Reentry of Nondividing Leukemic Cells into a Proliferative Phase in Acute Childhood Leukemia

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ABSTRACT Reentry of small leukemic blast cells into a proliferative phase was demonstrated in a 3 yr old girl with untreated acute lymphoblastic leukemia. Since the proliferating leukemic cell compartment in this disease is not self-maintaining, continual entry of cells into this compartment is necessary to prevent depletion of proliferating cells. In order to identify the source of replacement cells, the rate of change of tritiated thymidine–labeled cells in the proliferating compartment was observed by means of serial bone marrow samples under two conditions. In the first study period only 10% of small leukemic blast cells were labeled, and in the second study period 72% of this population had become labeled. During the first period the proliferating blast cells were rapidly replaced by unlabeled cells, while during the second period the replacement cells were coming largely from a labeled cell source. The only identifiable source of cells for maintenance of the proliferating population which was virtually unlabeled during the first period and largely labeled during the second period was the population of small leukemic blast cells.

The finding that the small blast cells are only temporarily nonproliferative could account for effectiveness of therapy directed primarily against a dividing cell population. Persistence of some cells with longer resting times into remission could provide a focus for subsequent relapse.

INTRODUCTION

Two characteristic phases of leukemic blast cell activity have been proposed for acute childhood leukemia: one actively proliferative and the other nonproliferative (1, 2). By means of in vivo labeling with tritiated thymidine, the cells in the actively proliferative phase have been identified as being large with fine nuclear chromatin. The cells which could not be demonstrated by these studies to be involved in proliferative activity were smaller cells with dense coarse nuclear chromatin. The proliferating blast cells divided one or more times in the bone marrow and then became smaller as they apparently stopped further division and joined the nonproliferating blast cell population.

The total proliferative activity of the marrow leukemic cells in acute leukemia is directly related to the proportion of these two populations and varies greatly from patient to patient, and in the same patient from one stage of the disease to another (3). The population of small leukemic cells may constitute as much as 95% of the marrow leukemic cells in some patients.

It is important to define the nature of these small leukemic cells because of their relationship to concepts of pathogenesis and therapy for this disease. The small cell may be an end stage cell destined for death as is a neutrophil. On the other hand, as the lymphocyte, it may be a resting cell, temporarily nonproliferating but capable of reentering a phase of cell division. The nonproliferating cells from mouse ascites tumors, for example, have been shown to be capable of resuming cell division when transplanted to a new host animal (4).

Gavosto, Pileri, Gabutti, and Masera (5, 6) have demonstrated that during steady-state conditions the large blast cell population is incapable of maintaining itself because loss of cells to the small blast cell population is greater than the number of cells produced by cell division. New cells must therefore continually enter the large blast cell compartment to prevent its depletion. Thus after a single injection of tritiated thymidine, labeled large blast cells are rapidly lost from the proliferating compartment with replacement by unlabeled

This work was presented at the Sixtieth Annual Meeting of The American Society for Clinical Investigation, May 1968.

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Received for publication 28 October 1968 and in revised form 26 March 1969.

cells (1, 5). These replacement cells could come from reentry of cells from the small blast cell population or could conceivably come from continual leukemic transformation of normal cells.

The following study was done to determine if small leukemic cells can return to a proliferating large cell phase. Changes in the per cent of labeled cells in the proliferating cell compartment were observed under two conditions: first when most small blast cells were unlabeled, and second when most small blast cells were labeled. If the proliferating cell compartment was maintained by reentry of the small blast cells, the rate of change in concentration of labeled large cells would be different under the two conditions. Recycling of unlabeled small blast cells into the labeled proliferating cell population would result in a progressive decrease in concentration of labeled cells. In contrast, recycling of labeled small blast cells would not decrease the concentration of labeled large cells.

METHODS

The patient in whom the study was done was a 3 yr old girl with untreated acute lymphoblastic leukemia. She had been asymptomatic until 10 days before admission when she developed unexplained fever. 4 days before admission a petechial rash was noticed by the parents. On admission she did not appear acutely ill and had a sparse petechial rash. The liver was felt 3 cm below the right costal margin, and the spleen was palpable 2 cm below the left costal margin.

Her hemoglobin was 8.4 g/100 ml, and the volume of packed red cells was 26%. The white blood count was 3300/mm^3 with 9% neutrophils, 80% lymphocytes, and 11% lymphoblasts. The platelet count was 41,000/mm^3, and the reticulocyte count was 2.4%. The bone marrow was 98% replaced by lymphoblasts.

