Direct Relationship Between Remission Duration in Acute Myeloid Leukemia and Cell Cycle Kinetics: A Leukemia Intergroup Study

By Azra Raza, Harvey D. Preisler, Roger Day, Zahida Yasin, Mike White, Joe Lykins, Cathi Kukla, Maurice Barcos, John Bennett, George Browman, Jack Goldberg, Hans Grunwald, Richard Larson, James Vardiman, and Ralph Vogler

Cell cycle characteristics including labeling indices (LI), duration of S-phase (Ts), and total cell cycle time (Tc) were determined in 54 standard-risk, newly diagnosed patients with acute myeloid leukemia following an infusion of bromodeoxyuridine. Remission induction therapy consisting of cytosine arabinoside and daunomycin was then administered to all patients, followed by three courses of consolidation to those who achieved complete remissions (CR). Older patients appeared to have more rapidly cycling cells (P = .003). No unique cell cycle characteristics were identified for patients who achieved remission versus those who had resistant disease. However, the pretherapy cell cycle characteristics were a strong prognosticator for remission duration. CR patients were divided into those whose leukemic cell Tc were above median (A) and below median (B). Among 14 B patients, median duration of response was 211 days, and all relapsed by day 600. Among 18 A patients, the median has not as yet been reached, with nine patients in continuous complete remission (log rank P = .007, Wilcoxon P = .04). We conclude that cell cycle characteristics of leukemic cells play a role in determining remission duration, perhaps because the leukemic cells of the former patients regrow slowly between courses of chemotherapy.

Estimation of the percentage of S-phase cells. After a diagnosis of standard-risk AML was established in every patient, BrdU was administered at a dose of 100 mg/m² in 50 mL of 0.9% normal saline IV over 1 h. Immediately at the end of the infusion, peripheral blood (PB) bone marrow aspirate (BMasp) and biopsy samples were obtained from every patient. The biopsy samples obtained at the end of BrdU infusion were fixed in Bouin’s solution and processed in plastic by using glycol methacrylate (GMA) as previously described. Sections were then processed by the monoclonal anti-BrdU antibody as described before, counterstained with hematoxylin and May-Grünwald stains, and at least 2,000 cells were counted from each biopsy section from at least five different fields by a single observer.

Determination of Ts. The post-BrdU infusion BMasp was double-labeled with ‘HTdr as described before. 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 ‘HTdr incorporation if at least five grains (if the background was less than 1 grain/cell, then three grains) were detected on its nuclear surface. The cells considered positive for double labeling demonstrated both fluorescence and silver grains over their nuclei. Ts was then calculated by the following formula as described by Wimber and

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Quastler: \( Ts = (DL + H5dt) \times /BrdU \), where DL is the number of cells demonstrating double labeling, \( H5dt \) is the number of cells only showing \( H5d \) incorporation, BrdU is the number of cells revealing only BrdU incorporation, and t is the time interval between the two labels. The time interval t in this formula was taken as 1 hour because that was the time lapse between the start of the first and the start of the second label.

**Determination of \( Tc \):** The length of \( Tc \) was determined by using the formula described before as follows: \( Tc = Ts \times GF/LI \), where GF is the growth fraction and LI is the percentage of S-phase cells obtained from plastic embedded biopsy samples. Because we were calculating the doubling times of only those cells actively engaged in cycle, the GF was assumed to be 100% in each case.

**Mechanics of sample transport.** The BrdU protocol INT-0050 is a Leukemia Intergroup study, providing for administration of the in vivo label locally at each member institution listed. All post-BrdU samples were processed initially at local institutions. For example, post-BrdU PB and BM samples were density-cut and double-labeled with \( H5d \) placed on coverslips, and fixed in 70% ethanol. These coverslips can be stored permanently in 70% ethanol. The BM biopsy samples were fixed and dehydrated locally and then transported to Roswell Park Memorial Institute (RPMI; Buffalo, NY) within a week. The PB and BMasp samples were transported regularly to RPMI. All monoclonal antibody (MoAb) applications, autoradiographic procedure, as well as GMA infiltration of biopsies and their processing were performed centrally at RPMI in the laboratory of Dr. Raza. All slides were interpreted by at least two individuals (Dr. Raza was one of them in every case) who blindly counted each other's slides. The majority of samples were assigned a coding number and individuals interpreting slides did not know the names of patients, the institution they were from, or outcome of therapy in any of the cases.

