Differences in Cell Cycle Characteristics Among Patients With Acute Nonlymphocytic Leukemia

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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 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 (T1) in myeloblasts and their total cell cycle time (Tc). The Tc varied from four to 49 hours (mean, 19 hours; median, 17 hours). Similarly, there were wide variations in Tc of individual patients ranging from 16 to 292 hours (mean, 93 hours; median, 76 hours). There was no relationship between Tc and the percentage of S-phase cells, but there was a good correlation between Tc and T (r = .8).

Patients with relapsed acute nonlymphocytic leukemia (ANLL) appeared to have a longer Tc and T than those studied at initial diagnosis. A subgroup of patients at either extreme of Tc were identified who demonstrated clinically documented resistance in response to multiple courses of chemotherapy. We conclude that Tc and T provide additional biologic information that may be valuable in understanding the variations observed in the natural history of ANLL.

DEFINING THE PROLIFERATIVE characteristics of malignant cells is a fundamental necessity if one is to understand the natural history of a neoplasm. The leukemias provide an excellent model system for such studies. However, attempts to relate cell cycle characteristics in acute nonlymphocytic leukemia (ANLL) to both the course of the disease as well as the response to therapy have yielded conflicting results. This lack of correlation is surprising, since intuitively one would expect that proliferation would be an important determinant of response.

Several potential sources of such inconsistencies are readily identifiable. First, labeling index (LI) studies measuring the percentage of S-phase cells were performed on bone marrow (BM) aspirates in vitro that might yield inaccurate results due to a variable hemodilution of the sample. Second, the determination of the durations of S-phase (Tc) and total cell cycle time (Tt) is available on a very small number of patients, the majority of whom had brief life expectancies, so that correlations with the course of the disease could not be sought satisfactorily. Most importantly, however, were the problems that existed in the methodology available for such studies. Reviewing these concerns, Perry concluded in 1976 that since “the present approaches are either too slow or impractical, new techniques are needed to assess cell cycle times, growth fraction and other important parameters.”

We have utilized a new approach by combining immunofluorescence and autoradiography to determine the cell cycle characteristics on 40 leukemic patients. This paper describes the relationship of these parameters to the clinical course and the response to therapy in patients with ANLL.

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 established, and the diagnosis was confirmed in 37 patients by both the clinical course and the response to therapy. Patients with relapsed acute nonlymphocytic leukemia (ANLL) appeared to have a longer Tc and T than those studied at initial diagnosis. A subgroup of patients at either extreme of Tc were identified who demonstrated clinically documented resistance in response to multiple courses of chemotherapy. We conclude that Tc and T provide additional biologic information that may be valuable in understanding the variations observed in the natural history of ANLL.

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 decrease 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 × 109 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.

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. 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

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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 there showed any brown staining overlying the nucleus. Control samples were treated with only the primary or only the secondary antibody or just 3-aminom-9-ethylcarbazole (AEC). There was no positive labeling detected in any of the control samples.