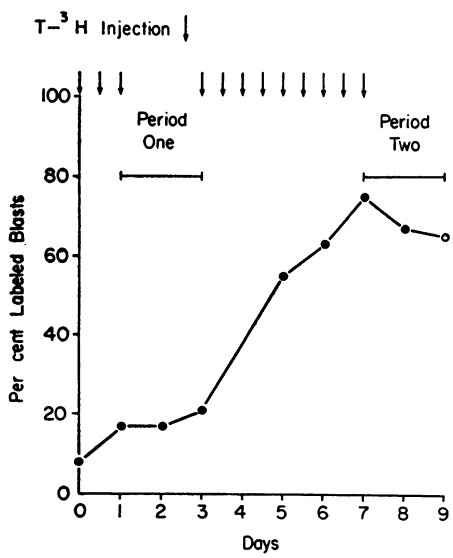

FIGURE 1 Experimental design and labeling indices of marrow during tritiated thymidine administration.

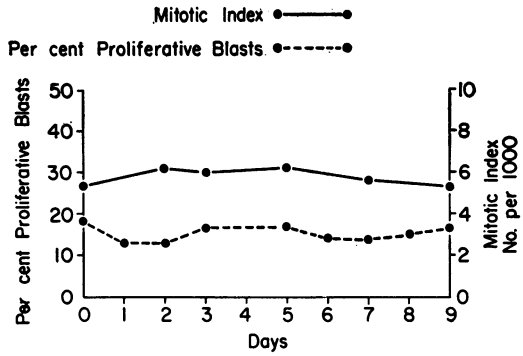

FIGURE 2 Steady state of marrow proliferative activity.

Informed consent was obtained from the parents for the study. No medications or blood transfusions were given before or during the course of the study.

The experimental design is illustrated schematically in Fig. 1. On the 1st day, three injections of tritiated thymidine (specific activity 360 mc/mmole) were given intravenously at 12-hr intervals, 100 μc/kg body weight. Serial bone marrow samples were obtained during the following 48 hr observation period. A second course of nine injections was then given in a similar manner during the 4th–7th day, inclusively. Serial bone marrow samples were obtained during this second course of injections and throughout the 48 hr observation period thereafter. The total dose of tritiated thymidine was 1.2 mc/kg body weight.

Radioautographs were prepared from the marrow samples with Kodak AR 10 stripping film (7). The film was exposed for 22 days. After they were developed, the radioautographs were stained with Wright's stain. Cells with five or more grains over the nucleus were considered labeled. 2000 blast cells were counted per marrow sample. Classification as to proliferative (large) or nonproliferative (small) cells was made according to nuclear size and the character of the nuclear chromatin pattern. For the two samples obtained at the beginning of the two observation periods nuclear size was measured by a micrometer, and the per cent of cells labeled within each nuclear size category noted, based on 200 blast cells per sample. The labeled mitotic figure data were based on counting 50 mitoses per sample. The mitotic indices were determined by the acetocarmine method of Japa (8) from counting 10,000 nucleated cells. Mean grain counts and grain count distributions were obtained from 200 to 400 labeled cells in each size category per sample. A base line labeling index was obtained before the study by in vitro incubation of marrow with tritiated thymidine, 1 μc/ml for 1 hr at 37°C. In vitro and in vivo labeling indices are similar (3).

RESULTS

The over-all labeling of marrow cells throughout the entire study period is shown in Fig. 1. The base line labeling index was 8% which increased to 17% after the three injections of tritiated thymidine. There was little change thereafter until the second series of injections was begun when an increase to a peak value of 75% was observed. After the injections were discontinued, a slight decrease in per cent of labeled cells occurred.