**Clinical data.** Fifty-four standard-risk, newly diagnosed AML patients are the subject of this report. The term standard risk refers to all previously untreated patients with AML (FAB, M1, M2, M3, M4, M5, M6, M7). Patients in any of the groups listed below are considered to be high-risk patients and are treated on a different chemotherapy regimen. They will be the subject of a subsequent report. (1) Patients who were older than 70 years of age. (2) Patients with overt congestive heart failure and patients with uncontrollable ventricular arrhythmias, both of which precluded such individuals from receiving anthracyclines. (3) Patients whose performance status was 2 (completely bedridden). (4) Patients with AML secondary to chemotherapy, radiation therapy, exposure to toxic agents, or patients with a history of myelodysplastic syndrome. Induction therapy consisted of daunomycin (DNR) 50 mg/m²/d (35 mg/m²/d for patients older than 60 years) by continuous IV infusion on days 1, 2, and 3, and cytosine arabinoside (araC) at 100 mg/m²/d by continuous IV infusion on days 1 through 7 (7 + 3). Patients whose BMasp contained \( \geq 10\% \) abnormal cells on day 0 were given 3 additional days of araC (10 + 3). Complete remission (CR) was defined according to the Cancer and Leukemia Group B (CALGB) criteria described before. 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 (resistant disease or RD) and those patients who died during induction therapy or during the hypoplastic phase induced by chemotherapy ("others"). The CR rate on this protocol (which is being performed at the present time) is 60.4%, with 15% patients failing due to RD and 8% dying during induction therapy. Following achievement of CR, all patients were administered three courses of intensive consolidation therapy. Courses 1 and 3 consisted of araC 50 mg/m²/d for 3 days by continuous infusion and araC 100 mg/m²/d \( \times 7 \) days). Course no. 2 consisted of araC at 3 Gm/m² IV every 12 hours for 4 days (reduced to 2 Gm/m² for patients older than 60 years).

**Statistical methods.** Clinical data were collected on INT-0066 locally by each participating institution and sent for entry into the Leukemia Intergroup (LIG) data collection’s office by Frontier Science, Buffalo, NY. The laboratory data were entered onto forms and sent for decoding of samples to the LIG operations office and then entered into the computer. All statistical tests performed were two-sided. All correlations presented herein are Spearman correlations. The statistical significance of correlations were tested with permutation tests. Relationships with clinical outcome were tested with Fisher exact tests. Two types of analyses were performed to relate cell cycle variables to remission duration. One type classified patients as high or low on each variable, and compared the resultant groups using log-rank tests and Gehan-Gilbert-Wilcoxon tests (hereafter referred to as Wilcoxon tests). In each case, "high" meant that the value in question was higher than the median among the 54 study patient values. The other type of analysis was to rank each variable and fit accelerated failure time models linear in the ranks. The distributions of remission durations were assumed to be Weibull distributions. The statistical analysis systems (SAS) procedures LIFETEST and LIFEREG were used for these analyses. Two patients for whom the duration of response was not available are excluded from these analyses (patient no. 141 in Table 1 is thereby excluded in Ts analysis, but \( Tc \) is unavailable for this patient. The other patient who was excluded is no. 121).

**RESULTS**

**Measurements of cell cycle parameters in vivo.** Fifty-four standard-risk, newly diagnosed AML patients were studied. Figures 1A and B show post-BrdU infusion BMasp and biopsy sections from an AML patient, treated with the anti-BrdU antibody followed by a secondary antimouse antibody conjugated to peroxidase. Reddish-brown staining is clearly visible over the nuclei of S-phase cells. Table 1 gives details of the cell cycle parameters for all 54 patients. As can be seen from this table, the percentage of S-phase cells or the LI was available from BMasp in 49 patients and from BM biopsy in 50 patients. The mean LI from BMasp was 11.1% (median = 9%, range 2% to 40%), while the mean LI from BM biopsies was 27.3% (median = 27.5%, range 9% to 47%). These differences in LI between aspirates and biopsies can be most readily explained by the variable hemodilution of the aspirate, which often occurs.

Measurements of the Ts are available for 48 patients (Table 1). The mean Ts of the group was 14.7 hours (median = 13.5 hours, range 6 to 43 hours). Tc measurements are available in 45 patients. The mean Tc was 59.8 hours with a median of 48 hours and range of 18 to 211 hours.