**Determination of T_\(_i\)**. T\(_i\) was determined in 40 leukemia patients as follows. The post-BrdU infusion BM aspirate was processed in Ficoll-Hypaque as described before. Cells at a concentration of 1 x 10^9/mL were resuspended in RPMI 1640 and 10% FCS. One hundred microliters of [methyl-\(^{3}H\)]-thymidine (stock solution, 20 \(\mu\)Ci/mL; specific activity, 60 Ci/mmol; Amersham Corp, Arlington Heights, IL) was added to 1 mL of the cell suspension along with 10 \(\mu\)L of 10^-4 mol/L fluoresceoxyuridine (FUdR) and incubated for one hour at 37°C in 5% CO\(_2\). 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 for 48 hours at ~70°C. Kodak D19 developer was used to develop the samples, and after the samples were fixed, the coverslips were mounted onto glass slides with Elvanol 51-50 (Dupont) and examined under a fluorescence microscope. At least 2,000 positively labeled cells were counted per slide by a single observer. A cell was considered positively labeled for BrdU incorporation if there was any fluorescence detected over its nucleus and positive for \(^{3}H\)-thymidine (\(^{1}H\)TdR) incorporation if at least five or more silver grains were detected on its nuclear surface. The cells considered positive for double labeling demonstrated both fluorescence and silver grains over their nuclei.\(^{1}H\)TdT was then calculated by the following formula as described by Wimber and Quastler: T\(_i\) = (DL + \(^{1}H\)TdT) \(\times T\)/BrdU, where DL is the number of cells demonstrating double labeling, \(^{1}H\)TdT is the number of cells only showing \(^{1}H\)TdR incorporation, BrdU is the number of cells revealing only BrdU incorporation, and t is the time interval between the two labels. The interval t in patients who received a two-hour BrdU infusion was taken as two hours, and for individuals who received a one-hour infusion of BrdU, it was one hour. The reason we were able to keep this interval constant was because of the extreme care we exercised in obtaining the samples immediately at the end of the BrdU infusion and subsequently handling all samples on ice. Even cell washes were conducted in refrigerated centrifuges so that all metabolic activity in the cells remained arrested until the start of incubation with the second label (\(^{1}H\)TdT) at 37°C.

**Determination of T_\(_i\)**. Once the values for T\(_i\) and the percentage of S-phase cells obtained from the BM biopsy samples were available, the length of T\(_i\) was determined by using the formula described before as follows:

\[
T\(_i\) = T\(_i\) \times GF/LI,
\]

where GF is the growth fraction and LI is the percentage of S-phase cells as obtained from the biopsy samples. Since we were calculating the cycling times of only those cells that were actively in cycle, the GF was assumed to be 100% in each case.

**Clinical data**. Prior to administering the BrdU infusion, a diagnosis of acute leukemia was established in every case. Of the 40 cases being reported in this paper, 37 had ANLL with a French-American-British (FAB) staging of M0 through M7, and three patients had blastic crisis of CML. Twenty-six patients had newly diagnosed ANLL, and 11 patients were studied at the time of relapse of ANLL. Of the 26 newly diagnosed ANLL patients, 24 received remission induction therapy, whereas two patients refused chemotherapy and only received supportive care and/or 1 g hydroxyurea orally everyday until the time of death. Twelve patients received remission induction therapy with cytosine arabinoside (araC) at 100 mg/m\(^2\)/d by continuous intravenous infusion and adriamycin at 30 mg/m\(^2\)/d intravenously for seven (7 + 3) or ten (10 + 3) days plus three days respectively. Twelve patients received high-dose araC at 3.0 g/m\(^2\)/d by intravenous infusion over a period of 75 minutes every 12 hours for six days (HDaraC), and one patient also received three days of daunorubicin (DNR) at 45 mg/m\(^2\)/d intravenously for the last three days after HDaraC therapy (HDaraC + DNR). Of the 24 newly diagnosed ANLL patients who received the remission induction therapy described earlier, eight entered complete remission (CR), 11 had resistant disease (RD), and five patients died during induction ("other" failures). The treatment failures were classified according to our previously described classification system, which provides for recognizing those individuals who failed therapy because of persistent leukemia (RD) v patients who died during induction therapy or during the hypoplastic phase induced by chemotherapy ("others"). CR was defined according to the Cancer and Leukemia Group B (CALGB) criteria described before.22 All of the 11 ANLL patients studied at the time of relapse received remission induction therapy. Seven were treated with HDaraC, and four received mitoxantrone, 12 mg/m\(^2\)/d, intravenously for five days. Two of these latter patients also received 5-azacytidine at 100 mg/m\(^2\)/24 hr by continuous intravenous infusion for three days after five days of mitoxantrone therapy. Four patients achieved CR (3/7 CRs in the HDaraC-treated group and 1/4 CR in the mitoxantrone-treated group), five had RD (2/7 in the HDaraC-treated group and 3/4 in the mitoxantrone-treated group), and two patients treated with HDaraC died during induction or were "other" failures.