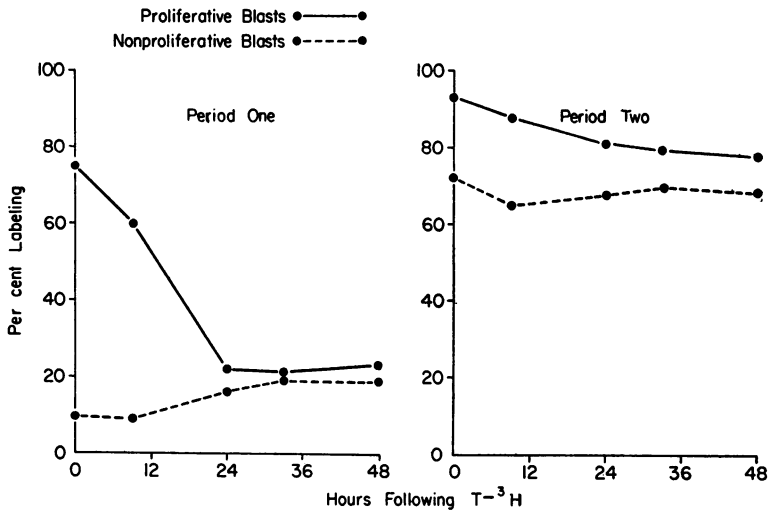

FIGURE 3 Labeling of proliferative (large) and nonproliferative (small) blast cells of marrow.

There was little variation in marrow proliferative activity during the study period as shown in Fig. 2, a finding indicating a steady state. The average mitotic index with 1 SD was 5.8 ±2.3/1000, and the variations observed were not statistically significant. The size of the proliferative population as indicated by the proportion of large blast cells varied insignificantly around a mean value with 1 SD of 15 ±3%.

Changes in the per cent of labeled cells in the two compartments are shown in Fig. 3. At the beginning of the first observation period, 75% of the large and 10% of the small blast cells were labeled. A rapid decrease in labeling of large blasts to 22% had occurred by 24 hr, and then the labeled cell concentration remained constant. As these labeled large blast cells became smaller and entered the small blast cell population, labeling of this latter group of cells increased to 20%.

At the beginning of the second observation period 93% of the large and 72% of the small blast cells were labeled. The small blast cell compartment had gradually accumulated labeled cells emigrating from the proliferating compartment during the prolonged preceeding period of label injection. In marked contrast to the observations during the first observation period, labeling of the proliferating compartment decrease only slightly to 78% at the end of the 48 hr second observation period.

The time course for labeled mitotic figures is shown in Fig. 4. During the first period, labeling of mitotic figures had decreased from 92 to 12% by 32 hr as unlabeled mitotic figures rapidly appeared. There was a subsequent increase in labeling to 24% at 48 hr. In contrast during the second period only a slight decrease in labeling from 96 to 73% had occurred by 48 hr.

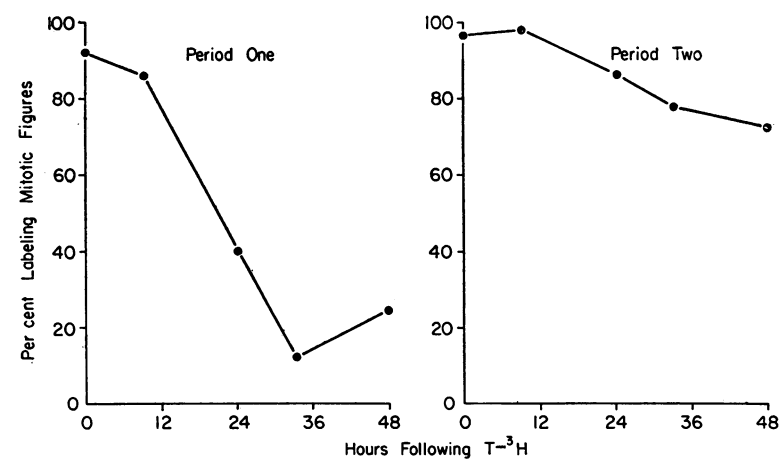

FIGURE 4 Time course of labeled mitotic figures of marrow.

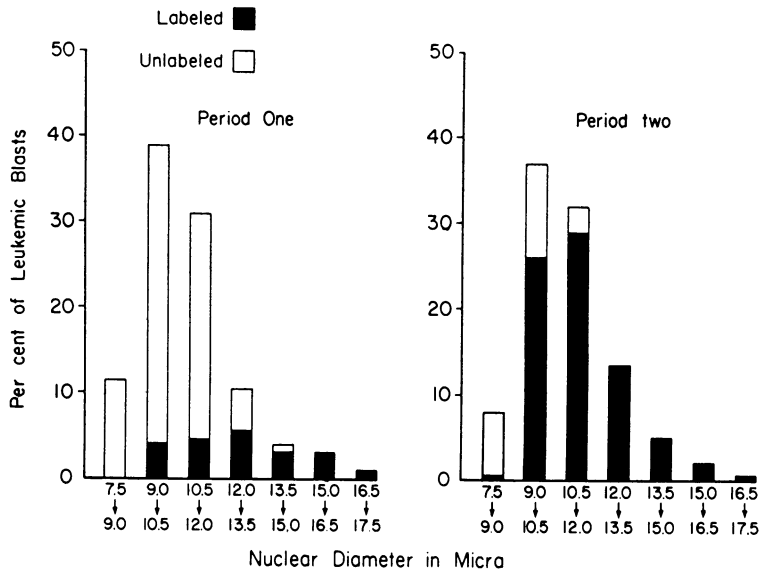

FIGURE 5 Size distribution and labeling of blast cells in marrow.