**Relationship of cell cycle parameters to patient and leukemic cell characteristics.** An assessment was made to determine whether any relationships existed between the cell cycle parameters and the characteristics of the patients or their leukemic cells. Among the 54 patients, 26 were male and 28 female. Table 2 shows the relationship between the four cell cycle kinetic parameters and age and sex in these patients. No relationship was discernable between the cell cycle parameters and sex, but there was a statistically significant relationship with age. Increasing age was associ-
### Table 1. Cell Cycle Characteristics and Clinical Data on Newly Diagnosed, Standard-Risk AML Patients Treated on INT-0066

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**Mean** | 11.10 | 27.26 | 14.69 | 59.84 |
**Median** | 9.00 | 27.50 | 13.50 | 48.00 |
**Range** | 2-40 | 9-47 | 6-43 | 18-211 |

Abbreviations: %LI Asp, percentage of Ts cells from BMasp; %LI Bx, percentage of Ts cells from bone marrow biopsy; Tx Outcome, treatment outcome; UPN, unique patient number.
The French-American-British (FAB) classification of the leukemia of every patient was available. Table 3 shows the distribution of each FAB category into these two groups as well. While no statistically significant differences were noted, it is interesting to note that all four FAB M3 patients had above median Tc values.

Relationship between cell cycle parameters and outcome of therapy. All patients received araC/DNR remission induction therapy following the BrdU infusion. The CR rate for all patients treated on this protocol has consistently been between 70% to 80%. For the 54 patients being reported here in whom cell cycle studies were performed, there were 40 CRs, 9 RD failures, and 5 patients died early in therapy or during the period of marrow hypoplasia. None of the cell cycle variables examined showed any statistically significant relationship to induction outcome, including the doubling time as shown in Fig 2. The data demonstrate that patients with rapidly cycling cells and patients with slowly cycling cells can achieve CRs when treated with the remission induction regimen used in this study.

Relationship of cell cycle parameters and duration of remission. To determine whether there was any relationship between the pretherapy cell cycle characteristics of leukemic cells and the duration of remission, the median value of each cell cycle parameter was used to divide patients into a slowly proliferative group (above median value [A] and a rapidly proliferative group (below median [B]). These median values were then plotted on a survival function curve. A highly significant relationship between remission duration and cell cycle time was identified (Fig 3). All of the patients in long-term remission had pretherapy leukemia cell cycle times which were greater than the median value. Nine of the 18 patients in this group continue to be in remission. In contrast, all 14 remission patients whose leukemic cell cycle times were less than the overall median value relapsed by day 600 with a median duration of remission of 210 days. These differences are statistically significant (log rank $P = .007$, Wilcoxon $P = .042$). Hence, only patients whose leukemic cells at the time of diagnosis were slowly cycling have long remissions. Interestingly, of the nine patients on curve A in Fig 3, two had acute promyelocytic leukemia (FAB M3) and the longest survival is an M3 patient.

The Ts was also related to remission duration, but was not statistically significant (log rank $P = .10$, Wilcoxon $P = .29$) while neither the pretherapy LI of the BMasp or biopsy were related to remission duration.

DISCUSSION

Two conditions must be satisfied to determine the clinical relevance of the cell cycle characteristics of malignant diseases. First, a reliable quantitative measurement of the proliferative characteristics of the tumor should be available. Assessment of only the percent of cells in S-phase is not adequate. Second, the relationship of these parameters to both the outcome of therapy and the course of the disease should be sought in a group of patients who have a relatively comparable disease and who receive the same treatment. In the present report, we have accomplished both goals by measuring, in addition to the LI, the Ts and the Tc of the malignant cells in a uniform group of patients who received the same therapy. Before discussing the clinical relevance of cell cycle parameters in AML, it is appropriate to briefly discuss some aspects of the methodology used in our studies. (However, this is not a method report, and indeed, detailed descriptions of the double-label technique have been dealt with extensively in the past. For detailed answers, please see references 7, 9, and 10).

One question that may be raised by those not familiar with the methods used in these studies is whether we have measured the characteristics of leukemic cells or of residual normal cells in the bone marrow. In reality, this question asks whether measurements of normal residual stem cells and normal "differentiating" cells could have affected the assessment of leukemic cell cycle parameters. Normal "stem" cells

Fig 1. (A) BMasp obtained from an AML patient immediately at the end of a 1-hour infusion of BrdU and treated with anti-BrdU antibody shows marked brown staining of cells actively engaged in DNA synthesis. (B) Immediate post-BrdU infusion bone marrow biopsy section treated with the anti-BrdU antibody shows a number of blasts synthesizing DNA. A megakaryocyte is also in Ts.

