Differences in Cell Cycle Characteristics Among Patients With Acute Nonlymphocytic Leukemia

By Azra Raza, Yogesh Maheshwari, and Harvey D. Preisler

The proliferative characteristics of myeloid leukemias were defined in vivo after intravenous infusions of bromodeoxyuridine (BrdU) in 40 patients. The percentage of S-phase cells obtained from the biopsies (mean, 20%) were significantly higher \( P = .00003 \) than those determined from the bone marrow (BM) aspirates (mean, 9%). The post-BrdU infusion BM aspirates from 40 patients were incubated with tritiated thymidine in vitro. These double-labeled slides were utilized to determine the duration of S-phase \( (T_s) \) in myeloblasts and their total cell cycle time \( (T_c) \). The \( T_s \) varied from four to 49 hours (mean, 19 hours; median, 17 hours). Similarly, there were wide variations in \( T_c \) of individual patients ranging from 16 to 292 hours (mean, 93 hours; median, 76 hours). There was no relationship between \( T_c \) and the percentage of S-phase cells, but there was a good correlation between \( T_c \) and \( T_s \) \( (r = .8) \). Patients with relapsed acute nonlymphocytic leukemia (ANLL) appeared to have a longer \( T_s \) and \( T_c \) than those studied at initial diagnosis. A subgroup of patients at either extreme of \( T_s \) were identified who demonstrated clinically documented resistance in response to multiple courses of chemotherapy. We conclude that \( T_s \) and \( T_c \) provide additional biologic information that may be valuable in understanding the variations observed in the natural history of ANLL.

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MATERIALS AND METHODS

A total of 40 patients received bromodeoxyuridine (BrdU) infusions; 37 had a diagnosis of ANLL, and the others were in blastic crisis of chronic myeloid leukemia (CML). The BrdU protocol was reviewed and approved by the Roswell Park Memorial Institute’s Clinical Investigation Committee as well as the National Cancer Institute (NCI) and the Federal Drug Administration. The BrdU used in this study was supplied by the NCI. Informed consent as per guidelines required by the NCI and FDA was obtained from every patient prior to the administration of BrdU. The methods used in these studies will be described separately in the following sections.

Estimation of the percentage of S-phase cells. After the diagnosis was established, BrdU was administered at a dose of 200 mg/m² in 100 mL of 0.9% normal saline. The BrdU was infused intravenously over a period of two hours by using a constant-rate infusion pump in the first 20 patients. The remaining patients received BrdU at 100 mg/m² intravenously over a period of one hour. The reason for a decrease in the BrdU dose as well as the infusion time was to infuse the least possible amount of a potential mutagen to patients without jeopardizing results. After the infusion, peripheral blood (PB), BM aspirates, and biopsy samples were obtained from each patient. All samples were handled on ice. Five milliliters of BM was aspirated into a plastic syringe containing 2 mL of 6% sodium citrate. The specimen was layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) (specific gravity, 1.077) and centrifuged in a refrigerated centrifuge at 1200 g for 30 minutes. The light-density cells were recovered, washed in RPMI 1640 cell culture medium, and resuspended at a concentration of 1 × 10⁷ cells/mL in RPMI 1640 and 10% fetal calf serum (FCS). All washes were conducted in refrigerated centrifuges, and extreme care was taken to always handle specimens in ice. The samples were then processed by the monoclonal anti-BrdU antibody (RPMB 1) as described before.14,15

The biopsy samples obtained at the end of BrdU infusion were fixed in Bouin’s solution (picric acid, formalin, acetic acid, and water) and processed in plastic by using glycol methacrylate as previously described.16 Two-micrometer-thick sections of the long-core biopsy samples were obtained by using a tungsten carbide-edged microtome and placed on Alcian blue-coated coverslips. Air-dried sections were then processed by the RPMB technique as described before. After processing, the coverslips were counterstained with hematoxylin and May-Grünwald. At least 2000 cells were counted per high-power field.