During the study a maximum of 72% of the small blast cells became labeled. Graphs of cell size distribution and degree of the labeling achieved in the various size groups in the initial marrow samples of each observation period are shown in Fig. 5. The distribution of nuclear diameters was similar at the two times, but the distribution of label was different. During the second period labeling of blasts with nuclear diameters of 12 μ or more increased to 100%, and an increase in the labeling of intermediate sizes from 9 to 12 μ also occurred. The smallest blast cells with nuclei less than 9 μ in diameter, on the other hand, labeled to less than 1% after the second period of tritiated thymidine injections.

Mean grain counts of leukemic blasts during the observation periods are shown in Fig. 6. Initial mean grain counts of large blast cells were significantly higher in period 2 than period 1. The mean grain counts of large blast cells decreased to a plateau by 24 hr in each period. There was little change in mean grain count of small blast cells. Grain count distribution curves for the zero time and 48-hr marrows of each observation period are shown in Figs. 7 and 8. In each case from the zero time to the 48 hr marrows there was a disappearance of cells with high grain counts and a shift of the distribution curve toward lower grain counts. This finding was most marked for large blast cells and especially in period 2. In period 2 the grain count distribution in small blast cells at time zero was similar to the distribution in large blast cells at 48 hr. Cells with three and four grains over the nucleus were not considered labeled and therefore are not shown in Figs. 7 and 8. Such cells made up only 1–3% of the large blast cells in any of the marrow samples.

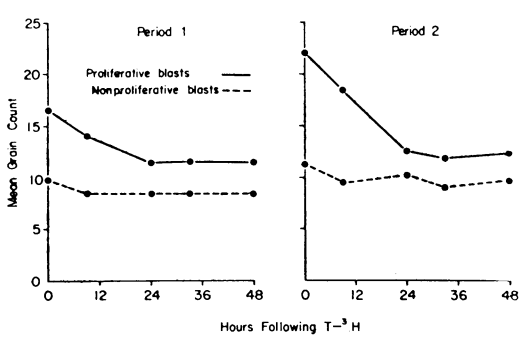

FIGURE 6 Mean grain counts of leukemic blast cells of marrow.

DISCUSSION

For the purpose of this study it was important that a relatively steady state of equilibrium between the large blast cell and small blast cell populations was present. From observations of the per cent of large blast cells in the marrow, the mitotic indices, and the distribution of cell sizes during the period of the study, no changes in the state of the marrow leukemic cells occurred. There also was no change in clinical or hematological findings during this time.

The change in labeling of the proliferating blast cell compartment was different during the two observation periods. In the first period, the per cent of labeled cells decreased rapidly. As the labeled large blast cells entered the small blast cell population they were replaced by unlabeled cells. In the second period, however, the per cent of labeled cells in the large blast cell compart-

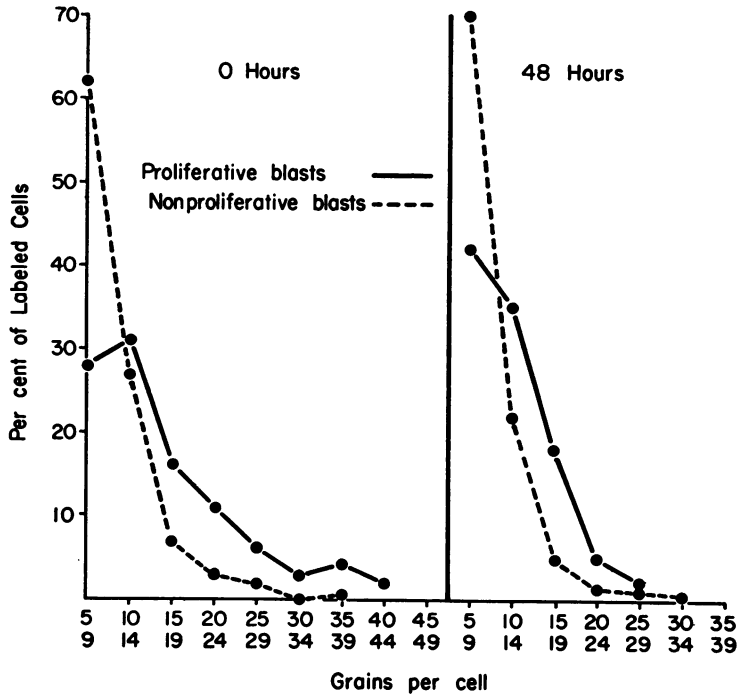

FIGURE 7 Grain count distribution (period 1).

