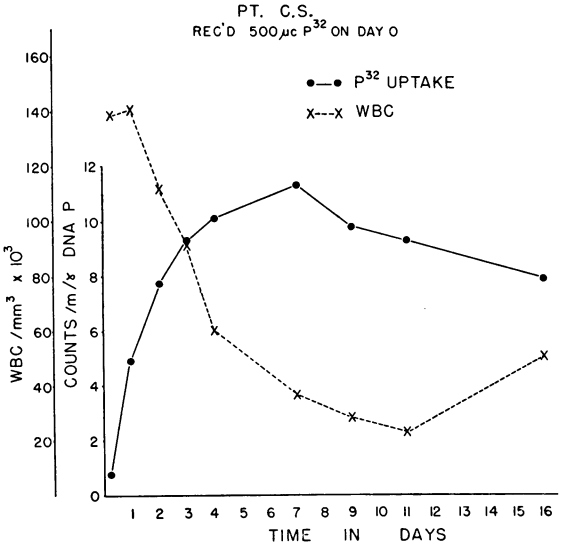

FIG. 5. *In vivo* INCORPORATION OF P^{32} INTO DNA OF LEUKEMIC CELLS (ACUTE MYELOBLASTIC LEUKEMIA) AFTER A SINGLE INTRAVENOUS DOSE OF P^{32}. Note the continued rise in specific activity for the first 7 days and the continued presence of label in spite of reduction in cell numbers in peripheral blood due to 6-MP therapy between Days 1 and 10. The curve indicates mixing of cells labeled on Day 0 with unlabeled cells in the blood and persistence of some labeled cells in spite of therapy.

cells (immature precursors) which were highly labeled were determined. The large cells were further categorized as to the extent of cellular degeneration, as manifest by loss of cellular integrity and basket-cell forms, occurring over the 24-hour incubation. It will be noted that there was no significant difference between the controls and the irradiated samples at any dose level in so far as the extent of labeling was concerned.

DISCUSSION

The evidence presented suggests, contrary to popular opinion, that the acute leukemic cell requires a longer time for DNA replication and proliferation than most normal hemic precursors and the proliferating cells in CML and CLL. If this is so, then an attempt must be made to relate this observation to other features of the disease.

Previous studies of patients with acute leukemia, employing P^{32} labeling of leukocytes *in vivo,* have shown a prolonged persistence of the DNA label (1, 2, 5). An example of this in a patient with established myeloblastic leukemia (Patient 3, Table I) is shown in Figure 5. The fall in white blood cell count shown was a result of 6-MP therapy, with no concomitant fall in the DNA label in the population as a whole. This phenomenon could be explained in several ways: *1*) the cells that incorporate the label soon after its injection remain in the circulating blood for a long time, without dividing; *2*) the labeled cells divide many times, the DNA label being retained by successive generations; *3*) the labeled DNA is reutilized as such, hence, is not lost with the death of the labeled cell.

This last possibility would seem unlikely as a major factor in DNA metabolism, since labeling would have been blocked by the reutilized, unlabeled DNA at the time the isotope was given. The data presented in this report and in those of others (6) would support the first explanation, since they do not indicate a rapid generation time for the bulk of leukemic blasts in the circulation. The type of data shown in Figure 5—i.e., the persistence of the DNA label, in spite of interference with growth processes by 6-MP—is difficult to explain by any mechanism other than prolonged survival of some of the immature cells which initially acquired the DNA label.

Our studies on leukemic cells in marrow preparations, like those of Gavosto, Maraini and Pileri (6), show similar results with cells from the marrow environment. Therefore, the apparently paradoxical situation exists in some acute leukemias of a rapidly expanding cell population, but a slow reproductive rate per cell. Indeed, the majority of acute leukemic cells, capable of eventual division, are in the "resting" or G-1 (interphase) state at any one time. When one considers the enormous bulk of potentially divisible leukemic cells, and the fact that, with logarithmic growth, one cell will provide 4.295×10^9 cells after 32 divisions, it is not difficult to reconcile these findings. The intermitotic interval of acute leukemic cells could be many times longer than that of normal precursors and still retain a marked growth advantage because of: *1*) the massive numbers of potentially divisible cells; *2*) the failure of maturation of leukemic blasts and retention of "stem cell" proliferative capacity; and *3*) the indefinite longevity of the leukemic blast, as contrasted with the short survival time of normal granulocytes.

