Cell Cycle Characteristics, Maturation, and Phagocytosis
In Vitro of Blast Cells From Patients With
Chronic Myelocytic Leukemia

By J. Whang-Peng, S. Perry, T. A. Knutsen, and J. J. Cart

Cell cycle characteristics, phagocytosis and maturation of immature white blood cells from seven patients with Ph'-positive chronic myelocytic leukemia were studied in vitro. In five patients whose cells contained only one Ph' chromosome there were two in early stable phase, one in transitional phase, and two in blastic crisis. The other two patients had cell lines with more than one Ph' chromosome in addition to cells containing one Ph' chromosome. Cell cycle times in the seven patients as determined with tritiated thymidine ranged from 48 to 59 hr regardless of stage of the disease with one exception (32 hr in a patient who died within 1 wk). A small percentage of myeloblasts was arrested in early S or G2 for the duration of the study of up to 1 wk. Maturation in vitro was observed with appearance of the first labeled metamyelocytes at 2 hr and first labeled mature segmented and nonsegmented neutrophils at 10 hr (with the median time of 22 hr of in vitro culture). Results of this study suggest that cell precursors in blastic transformation have a diminished capability to mature despite the early presence of mature neutrophils in vitro. Early appearance of labeled mature neutrophils and irregularity of the time sequence are characteristic of cells from patients in blastic transformation. Immature granulocytic precursors were shown capable of phagocytosis in vitro, suggesting that although nuclear maturation was delayed, membrane and cytoplasmic function was intact. This dissociation may be characteristic of leukocytes from patients with chronic myelocytic leukemia.

There have been a number of reports concerning cell cycle characteristics of immature myeloid leukocytes from patients with chronic myelocytic leukemia (CML), and there have been a few studies of blast cells from patients in the blastic stage of the disease. Most of these studies have been done in vivo following the administration of tritiated thymidine (3H-TdR).1-5 We have had the opportunity to study, in vitro, the cell cycle characteristics, and the maturation and phagocytotic activity of blast cells ob-

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J. Whang-Peng, M.D.: Senior Investigator, Human Tumor Cell Biology Branch, Clinical Trials, National Cancer Institute, National Institutes of Health, Bethesda, Md. S. Perry, M.D.: Associate Scientific Director for Clinical Trials, Chemotherapy, National Cancer Institute, National Institutes of Health, Bethesda, Md. T. A. Knutsen, M.T. (ASCP): Medical Technologist, Human Tumor Cell Biology Branch, Clinical Trials, National Cancer Institute, National Institutes of Health, Bethesda, Md. J. J. Cart, Ph.D.: Head, Mathematical Statistics and Applied Mathematics Section, Biometry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md.
tained from the peripheral blood of patients with Ph1-positive CML. The results of these studies are described in this paper.

MATERIALS AND METHODS

In a syringe containing 800 units of heparin, 40–50 ml of whole blood was collected by venipuncture from two females and five male patients with CML admitted to the Clinical Center, National Institutes of Health. The diagnosis of CML was made on the usual basis and all the seven patients in the study were Ph1 chromosome-positive. Two patients were in the stable phase of the disease, one patient was in a transitional phase, and four patients were in blastic transformation. The blood was allowed to settle by gravity in 40-ml test tubes at 37°C for 30–60 min. After the red cells had settled out, the leukocyte-rich plasma was added to Eagle’s medium with 30% autologous plasma in a proportion of 1 to 5. The cell suspension was incubated at 37°C for 1 hr with 3H-TdR (New England Nuclear Corp., Boston, Mass., specific activity 15.9 Ci/mole), 1.0 μCi/ml culture. The cells were then washed three times with cold Eagle’s medium containing 30% calf serum, penicillin-streptomycin, and L-glutamine.

After the final washing, the cells were resuspended in Eagle’s medium containing 30% calf serum and penicillin-streptomycin at a final cell concentration of 1 × 10^6 cells/ml. Up to 80 identical 6-ml cultures were set up for each patient and the cultures were harvested at different intervals (every 2 hr when possible). Then 1 ml of each culture was fixed in 8:1 absolute alcohol:glacial acetic acid for evaluation of maturation, and the remaining 5 ml of the cultures was harvested for chromosome preparations and for mitotic labeling studies. Radioautographs were prepared from both the maturation and chromosome preparations with AR-10 stripping film and exposed for 1 wk, then developed and stained with Giemsa. Polystyrene latex particles were added to some of the cultures, 0.2 ml of a 1:100 dilution (polystyrene latex, 10% solids, particle diameter 1.1 μ, Dow Chemical Co., Midland, Mich.), 4 hr before harvesting, in order to study the phagocytic activity of the cells.