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were counted from each biopsy section from at least five different fields by a single observer. A cell was counted as positively labeled if it showed any brown staining overlying the nucleus. Control samples were treated with only the primary or only the secondary antibody or just 3-amino-9-ethylcarbazole (AEC). There was no positive labeling detected in any of the control samples.

**Determination of T<sub>r</sub>**. T<sub>r</sub> was determined in 40 leukemic patients as follows. The post-BrdU infusion BM aspirate was processed in Ficoll-Hypaque as described before. Cells at a concentration of 1 x 10<sup>5</sup>/mL were resuspended in RPMI 1640 and 10% FCS. One hundred microliters of [methyl-<sup>3</sup>H]-thymidine (stock solution, 20 μCi/mL; specific activity, 60 Ci/mmol; Amersham Corp, Arlington Heights, IL) was added to 1 mL of the cell suspension along with 10 μL of 10<sup>-4</sup> mol/L fluorodeoxyuridine (FUDR) and incubated for one hour at 37°C in 5% CO<sub>2</sub>. After the incubations were completed, cells were washed thrice in cold thymidine and once in phosphate-buffered saline. Three drops of this suspension were placed on Alcian blue-coated coverslips and fixed in 70% ethanol for ten minutes. These coverslips were first processed by the RPMB technique described before and then coated with Nuclear Track Emulsion (NTB2; Eastman Kodak, Rochester, NY) and exposed in the dark.

...as obtained from the biopsy samples. Since we were calculating the growth fraction and LI is the percentage of S-phase cells as obtained from the biopsy samples. Similarly, a two-tailed t test as well as Mann-Whitney and Spearman correlations were performed to compare the T<sub>r</sub>, T<sub>s</sub>, LI from BM aspirates, LI from BM biopsy samples v other clinical or biologic parameters such as the FAB type, height of the WBC count at presentation, cytogentic, and so on.

**RESULTS**

**Estimation of S-phase cells in vivo.** Figure 1 shows an RPMB-processed BM aspirate sample after BrdU infusion in patient. The use of immunoperoxidase-conjugated secondary antibody and counterstaining permits evaluation of the morphology of the S-phase cells. Using these slides, we were able to assess the percentage of S-phase cells from...
matched BM aspirates and plastic-embedded biopsy samples in individual patients. These data are presented for the 40 leukemic patients in the first two columns of Table 1. Clearly, higher LI values were obtained from the biopsy samples (mean, 23%) than from the respective BM aspirates (mean, 9%) in individual patients. The difference in the means of the two groups as determined by the t test was statistically significant with a P value of .00003. In addition to this striking difference, it is important to note in Table 1 that there is no consistent relationship between the two values in individual patients. For example, patient 1 (Table 1) had 4% S-phase cells in the aspirate and 16% in the biopsy sample, whereas patient 2 had a LI of 15% in the aspirate and 17% in the biopsy sample.

Measurement of cell cycle times. Tc, as well as T, were determined on 40 leukemic patients. Table 1 provides this information in detail. Thirty-seven patients had a diagnosis of ANLL, 26 having been studied at the time of initial diagnosis and 11 at the time of relapse. Three patients were studied in blastic crisis of CML. If all the leukemic patients studied are considered together, one can see from Table 1 that there are wide variations in both Tc as well as T in ANLL patients, although the mean and median values for each correspond well with what has been reported in the literature (mean Tc, 19 hours; median, 17 hours; and mean T, 93 hours; median 76 hours). The Tc ranges from four to 49 hours, and the T ranges from 16 to 292 hours. Table 1 also compares the cell cycle characteristics of newly diagnosed and relapsed ANLL patients as well as three individuals studied during blastic crisis of CML. The last three patients demonstrate shorter Tc and T as compared with the ANLL group. Although not statistically significant because of the few patients studied, it appears that both Tc and T of the leukemic cells in relapsed individuals are longer than that for newly diagnosed ANLL (mean of 22 hours v 18 hours for Tc, and mean of 117 hours v 87 hours for T, respectively).