**Control samples**. Samples of PB, BM aspirates, and biopsies were obtained from all 40 patients immediately at the end of the BrdU infusion. All samples were placed on ice and transported to the laboratory in the same building. Subsequent cell separation procedures as well as cell washes were conducted in refrigerated centrifuges with cold media. Since minor delays ranging from a few minutes to an hour could in theory occur, we performed the double labeling of samples kept on ice at various times. Immediate post-BrdU infusion BM samples were incubated with \(^{1}H\)TdT, and the cell kinetic data thus obtained were compared with data obtained from the same specimen that was placed on ice for 15, 30, 60, and 120 minutes and then incubated with \(^{1}H\)TdT. Results indicate that no differences were noted for delays of up to two hours as long as the samples were kept on ice. Long delays, however, never occurred in our experience because the protocol was very strictly followed, all specimens being incubated with \(^{1}H\)TdT within 30 minutes.

**Statistical methods**. Analysis was performed by using the terminal analysis package, a set of interactive computer programs. A two-tailed \(t\) test was performed to compare the means of the percentage of cells in S-phase from the bone marrow aspirates and the BM biopsy samples. Similarly, a two-tailed \(t\) test as well as Mann-Whitney and Spearman correlations were performed to compare the T\(_i\), T\(_i\), 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, cytogenticics, and so on.

**RESULTS**

**Estimation of S-phase cells in vivo**. Figure 1 shows an RPMB-processed BM aspirate sample after BrdU infusion in a 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.** $T_s$ as well as $T_c$ 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 during blastic crisis of CML. These last three patients demonstrate shorter $T_s$ and $T_c$ as compared with the ANLL group. Although not statistically significant because of the few patients studied, it appears that both $T_s$ and $T_c$ of the leukemic cells in relapsed individuals are longer than that for newly diagnosed ANLL (mean of 22 hours $\pm$ 18 hours for $T_s$ and mean of 117 hours $\pm$ 87 hours for $T_c$, respectively).

Since $T_s$ is a derivative of both $T_c$ and LI, we determined its relationship to these two parameters separately. Although there was no correlation between $T_c$ and LI ($r = .4$), there appeared to be a statistically significant correlation ($r = .8$)
between T_s and T_e. Figures 2A and 2B show these comparisons. We also assessed the relationship between T_s and T_e 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 between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value between any of these values.

**Relationship of cell cycle characteristics to clinical course of the disease in ANLL.** Although the means of T_s and T_e 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_e for patients studied at the time of initial diagnosis of ANLL v the outcome of remission induction therapy are presented in Table 2. It appears at a glance that both T_s and T_e are shorter (mean T_s, 16 hours; mean T_e, 66 hours) in patients who achieved CR as compared with those who had RD (mean T_s, 21 hours; mean T_e, 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 speci-
and 8 have 7% S-phase cells in the BM aspirate and 25% cells in their biopsy specimens, yet their Tc (49 hours) and Te (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, i.e., LI as well as Te and Tc, 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.35,37,39-43 Because the dose level and schedule of [3H]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 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 [3H]Tdr, we used our previously described double-label technique to measure the Te and Tc in 40 leukemic patients. The interval 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 ([3H]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 Te 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 Te 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 Te longer than 80 hours tend to respond unfavorably to chemotherapy. In fact, of six ANLL patients who had an extraordinarily long Te (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 Te do not respond well either. For example, the patient with Te 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 Te 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 Te 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 Te 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 Te and Tc compared with the ANLL group, an observation consistent with the fact that, although chemotherapy often produces marrow aplasia, rapid regrowth of blast crisis clones often occurs. Similar to previously reported results,3,39-40 both the Te and Tc in relapsed ANLL patients were longer than that for newly diagnosed ANLL (22 v 18 hours for Te and 177 v 87 hours for Tc, 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 Te 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 Te. This assumption was based on...
the observations reported in three elegant studies (17, 40, 41) that have shown that the noncycling or G0 compartment of cells in ANLL is relatively small. For example, Clarkson et al administered 3H-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 Tc as well as the Tw of individual patients. A subgroup of individuals was identified with either a very long or very short Tw 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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Cell cycle analysis in ANLL

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