Giemsa-stained cells were categorized according to the criteria of Diggs et al. The morphology and labeling in different stages of maturation sequence of granulocytes have been described in a preliminary report: myeloblasts that vary in diameter from 15 to 20 μ with moderate amounts of bluish nongranular cytoplasm, a round or lobulated nucleus that stains predominantly red, and usually two or more nucleoli; promyelocytes with a greater amount of cytoplasm than the myeloblasts, less well-defined nucleoli than the myeloblasts, and coarser chromatin strands; neutrophilic myelocytes usually smaller than the promyelocytes and with a relatively larger amount of cytoplasm, round nuclei, usually indistinct or absent nucleoli, and unevenly stained and thickened chromatin strands; neutrophilic metamyelocytes with a slightly indented smaller nucleus, small, pinkish-blue cytoplasmic granules, and less well-defined chromatin structures than the myelocytes; neutrophilic band forms slightly smaller than the metamyelocytes, and a nucleus showing degenerative changes, with usually a dark, pyknotic mass at each pole, and small cytoplasmic granules evenly distributed and staining various shades of pink and blue; segmented neutrophils with definite lobes, connected by a very narrow filament or strand, and light-pink cytoplasm containing small, numerous, and evenly distributed granules, light pink to bluish-black; eosinophils containing relatively large eosinophilic spherical granules and a bilobulated nucleus; basophils with round, indented, band, or lobulated nuclei and dark and metachromatic granules; abnormal mature neutrophils with a small, dense, round, nonlobulated nucleus that has coarse, lumpy chromatin.

The mitotic index was determined by counting the number of mitoses in 5000 nucleated cells and is expressed per 1000 cells. Phases of the mitotic cycle were determined from the labeled mitosis curve in the usual way. The interval between two successive mitotic peaks was taken as the generation time (T_g). The mean duration of S was estimated from the time between the midpoints of the rising and falling curves of per cent labeled mitoses;
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Fig. 1.—Labeled mitosis curve in in vitro cultures from five patients with one Ph¹ chromosome.

median $G_2$ was defined as the interval between administration of $^3$H-TdR and the time when 50% of mitoses are labeled; the duration of $M$ was determined from the equation $T_M = \text{mitotic index (MI)} \times \text{mean generation time (T_G)}/0.693$.

RESULTS

The time of appearance of labeled mitoses after pulse labeling of the cells, in vitro, from five patients is shown in Fig. 1. Each had 46 chromosomes with one Ph¹ chromosome, and the estimates for the various phases of the cell cycle in these individuals are listed in Table 1.

Two of the patients in blastic crisis had one Ph¹ chromosome, as well as additional cell lines with more than one Ph¹ chromosome. Patient B.P. had three different cell lines in the cultures harvested at the beginning of the experiment: 78% of the cells had 46 chromosomes with three Ph¹ chromosomes and were missing two chromosomes in the F group (Fig. 2A); 2% of the cells

<table>
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<th>Table 1.—Cell Cycle Times</th>
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<tr>
<td>Patient</td>
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<td>---------</td>
</tr>
<tr>
<td>H.A.</td>
</tr>
<tr>
<td>SB.</td>
</tr>
<tr>
<td>F.W.</td>
</tr>
<tr>
<td>M.L.</td>
</tr>
<tr>
<td>B.H.</td>
</tr>
<tr>
<td>B.P.</td>
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<tr>
<td>L.E.</td>
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Fig. 2.—(A) Metaphase and karyotype of a cell with 46 chromosomes including three Ph' chromosomes from patient B.P. (B) Metaphase and karyotype of a cell with 46 chromosomes including one Ph' chromosome from patient B.P.

had 45 chromosomes with two Ph' chromosomes and were missing one chromosome in the F group; and 18% of the cells had 46 chromosomes with one Ph' chromosome (Fig. 2B). The percentage of each cell line fluctuated at each harvest time (Fig. 3) although the cells with three Ph' chromosomes remained at 78% of the total for the duration of the cultures except for the hours from 50 to 90. Phases of the cell cycle (Table 1) were difficult to estimate with precision but T_0 was taken as the interval between the major peaks. Patient L.E. (Fig. 3) had two different cell lines: 10% of the cells had 46 chromosomes with one Ph' chromosome and 90% of the cells had 49 to 52 chromosomes with two Ph' chromosomes; in the latter group 72% had 52 chromosomes with two Ph' chromosomes, two extra chromosomes in both groups C and G, and one extra chromosome in both groups D and F. Again the percentage of the two cell lines fluctuated with the time of harvest; after
40 hr of incubation, all the metaphases had two Ph¹ chromosomes. Cell cycle characteristics for this patient are shown in Table 1. Unfortunately, generation times for each of the different chromosome classes could not be determined since overlying radioautographic grains made it impossible to do precise chromosome analyses.