An antibody shows marked brown staining of cells actively engaged in the end of a 1-hour infusion of BrdU and treated with anti-BrdU.
Cell kinetics in AML

Table 2. Cell Cycle Characteristics of Myeloblasts Versus Clinical Characteristics of Standard-Risk, Newly Diagnosed Patients Being Treated on INT-0066

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<td>.06</td>
<td>.32</td>
<td>.71</td>
<td>.72</td>
</tr>
<tr>
<td>% Abnormal cells (blood)</td>
<td>-.12</td>
<td>-.12</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>% Abnormal cells (bone marrow)</td>
<td>.39</td>
<td>.38</td>
<td>.92</td>
<td>.59</td>
</tr>
<tr>
<td>% Abnormal cells (bone marrow)</td>
<td>-.33</td>
<td>-.23</td>
<td>.02</td>
<td>.17</td>
</tr>
<tr>
<td>Platelet count</td>
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<td>.87</td>
<td>.26</td>
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<tr>
<td>Biopsy cellularity</td>
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<td>.22</td>
<td>.25</td>
<td>.13</td>
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<tr>
<td>Leukemic cell mass</td>
<td>.008</td>
<td>-.05</td>
<td>-.02</td>
<td>-.07</td>
</tr>
<tr>
<td>Normalized LDH</td>
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<td>.73</td>
<td>.86</td>
<td>.65</td>
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<tr>
<td>Leukemic cell mass</td>
<td>-.006</td>
<td>.11</td>
<td>.12</td>
<td>-.06</td>
</tr>
<tr>
<td>% Abnormal cells (bone marrow)</td>
<td>.96</td>
<td>.47</td>
<td>.42</td>
<td>.70</td>
</tr>
<tr>
<td>% Abnormal cells</td>
<td>-.03</td>
<td>.09</td>
<td>.20</td>
<td>.07</td>
</tr>
<tr>
<td>% Abnormal cells</td>
<td>.87</td>
<td>.50</td>
<td>.16</td>
<td>.61</td>
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<tr>
<td>% Abnormal cells</td>
<td>-.23</td>
<td>.01</td>
<td>.03</td>
<td>-.02</td>
</tr>
<tr>
<td>day 6 (bone marrow)</td>
<td>.13</td>
<td>.93</td>
<td>.83</td>
<td>.86</td>
</tr>
<tr>
<td>Normalized LDH</td>
<td>.11</td>
<td>-.001</td>
<td>.04</td>
<td>-.08</td>
</tr>
<tr>
<td>Fibrinogen level</td>
<td>.42</td>
<td>.99</td>
<td>.79</td>
<td>.59</td>
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<tr>
<td>Albumin level</td>
<td>.06</td>
<td>.00</td>
<td>.19</td>
<td>-.08</td>
</tr>
<tr>
<td>Leukemic cell mass</td>
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<td>.99</td>
<td>.19</td>
<td>.58</td>
</tr>
<tr>
<td>% Abnormal cells</td>
<td>.07</td>
<td>.17</td>
<td>.28</td>
<td>.13</td>
</tr>
<tr>
<td>Albumin level</td>
<td>.02</td>
<td>.23</td>
<td>.05</td>
<td>.37</td>
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Leukemic cell mass is the percentage of leukemic cells in marrow aspirate times biopsy cellularity. Normalized LDH is the patient’s LDH level divided by the highest "normal" value at that institution (laboratory).

represents less than 0.01% of normal bone marrows, and in leukemic marrows their frequency is presumed to be even lower. Because the percentage of S-phase cells labeled by BrdU in the biopsy sections of leukemic marrows is generally 20% or more (see Table 1), even if the normal progenitor cells had an LI of 50%, they would at most represent only 1/1,000th of the labeled cells. This small number could hardly affect the cycle cell calculations. As far as the question of whether “maturing” normal cells were confused with leukemic blasts is concerned, the labeling technique used insures against this. Visual evaluation of every cell that is being examined permits us to distinguish very clearly between leukemic blast cells and more mature cells such as myelocytes, red blood cell precursors, megakaryocytes, and so on (see Fig 1). Thus, the fact that cell cycle studies are addressed to floridly leukemic marrows before any therapy, together with the ability to visually distinguish between blast cells and the few mature residual normal cells, precludes any confusion between leukemic versus normal cells.