This interpretation would be consonant with the findings of a low mitotic index and stathmokinetic index in acute leukemia (7), without assuming that most acute leukemic cells cannot divide at all. If the mitotic time (i.e., the time the cell is in visible mitosis) of leukemic cells undergoing division is approximately the same as that for normal cells (45 minutes), the markedly prolonged G-1 period would reduce proportionately the number of mitoses visible at any one moment.

The findings, with respect to DNA synthesis by immature cells in CML blood, are similar to those of others and are comparable to the kinetics of normal granulocytic precursors in the marrow. This "extension" of the marrow precursors into the blood and tissues in CML would explain both the *in vitro* findings reported here and the *in vivo* data previously presented (1, 2, 5). Similarly, from a kinetic viewpoint, the picture in CLL seems to be an expansion of the normal proliferative lymphatic activity. In both CML and CLL, there is found a relatively small portion of immature cells, rapidly growing, maturing, functioning, and dying, along roughly normal lines. Both of these conditions would be expected to respond quickly to X-ray or radiomimetic drugs. Acute leukemia, on the other hand, would be expected to be resistant to this type of therapy, since the majority of the cells capable of proliferation are in a "resting" state at any one moment. However, when acute leukemic cells are eliminated by some other means (i.e., adrenal steroids, antimetabolites), the remaining leukemic cells are "outgrown" by the remaining normal precursors, which have an inherently shorter generation time. Eventually, the leukemic cells will reassume numerical superiority.

These data lend support to the widely accepted view that the acute leukemias represent a basic disturbance in cell maturation and are quite different from the chronic leukemias in pathogenesis.

The data on the kinetics of H^3TDR and P^{32} labeling of DNA indicate a difference in the rate of uptake of the two labels into DNA (Figure 1). Since this difference has been observed in all cell systems studied to date (including normal canine marrow and lymph), it is probably not related to peculiarities of a particular type of cell. The slower uptake rate for P^{32} in contrast with H^3TDR may reflect an intracellular phosphate pool which is large relative to that for TDR. The latter is probably small, since TDR is not on the normal pathway of endogenous thymidylic acid formation. However, this does not explain the rapid attainment of maximum uptake of H^3TDR with little further increase in specific activity with prolonged incubation. This phenomenon may well reflect an alteration in DNA synthesis induced by the added thymidine. Thymidine enters the DNA polymer, along with the deoxynucleosides, deoxycytidine, deoxyguanosine, and deoxyadenosine, after phosphorylation to the triphosphates. Thymidine is shunted into the system, circumventing the methylation of deoxyuridilic acid to deoxythymidylic acid, as Friedkin and Kornberg have shown (8, 9). The latent availability of endogenous thymidine, therefore, makes its presence rate-controlling in the sense that when it becomes available, it is rapidly phosphorylated and polymerized into DNA with the other three nucleotide-triphosphates. If this is so, then all cells studied thus far have some intracellular pools of deoxycytidine, deoxyguanosine, and deoxyadenosine. Recent work by Canellakis and co-workers (10), Bollum and Potter (11), Hiatt and Bojarski (12), and Weissman and associates (13) has shown that the presence of the kinases responsible for the phosphorylation of thymidine and the subsequent polymerization of DNA is characteristic for growing tissue, in contrast to nonproliferating tissue. These studies indicate an important role of thymidine and the enzymes concerned with its conversion to the triphosphate in the last stage of DNA synthesis. The work of Weissman, Smellie and Paul (13) suggests that thymidine may induce the appearance of these kinases. Additional evidence for this from another approach is the work of Greulich, Cameron and Thrasher, which shows an increase in mitotic index in mouse intestinal mucosa after thymidine injection (14).