Both labeled and unlabeled neutrophilic precursors were capable of phagocytosis. Further, it is of interest that cells with latex particles were capable of mitosis.

The time of appearance of the first labeled metamyelocytes and granulocytes are shown in Table 2. In patient F. W., who was in transitional phase, labeled metamyelocytes were seen at 2 hr after pulse labeling with ³H-TdR. The four patients with blastic crisis first had labeled metamyelocytes at 6, 12, 14 and 20 hr, and the two patients in a stable phase did not show labeled metamyelocytes until 24 hr and 22 hr after ³H-TdR labeling. The earliest appearance of labeled granulocytes was seen at 10 hr in patient F.W., the same patient in whom the first metamyelocyte was seen. They appeared in the stable-phase patients (H.A. and S.B.) at 48 and 47 hr, and in the patients in blastic crisis (M.L., B.H., B.P., and L.E.) at 16, 48, 22, and 16 hr. The two patients in a stable phase demonstrated better granulocyte maturation than the patients in a more advanced state of their disease; 40 and 60% of labeled cells were mature neutrophils by the fifth day of in vitro cultures, whereas three of the four patients in blastic crisis had only 5, 18, and 26% mature neutrophils by the fifth day.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage of Disease</th>
<th>Duration of Disease</th>
<th>Survival After Experiment</th>
<th>No. of Phls</th>
<th>Peripheral Blood</th>
<th>L.I. (Per Cent)</th>
<th>Appearance Times</th>
<th>Labeled Granulocytes (Per Cent)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cell Count (per cu mm)</td>
<td>Blasts (Per Cent)</td>
<td>Metamyeloctye*</td>
<td>Day: 1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>H.A.</td>
<td>Early</td>
<td>1 mo</td>
<td>30 mo +</td>
<td>1</td>
<td>33,700</td>
<td>1</td>
<td>7</td>
<td>24 48 0 4 24 48 60 — — —</td>
</tr>
<tr>
<td>S.B.</td>
<td>Early</td>
<td>1 wk</td>
<td>22 mo +</td>
<td>1</td>
<td>334,000</td>
<td>1</td>
<td>6</td>
<td>22 47 0 2 4 28 40 — 41 —</td>
</tr>
<tr>
<td>F.W.</td>
<td>Transitional</td>
<td>18 mo</td>
<td>3 mo</td>
<td>1</td>
<td>23,800</td>
<td>9</td>
<td>8</td>
<td>2 10 8 4 18 34 38 — 44 —</td>
</tr>
<tr>
<td>M.L.</td>
<td>Blastic crisis</td>
<td>114 mo</td>
<td>3 mo</td>
<td>1</td>
<td>39,000</td>
<td>78</td>
<td>12</td>
<td>14 16 2 4 3 7 — — — 18</td>
</tr>
<tr>
<td>B.H.</td>
<td>Blastic crisis</td>
<td>2 wk (?)</td>
<td>3 mo</td>
<td>1</td>
<td>161,000</td>
<td>23</td>
<td>6</td>
<td>6 48 0 2 6 17 26 — — —</td>
</tr>
<tr>
<td>B.P.</td>
<td>Blastic crisis</td>
<td>53 mo</td>
<td>1 mo</td>
<td>1</td>
<td>218,000</td>
<td>19</td>
<td>18</td>
<td>20 22 2 4 4 4 5 10 9 —</td>
</tr>
<tr>
<td>L.E.</td>
<td>Blastic crisis</td>
<td>26 mo</td>
<td>1 wk</td>
<td>1 2 3</td>
<td>150,000</td>
<td>20</td>
<td>5</td>
<td>12 16 2 2 8 28 43 — — —</td>
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* Appearance of first labeled cells, in hours.
L.I., labeling index.
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DISCUSSION

There have been relatively few determinations of cell cycle phases of immature myeloid cells from patients with CML. The first studies were those reported by Killmann et al., who estimated on the basis of grain count analysis that the generation time of blast cells was 53–57 hr in a patient with CML in possible blastic transformation, and 45–51 hr in a patient in a more typical blastic crisis phase. Computed DNA synthesis times were less than 3.3 and 6.2 hr respectively.