ment decreased little. During this time most of the cells entering the large blast cell compartment to replace departing cells must have been labeled.

The time course for labeled mitotic figures confirmed these observations. During the first period the per cent of labeled mitotic figures decreased rapidly in the 1st 32 hr. The increase of labeling seen at 48 hr most likely marked the appearance of a second generation of mitoses from cells labeled initially 60–70 hr before. This measure of generation time is in agreement with previous studies (3). During the second period there was little decrease in per cent of labeled mitotic figures, a finding indicating that cells going through mitosis at this time were coming from a labeled cell source.

The potential and only identifiable source of cells for maintenance of the large blast cell population which was virtually unlabeled during the first observation period and largely labeled during the second observation period was the population of small blast cells. Of interest is the observation that labeling of these small blast cells remained constant during the second period, as if labeled cells entered and left the compartment at equal rates. A state of equilibrium between large and small cell compartments also seemed to have been reached during the 2nd 24 hr of the first observation period. From these studies it is evident that the majority of small blast cells were capable of reentry into a phase of cell division. Thus these cells are not permanently incapable of cell division but only temporarily nonproliferative. The conditions which allow for return of these cells into a proliferative phase were not apparent from this study.

The absence of label in some cells at the end of the study indicates that not all leukemic cells had gone through the proliferative cycle during that time period. These cells with relatively long resting times between proliferative cycles were mostly the smallest leukemic blasts. The slight increase in unlabeled large blast cells and mitotic figures during the second observation period probably is accounted for by the entry of some of these unlabeled cells into cell division at this time.

The interpretation of grain count data is difficult, mainly because of the loss of labeled cells below the background count after cell division. The initial mean grain counts of large blast cells were significantly greater in period 2 due to multiple labeling of some blast cells during the prolonged exposure to T-^{3}H. As expected in a dividing cell population, the mean grain counts of large blast cells decreased during each observation period. Decreasing grain counts with cell division were also reflected in the grain count distribution curves. As large blast cells with high grain counts disappeared the curve shifted toward cells of lower grain count. As the large blast cells became smaller, the same shift in grain count distribution was evident to a lesser degree in small blast cells. The grain count distribution of large blast cells in the 48 hr marrow of period 2 was consistent with the grain count distribution of small

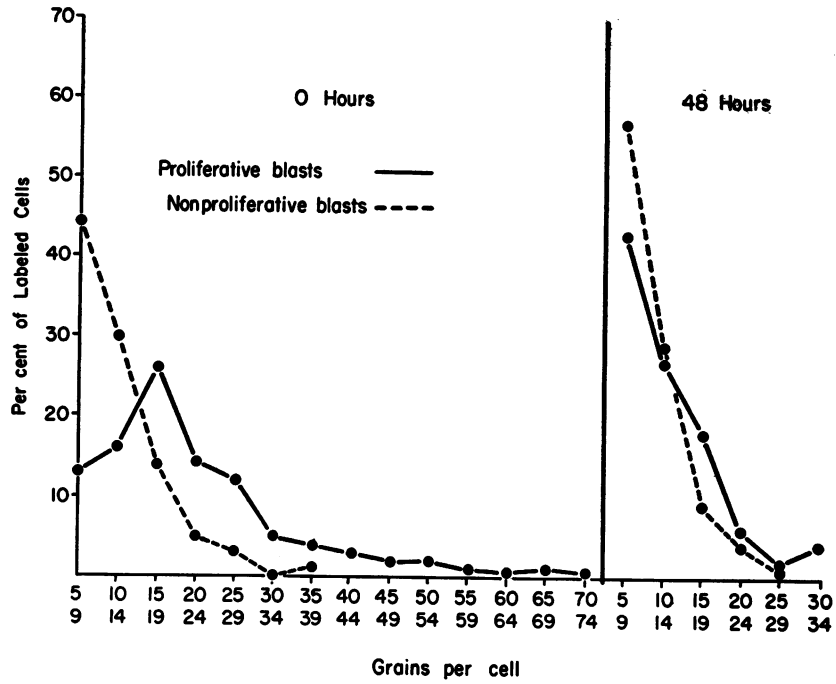

FIGURE 8 Grain count distribution (period 2).

blast cells at the beginning of period 2. This evidence supports the concept that labeled small blast cells in period 2 did reenter the proliferating compartment.