A second important point to be noted in these studies is the fact that by labeling S-phase cells in vivo we are able to measure the BM biopsy LI rather than being dependent on an aspirate LI, which can often be spuriously low due to hemodilution. Without this precise measurement of the percentage of S-phase cells, calculations of Tc cannot be made accurately. No previous study in the literature besides our own has used the biopsy LI to calculate Tc.

Finally, our assumption that GF was 100% was based partly on the fact that there is no reliable method of estimating this value accurately, and partly on the basis of

Table 3. Tc of Myeloblasts Versus FAB Classification in Patients Treated on INT-0066

<table>
<thead>
<tr>
<th>FAB</th>
<th>n</th>
<th>Mean Tc (h)</th>
<th>Median Tc (h)</th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>M1</td>
<td>7</td>
<td>70.14</td>
<td>52.0</td>
<td>4</td>
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<tr>
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<td>52.3</td>
<td>41.0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>M3</td>
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<td>98.7</td>
<td>77.5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>M4</td>
<td>14</td>
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<td>51.0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
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<td>47.0</td>
<td>2</td>
<td>3</td>
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<td>5</td>
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<td>40.0</td>
<td>2</td>
<td>3</td>
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</table>

Abbreviations: A, number above median Tc; B, number below median Tc.
Fig 2. Duration of cell cycle time versus outcome of remission induction therapy on INT 0066.

Fig 3. Relationship between Tc (split into two groups: A, above median cell cycle time; B, below median cell cycle time) and remission duration in AML patients treated on INT 0066. Note FAB categorization of all long-term survivors on curve A.

cycle-specific proteins. However, no independent means are presently available to confirm these data. In the final analysis, estimations of biologic parameters such as GF are validated only if they demonstrate substantial correlations with clinical course of the disease or outcome of therapy. In this respect, because some of the parameters, especially those of Tc, are significantly related to clinical course of AML in our study, it appears that even if an error is introduced by our method of calculations it is likely to be a small one.

Having addressed these potential methodologic questions, let us now review the data that have been presented in this report. Not surprisingly, as we have previously reported, even in this relatively homogeneous group of newly diagnosed, standard-risk AML patients, the cell cycle characteristics of myeloblasts vary greatly from patient to patient (Table 1). It is of interest that the leukemic cells of older individuals cycle more rapidly than those of younger patients. This observation may in part account for the poorer prognosis of older individuals. Also interesting is the suggestion that the leukemic cells of patients with FAB M3 have longer cell cycle times than the leukemic cells of other FAB categories (Table 3), which is perhaps a reflection of the advanced state of maturation of these cells. In other studies we demonstrated that the cell cycle times of HL60 cells increase as they mature.

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No unique cell cycle characteristics helped distinguish between those patients who achieved CR versus those who failed remission induction therapy. Because of the high CR rate of 76% on the treatment protocol INT0066, very few treatment failures are available for comparison (33 CRs v 9 RDs). Perhaps with greater numbers of cases being studied, we will be able to identify a relationship between cell cycle time and remission induction outcome. In contrast, we have identified a strong relationship between cell cycle time and remission duration, with the only patients remaining in continuous remission being those whose leukemic cells proliferate slowly.

Relapse in an AML patient who initially achieved CR must be a complex phenomenon being related to the amount of residual disease, the proliferative potential of the surviving leukemic cells, the rate of regrowth of these cells, the effect of consolidation therapy on cell numbers, and their level of differentiation. The lack of relationships between cell cycle time and remission induction outcome, and the strong relationship of cell cycle time and remission duration seems, at first glance, to be paradoxical. However, it becomes understandable if one interprets the remission induction outcome data to indicate that when araC/DNR is administered, the killing of leukemic cells is independent of cell cycle parameters. If this is the case then the most likely explanation for the observed relationship between remission duration and leukemic cell cycle times is that the cell cycle time plays a major role in determining the rate of leukemic cell regrowth between courses of consolidation therapy and subsequent to the cessation of all therapy. If this hypothesis is
correct, then major efforts should be directed at reducing the rate of regrowth of leukemic cells between courses of therapy. Administration of retinoic acid, α-interferon, etc between courses of consolidation therapy may be useful in this regard.

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Direct relationship between remission duration in acute myeloid leukemia and cell cycle kinetics: a leukemia intergroup study

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