Since Tc is a derivative of both T and LI, we determined its relationship to these two parameters separately. Although there was no correlation between T and LI (r = -.4), there appeared to be a statistically significant correlation (r = -.8)
between $T_s$ and $T_c$. Figures 2A and 2B show these comparisons. We also assessed the relationship between $T_s$ and $T_c$ and the FAB type, the cytogenetic karyotype, and the height of the WBC count at presentation. There was no unique relationship between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value of the difference between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value of the difference between $T_s$ and $T_c$ was 72 hours. Similarly, in ten newly diagnosed ANLL patients with FAB-M4, the median values for $T_s$ and $T_c$ were 15 and 70 hours, respectively.

Relationship of cell cycle characteristics to clinical course of the disease in ANLL. Although the means of $T_s$ and $T_c$ for all leukemic patients studied approached closely with what has so far been reported in the literature, it is clear that there are distinct differences between individual patients. For example, the leukemic cells of seven patients had $T_s$ values that differed from the population mean value by more than 1 SD (ie, either less than 23 hours or greater than 157 hours). All seven patients (Table 1, patient numbers 4, 7, 8, 20, 29, 32, and 34) failed to enter CR after chemotherapy, six because of persistent leukemia after therapy and one because of early death. Five of the six treatment failures had a very long $T_s$ (173, 196, 203, 213, 282 and 292 hours, Table 1), whereas one had a very short $T_s$ (16 hours). Although chemotherapy produced marrow aplasia in the latter patient, leukemic cells rapidly repopulated the marrow.

The values of $T_s$ and $T_c$ for patients studied at the time of initial diagnosis of ANLL and the outcome of remission induction therapy are presented in Table 2. It appears at a glance that both $T_s$ and $T_c$ are shorter (mean $T_s$, 16 hours; mean $T_c$, 66 hours) in patients who achieved CR as compared with those who had RD (mean $T_s$, 21 hours; mean $T_c$, 104 hours). Of note in the RD group is that two patients had a very long $T_s$ (196 and 283 hours), whereas two patients had a very short $T_s$ (16 and 38 hours), which suggests a possible role of cell cycle-related “kinetic resistance” to therapy in these individuals.

### DISCUSSION

The proliferative characteristics of malignant cells were studied in 40 leukemic patients. The data presented in this paper led to three important observations. First, the percentage of S-phase cells was much higher in BM biopsy specimens than in simultaneously obtained BM aspirates. Second, there was great variability in cell cycle times of individual patients ranging from very short to very long durations of both the S-phase and the cycling times of cells. Finally, there was no direct relationship between the length of the cell cycle and the percentage of cells actively synthesizing DNA.

In previous studies, we have demonstrated that the in vitro BrdU LI were similar to the in vitro 3HTdr LI.21 In the present study, the in vivo BrdU labeling of BM aspirates provide data comparable with studies that have used 3HTdr in vivo. For example, the mean LI in BM aspirates after a BrdU infusion in this study is 9% compared with the mean LI after a 3HTdr infusion reported in the literature ranging between 5.9% to 7.2%.22,23 Similarly, the observation of a higher LI from matched BM biopsy samples of these patients confirmed the findings of previous investigators21,22 who have addressed the question of hemodilution of the aspirates.