More recently Ogawa et al., utilizing essentially the same approach as previous investigators, studied three patients with CML, two of whom were Ph1 chromosome-positive, but no satisfactory preparation was obtained in the third. Median grain-count halving times were 88 and 102 hr for the myeloblasts of the marrow and blood, respectively. Marrow myeloblasts of the third patient were found to have a grain count halving time of 40 hr. For comparison, cell cycle times for normal myeloblasts in vivo have been estimated at 24 hr, and for blasts in acute myelocytic leukemia, at 50–60 hr.

A few studies of in vitro DNA synthesis in immature cells from the peripheral blood have been published. Craddock and Nakai suggested, on the basis of 3H-TdR uptake, that leukemic blast cells may have a much longer generation time than normal or precursor cells in chronic leukemia. In the present study, cell cycle times of blasts studied in vitro ranged from 48 to 59 hr except for one patient in blastic crisis whose blasts divided at 32-hr intervals. Cells with more than one Ph1 chromosome had shorter generation times, and in patient L.E. the cell line with two Ph1 chromosomes appeared to outgrow the cell line with only one Ph1 chromosome. It is of interest that the labeling index of the metaphases with two Ph1 chromosomes approached 100%. However, because of a high death rate in this patient’s cultures, there may have been significant reutilization of labeled material.

DNA synthesis times in the present study ranged from 18 to 37 hr, which is greater than previous estimates in CML, but is probably not significantly different from estimated DNA synthesis times of 13 to 24 hr in normal myeloblasts. The cell cycle times did not appear to be related to the stage of the disease in our small series of patients, although the patient whose blasts had the shortest generation time died within 1 wk of the study, with a rapidly rising count. The two patients in the early, stable phase of their disease had the longest T1: 0.45 and 0.6 hr, which were the closest to the normal duration; three of the four blastic crisis patients had shorter T1 times: 0.18, 0.24, and 0.24 hr.

Considerable variation has been reported for both the interval between in vivo administration of 3H-TdR to the first appearance of labeled mature neutrophils and for the development of these cells in vitro from labeled precursors. Fliedner et al. studied 15 patients with a variety of hematopoietic and nonhematopoietic conditions. The interval to the appearance of the first labeled segmented granulocytes in the peripheral blood after intravenous 3H-TdR ranged from 96 to 144 hr except in one instance of bacterial infection in which this interval was shortened. This shorter emergence time was interpreted as indicating a faster than normal nuclear maturation with normal or
delayed cytoplasmic maturation. The possibility that variations in the release of cells into the blood might be responsible was considered unlikely, since previous studies by these investigators had indicated that release from the marrow reserve was a random process.

In the present in vitro study, the time to appearance of labeled mature neutrophils (segmented, lobulated, or round forms) more closely resembles data reported in the latter patient. The appearance time of first labeled neutrophils varied from 10 to 48 hr and the median time for all patients was 22 hr. Boll® reported that the maturation curve in vitro of inflammatory and CML cells both remained within the normal range. If this is true, then the longer time required for the first appearance of labeled normal neutrophils in the study by Fliedner et al. was most likely due to a delay in the release of these cells from the bone marrow compared to the neutrophils of patients with infection.

The two patients in the early and more stable stages of CML had longer maturation times (47 and 48 hr) and 40 to 60% of the labeled precursors matured into granulocytes. In three of the four blastic crisis patients the labeled mature neutrophils appeared within 24 hr but less than 26% of the labeled cells were mature neutrophils after 5 days of culture. These results indicate that cell precursors in blastic crisis have a diminished capability to mature despite the early presence of mature neutrophils in vitro. Clinically, the patients with a higher percentage of labeled mature neutrophils in the in vitro culture tended to have a better prognosis.

The shape of the nucleus of the mature neutrophil—segmented, bilobulated, or round—had no bearing on the time of appearance of the labeled cell and is in agreement with previous studies. Normally, the maturation sequence proceeds from metamyelocytes to band forms to mature neutrophils, but apparently this was not the case in the present study because there was no increase in labeled band forms observed prior to the appearance of labeled mature neutrophils. The early appearance of labeled mature neutrophils and irregularity of the time sequence may be characteristic of blastic cells from CML patients in blastic crisis.

Heavily labeled myeloblasts or lobulated blast forms were noted throughout the culture period. This was interpreted as arrest in either S or G2. The duration of the arrested phase was up to 1 wk of culture for some patients. This delay could be attributable to either an inherent defect in maturation or a deficiency of an essential factor requisite to normal maturation. The fact that labeled or unlabeled precursors are capable of phagocytizing latex particles as well as bacteria® supports the proposition that nuclear maturation is delayed while the cytoplasm matures normally. This divergence of differentiation may possibly be another characteristic of CML cells.

REFERENCES

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