The influence of label reutilization on the results of the study is difficult to ascertain. Salvage of label from deoxyribonucleic acid (DNA) occurs at the nucleoside level, as reutilization can be abolished by flooding the system with unlabeled thymidine (9, 10). Therefore it would be expected that cells labeled by reutilization of tritiated thymidine would have a uniform labeling of low grain count from a rather constant low level of label in the nucleoside pool (11). To minimize the effect of label utilization the background count was set somewhat high. Only cells with five or more grains over the nucleus were considered labeled. Large blast cells with three or four grains over the nucleus constituted only 1–3% of the proliferative compartment and showed no accumulation in the 48 hr marrows of the observation periods. From the grain count distribution curves it was impossible to distinguish a population of proliferating blast cells lightly labeled by reutilization from those lightly labeled due to cell division. Furthermore, if label reutilization accounted entirely for the observations of the second period, further increases in the total per cent of labeled cells would have been expected which did not occur. The observations of the second period as well as the first are best explained by an equilibrium between large and small blast cell compartments being reached. Thus, although label reutilization probably occurred to some degree, it was not a significant factor in the results of the study, nor does it affect their interpretation.

The nature of the resting phase of leukemic blast cells characterized by small nuclear size and dense, coarse nuclear chromatin requires further study. Baserga (12) has recently reviewed some of the concepts concerning the proposed G_0 phase of a proliferating cell cycle. In this phase, a cell maintains capacity for cell division but is neither actively preparing for mitotic division nor committed by cellular activity to a time schedule for future mitotic division. The phase must be definably different from a cell in the interphase between mitosis and DNA synthesis called G_1. In G_1 the mechanisms for the prereplicative phase of the mitotic cycle presumably remain intact. If the studies by Donachie (13) concerning replication of *Escherichia coli* bacteria prove to be applicable to mammalian cells, G_1 might be a phase of progressive cytoplasmic growth which, reaching a critical mass, triggers DNA synthesis.

The time sequence of response of rabbit lens cells to serum exposure resulting in the return of these cells to proliferative activity is in keeping with a period of conversion from G_0 to G_1 phases (14). These studies also indicate the prolonged periods of nonproliferation which can be followed by a return to proliferative activity. The studies of Lala and Patt (15) would indicate that in mouse ascites tumors late in population growth,

some cells enter a G_0 phase to be recalled to active proliferation after transplantation to a fresh host animal.

The variability of proportions of large and small leukemic cells observed in acute leukemia (3) might indicate that a similar conversion of some leukemic cells to a G_0 phase also takes place with population growth. Further studies are necessary to define the small leukemic cells as being in a state demonstrably different from a G_1 phase which might be variably long and become progressively longer with population growth. The recently reported observation (16) about the rapid increase in proliferative activity in marrow blast cell populations caused by extracorporeal irradiation support the concept that the controlling factors concerning these population changes are environmental and thus potentially available to manipulation.

The finding that most small blast cells resume devision within relatively short periods of time could account for the effectiveness of chemotherapy directed primarily against proliferating cells. Very long resting times for some blast cells might account for persistence of leukemic cells into remission (17, 18). Resting small blast cells surviving into remission and becoming resistant to chemotherapy could produce subsequent relapse on their resuming division.

As the small blast cell acts as a leukemic stem cell, the total leukemic cell population is capable of being self-maintaining. Therefore a concept of continued leukemic transformation of normal cells is not necessary to explain the course of this disease, and the leukemogenic agents would not need to be present at the time of clinical presentation of the patient.

Further approaches to therapy should include consideration of the resting cells. Therapy might be designed to increase susceptibility to present drugs by stimulating all leukemic cells to enter division. Another approach might be to maintain leukemic blast cells in an indefinite resting phase.

ACKNOWLEDGMENTS

This study was supported by research grants CA 04826 and FR 05535 from the National Institutes of Health. Technical help was provided by Virginia Fisher, Carol Whidden, and Ann Damon. Betty Powell assisted with preparation of the manuscript.

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