Many attempts have been made to relate the proliferative characteristics of leukemic cells as measured by the LI of BM aspirates with treatment outcome.20,21 The results have been conflicting. Obviously, one reason for this lack of relationship may be that the value for the percentage of S-phase cells was obtained from BM aspirates, which noted earlier may provide inaccurate information. Even if

### Table 2. Cell Cycle Studies on Newly Diagnosed Patients With Acute Nonlymphocytic Leukemia Who Received Remission Induction Therapy v Outcome of Treatment

<table>
<thead>
<tr>
<th>CR</th>
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<th>Others</th>
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<td>S. No.</td>
<td>$T_s$ (h)</td>
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<td>104</td>
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Abbreviations: CR, complete remission; RD, resistant disease; Others, death during induction; S. No., patient number; $T_s$, duration of S-phase in hours; $T_c$, duration of total cell cycle time in hours.
the LI were accurate, it is not the only determinant of the biologic behavior of leukemic cells since it provides no information about $T_r$ or $T_c$, which are far more important measurements than the LI alone. The most striking illustration of this can be observed in Table 1, where both patients 7 and 8 have 7% S-phase cells in the BM aspirate and 25% S-phase cells in their biopsy specimens, yet their $T_r$ (49 v 4 hours) and $T_c$ (196 v 16 hours) are entirely unrelated. One patient (no. 7) takes approximately eight days to double his leukemic burden, whereas the other (no. 8) is doubling his leukemic cell mass every 16 hours. Hence, in order to acquire a comprehensive view of the cell cycle characteristics in ANLL patients, all three characteristics, ie, LI as well as $T_r$ and $T_c$, must be taken into account.

Furthermore, conclusions regarding cell cycle characteristics in newly diagnosed ANLL patients have in the past been based on approximately 20 patients from nine different studies. Because the dose level and schedule of $^{3}H$Tdr infusions as well as the methodology of sample processing was quite different in each study, comparisons of such data are difficult. Since the methodology we used is considerably more efficient than the double-labeled autoradiography or the estimation of labeled mitosis used in previous studies, we were able to study large numbers of patients uniformly.

After administration of BrdU and in vitro incubation of BM aspirates with $^{3}H$Tdr, we used our previously described double-label technique to measure the $T_r$ and $T_c$ in 40 leukemic patients. The internal t between the two labels was maintained at a constant by obtaining the BM samples immediately at the end of the BrdU infusion and subsequently handling samples on ice. All cell washing procedures were conducted with cold media in refrigerated centrifuges so that metabolic activity in the cells remained arrested until the start of incubation with the second label ($^{3}H$Tdr) in vitro at 37°C.

Cell cycle characteristics were thus measured on 40 leukemic patients, 26 of whom were studied at the time of initial diagnosis of ANLL, 11 at the diagnosis of relapsed leukemia, and three individuals in blastic crisis of CML. Of the 26 newly diagnosed ANLL cases studied, 24 subsequently received remission induction therapy. It appears that 11 patients who survived therapy (CR + RD only) had a $T_r$ of less than 80 hours, and seven of these 11 patients achieved CR (Table 2). On the other hand, among eight patients who had a $T_c$ longer than 80 hours and who survived therapy (CR + RD), only one entered remission, and seven demonstrated clinical resistance. This seems to indicate that patients with a $T_c$ longer than 80 hours tend to respond unfavorably to chemotherapy. In fact, of six ANLL patients who had an extraordinarily long $T_c$ (173, 196, 203, 213, 282, and 292 hours, Table 2), five demonstrated resistance to chemotherapy, and one patient died during treatment.

On the other hand, patients at the other extreme of $T_c$ do not respond well either. For example, the patient with $T_c$ of 16 hours (no. 8, Table 1) achieved aplasia after chemotherapy, but his BM was repopulated by rapidly dividing leukemic cells. This patient is particularly interesting because he achieved aplasia twice after chemotherapy, and each time his BM was repopulated by leukemic cells. The regrowth rate of his leukemic cells as measured by the daily increase in myeloblasts from his PB both times was consistent with a $T_c$ ranging between 19 to 21 hours (manuscript in press). This value correlated strongly with a cycling time of 16 hours for the myeloblasts we had determined prior to administration of any chemotherapy. It must be remembered that for the calculation of $T_c$ we utilized the LI value from biopsy samples rather than BM aspirates. Had we used the LI value from the BM aspirate in this patient, the calculated $T_c$ would have been 57 hours rather than 16 hours. Since the clinical and biologic behavior of his leukemic cells was more consistent with a cycling time of 16 hours, this case validates the use of biopsy specimens for a more accurate assessment of the percentage of cells actively engaged in DNA synthesis.