This influence of thymidine on DNA synthesis must be considered in any study of DNA synthesis rate, and it is apparent that the *rate* of uptake of thymidine soon after inoculation of a cell population is not a measure of endogenous DNA synthesis. Rather it is a reflection of several factors, among which are: *1*) the extent of intracellular pools of deoxycytidine, deoxyguanosine, and deoxyadenosine; *2*) the activity of the

phosphorylating kinases within the cell; and *3*) the activity of DNA polymerase.

In spite of these difficulties in using H^3TDR incorporation into DNA as a rate indicator, there was good agreement between the P^{32} and H^3 DNA labeling after 3 hours' incubation, in all instances where the studies were done before the institution of therapy with 6-MP or other agents. In four instances in which the cells were collected soon after the institution of 6-MP therapy, the P^{32} uptake was suppressed more than was the H^3TDR uptake, an effect which might be expected from this drug. The results to date, therefore, indicate that the *extent* of DNA labeling with H^3TDR after 3 hours, rather than the rate at which H^3TDR enters DNA, reflects the ability of the cell to perform the last steps of DNA synthesis, in the absence of factors which would interfere with earlier steps in purine and pyrimidine metabolism.

The paucity of highly labeled cells in some acute leukemic populations is noteworthy in this regard. Assuming that this finding is not due to an artifact, as previously considered, it is difficult to explain on any basis other than a long over-all DNA synthetic period. Normal and CML myeloid precursors and normal and CLL lymphoid precursors seem to rapidly double their DNA content when thymidine becomes available. Acute leukemic cells, on the other hand, rapidly polymerize a small amount of DNA when thymidine is made available. Since each cell is believed to double its DNA content before division, the paucity of acute leukemic cells, which are highly labeled, may indicate that those acute leukemic cells which are nearest to the mitotic period (i.e., have almost completed DNA synthesis) have already polymerized most of the diploid amount of DNA and can take up only a small additional amount after exposure to thymidine. This interpretation implies a gradual build-up of polymerized DNA with a greatly prolonged total S period. An alternative explanation would be a failure of acute leukemic cells to enter division after DNA doubling and the development of polyploidy. Most studies of the per cell DNA content of acute leukemic cells, however, have shown a distribution similar to that for normal proliferating tissue (15), and this could be the case regardless of the duration of the S period.

Additional evidence of the low proliferative activity of some acute leukemic cells (in ALB and AMMB) has been obtained by assays of the extent of DNA polymerization in ultracentrifuged extracts of cells, employing a modification of the method described by Smellie, Kier and Davidson (16). The results, which will be reported separately, indicate a low activity of one or more enzymes concerned with nucleotide polymerization into DNA (thymidine kinase, thymidine-5′-phosphate kinase, thymidine-5′-pyrophosphate kinase or polymerase) in ALB and AMMB cells, as compared with CML cells and normal myeloid and lymphoid precursors.

It cannot be overemphasized that thymidine uptake into DNA reflects only the last steps of DNA synthesis and, in short-term incubations, does not depend upon on-going DNA synthesis at an earlier level. Indeed, polymerization of DNA will occur in *in vitro* systems without the presence of cells, as discussed above. Injury to the cell, which is sufficient to lead to its destruction, as by X-ray, will not necessarily curtail this phase of DNA synthesis. The work of Das and Alfert (17) indicates that H^3TDR incorporation is actually increased by irradiation. These authors employed onion-root tip cells, and the observations with canine lymphocyte precursors reported herein would support these findings. The relevance of these data to the present subject is to emphasize the pitfalls one may encounter in the interpretation of this phenomenon and the need to relate it to other parameters of cell viability and proliferative activity.

SUMMARY AND CONCLUSIONS

A technique for quantitating the uptake of P^{32} and H^3TDR into DNA of normal and leukemic blood cells *in vitro* is described. Correlation of the extent of DNA labeling by these two isotopes with cell morphology and radioautographic determination of cell labeling suggests that, in many instances, leukemic blast cells have a much longer generation time than do normal or chronic leukemic precursor cells. In some instances, the majority of leukemic blasts are in interphase (G-1) at any one moment. The slow proliferative rate and the failure of cell maturation and differentiation impart a prolonged "survival" time to

leukemic blasts compared with normal, "expendable" leukocytes.

Distinct differences between the rate of uptake of H^3TDR and P^{32} were observed in all cell systems studied. The implications of these differences and the influence of thymidine on DNA polymerization are discussed. The alteration of DNA synthesis rate at various stages of the leukemic process and the influence of therapy on the phenomenon are described briefly.

REFERENCES

1. Craddock, C. G., Jr. The physiology of granulocytic cells in normal and leukemic states. Amer. J. Med. 1960, **28**, 711.
2. Perry, S., Craddock, C. G., Jr., and Lawrence, J. S. Rates of appearance and disappearance of white blood cells in normal and in various disease states. J. Lab. clin. Med. 1958, **51**, 501.
3. Kirby, K. S. A new method for the isolation of deoxyribonucleic acids: Evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem. J. 1957, **66**, 495.
4. Cronkite, E. P., Bond, V. P., Fliedner, T. M., and Killmann, S-A. The use of tritiated thymidine in the study of haemopoietic cell proliferation *in* Ciba Foundation Symposium on Haemopoiesis, G. E. W. Wolstenholme and M. O'Connor, Eds. London, Churchill, 1960, p. 70.
5. Craddock, C. G. Production and distribution of granulocytes and the control of granulocyte release *in* Ciba Foundation Symposium on Haemopoiesis, G. E. W. Wolstenholme and M. O'Connor, Eds. London, Churchill, 1960, p. 237.
6. Gavosto, F., Maraini, G., and Pileri, A. Nucleic acids and protein metabolism in acute leukemia cells. Blood 1960, **16**, 1555.
7. Astaldi, G., and Mauri, C. Recherches sur l'activité proliférative de l'hémocytoblaste de la leucémie aiguë. Rev. belge Path. 1953, **23**, 59.
8. Friedkin, M. Speculations on the significance of thymidylic acid synthesis in the regulation of cellular proliferation *in* The Kinetics of Cellular Proliferation, F. Stohlman, Ed. New York, Grune & Stratton, 1960, p. 97.
9. Friedkin, M., and Kornberg, A. The enzymatic conversion of deoxyuridylic acid to thymidylic acid. Fed. Proc. 1959, **18**, 230.
10. Canellakis, E. S., Jaffe, J. J., Mantsavinos, R., and Krakow, J. S. Pyrimidine metabolism. IV. A comparison of normal and regenerating rat liver. J. biol. Chem. 1959, **234**, 2096.
11. Bollum, F. J., and Potter, V. R. Nucleic acid metabolism in regenerating rat liver. VI. Soluble enzymes which convert thymidine to thymidine phosphates and DNA. Cancer Res. 1959, **19**, 561.
12. Hiatt, H. H., and Bojarski, T. B. Stimulation of thymidylate kinase activity in rat tissues by thymidine administration. Biochem. biophys. res. Com. 1960, **2**, 35.
13. Weissman, S. M., Smellie, R. M. S., and Paul, J. Studies on the biosynthesis of deoxyribonucleic acid by extracts of mammalian cells. IV. The phosphorylation of thymidine. Biochim. biophys. Acta 1960, **45**, 101.
14. Greulich, R. C., Cameron, I. L., and Thrasher, J. D. Stimulation of mitosis in adult mice by administration of thymidine. Proc. nat. Acad. Sci. (Wash.). 1961, **47**, 743.
15. Hale, A. J., and Wilson, S. J. The deoxyribonucleic acid content of the nuclei of leukaemic leukocytes. Lancet 1960, **1**, 577.
16. Smellie, R. M. S., Kier, H. M., and Davidson, J. N. Studies on the biosynthesis of deoxyribonucleic acid by extracts of mammalian cells. I. Incorporation of [^{3}H]thymidine. Biochim. biophys. Acta 1959, **35**, 389.
17. Das, N. K., and Alfert, M. Accelerated DNA synthesis in onion root meristem during x-irradiation. Proc. nat. Acad. Sci. (Wash.) 1961, **47**, 1.