Table 1 also compares the cell cycle characteristics of newly diagnosed and relapsed ANLL patients as well as three individuals studied during blastic crisis of CML. The three CML patients demonstrate a shorter $T_r$ and $T_c$ compared with the ANLL group, an observation consistent with the fact that, although chemotherapy often produces marrow aplasia, rapid regrowth of blastic crisis clones often occurs. Similar to previously reported results, both the $T_r$ and $T_c$ in relapsed ANLL patients were longer than that for newly diagnosed ANLL (22 v 18 hours for $T_r$ and 177 v 87 hours for $T_c$, respectively). Therefore, selection of leukemic clones that are slowly cycling may be an additional explanation for the frequent resistance to chemotherapy observed in these patients.

It must be remembered, however, that the biologic properties of leukemic cells represent only one of several factors that help to determine response. It is only possible to say that some cell cycle characteristics may be identified that if present will result in an unfavorable response to therapy, but if absent does not guarantee CR. Therefore we may see patients who have cell cycle characteristics compatible with a favorable response to chemotherapy yet who fail to enter remission because of other reasons (such as metabolic drug resistance of leukemic cells or a very high leukemic cell mass at the time of treatment). The clinical implications of identifying a subset of patients who will manifest resistance to therapy either because of very slowly cycling cells or unusually rapid proliferation regardless of other favorable prognostic factors are then obvious. For example, if this information can be obtained prior to therapy, both remission induction as well as consolidation/maintenance regimens could be altered to suit the individual needs of patients. Thus patients with a long $T_c$ may benefit from prolonged treatment utilizing low doses of drugs. On the other hand, individuals with very rapidly dividing leukemias may be candidates for intermittent therapy with intensive courses of S-phase–specific agents. Finally, the duration of remission in patients who achieve CR may at least in part depend upon the regrowth rate of the residual leukemic cells; however, the present study is too young for comment on this aspect.

One last concern that needs to be addressed in the assessment of cell cycle characteristics is the question of the actual growth fraction in each patient. We assumed it to be 100% in the calculations of $T_c$. This assumption was based on
the observations reported in three elegant studies\(^{37,40,41}\) that have shown that the noncycling or G₀ compartment of cells in ANLL is relatively small. For example, Clarkson et al. administered \(^{3}H\)Tdr by continuous infusion for eight to ten days and demonstrated that 90% to 98% BM cells were labeled at the end of this period. This left a definite but insignificant proportion of 2% to 8% noncycling cells in the G₀ compartment. Although it would be ideal to measure even this small subcompartment in every patient, at the present time the risk of administering BrdU by continuous infusion for long periods of time is not justified. Second, it must be remembered that the methods we utilized in our studies as well as those in the past can only yield information about the average behavior of a cell population. No information can be obtained about the rate of DNA synthesis or duration of cell cycle time in individual cells. It is possible that the 2% to 8% unlabeled cells in the study of Clarkson et al were very slowly cycling rather than being truly quiescent. Therefore, similar to past experience and assuming all cells to be in cycle, we have estimated the average cell cycle times of leukemic cells in a population.

In summary, therefore, cell cycle characteristics were determined for 40 leukemic patients. There were distinct differences in both the Tₐ as well as the Tₚ of individual patients. A subgroup of individuals was identified with either a very long or very short Tₚ who demonstrated clinical drug resistance in response to multiple courses of chemotherapy. Because these measurements can now be obtained within a reasonable time frame, they will contribute greatly in understanding the biologic behavior and the natural history of acute leukemias as well as assist in tailoring therapy to suit the needs of individual patients.

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