Biochemical Abnormality of DNA Synthesis of Erythroblasts in Acute and Chronic Myeloid Leukemia

By Peter Ernst, Michele Baccarani, and Sven-Aage Killmann

Six patients with acute and chronic myeloid leukemia (AML and CML) showed a low incorporation of labeled DNA precursors into basophilic erythroblasts. Based on mitotic indices and the use of vincristine as a stathmokinetic tool, it was evident that brisk proliferation in this cell compartment took place. Microspectrophotometric determination of single-cell DNA content showed that a considerable fraction of the erythroid cells were in DNA synthesis without incorporating an exogenously supplied DNA precursor. Following inhibition of the de novo synthesis of nucleoside triphosphates, the cells showed capacity for precursor incorporation. It is suggested that a biochemical abnormality, which leads to a high intranuclear nucleoside triphosphate pool, may inhibit nucleoside kinases and hence the utilization of labeled nucleosides, as well as diluting the labeled precursors. The defect should probably be considered to be a biochemical expression of "leukemicness" of the erythroid cells. The frequency of the phenomenon described here cannot be determined from the present study. The patients are part of a series of 44 patients who were studied with the purpose to investigate the effect of cytostatic treatment on the cell cycle of the leukemic cells. Many of the patients had so little erythropoiesis left that studies as those described here could not be carried out. Therefore, the reported frequency of 6/44 of the observed phenomenon is almost certainly an underestimate. Consequently, cytokinetic studies of erythropoiesis in CML and AML, using tracer techniques alone, must be evaluated with caution.

Since the introduction of radioautography with labeled deoxyribonucleosides as markers of DNA synthesis, considerable knowledge of the kinetics of proliferating cell systems has been achieved. Although criticism of this method has been raised, labeling procedures are generally accepted to provide reliable information about cell proliferation. We have recently had the opportunity to study six patients with myeloid leukemia who had sufficient

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Abbreviations used in text: TDR, thymidine; UDR, deoxyuridine; MTX, methotrexate; ARA-C, cytosine-arabinoside; VCR, vincristine; \( I_{tr} \), percentage of cells flash-labeled with tracer; \( I_M \), percentage of cells in mitosis; MGC, mean grain count; \( G_1 \), pre-DNA synthesis phase of cell cycle; \( S \), DNA synthesis phase; \( G_2 \), post-DNA synthesis phase; \( U \)-cells, \(^3H\)-TDR-unlabeled cells (in vitro) with a DNA content corresponding to cells in DNA synthesis.

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erythropoiesis left to make a study of this cell system in human leukemia possible. In the following, data on in vitro \(^3\)H-thymidine (\(^3\)H-TDR) incorporation will be presented. They show that in leukemia \(^3\)H-TDR incorporation into proliferating erythroid precursors may severely underestimate the fraction of erythroid cells in DNA synthesis.

**MATERIALS AND METHODS**

Four patients with acute and two patients with chronic myeloid leukemia were studied; data concerning the patients are presented in Table 1. The data of one patient (patient 3) have been published earlier. Only one patient had received cytotoxic treatment before the study, and in this case the drug was discontinued 14 days before the study. Bone marrow aspirates were taken from the sternum or iliac crest and were divided into two aliquots. One fraction was smeared on glass slides without delay, and after staining with May-Grünwald-Giemsa, the mitotic index (\(I_M\), percentage of cells in mitosis) was determined. Mitotic cells include cells from late prophase to late telophase. The remaining fraction of the aspirate was incubated at 37\(^\circ\)C for 1 hr with \(^3\)H-TDR (specific activity 1.9 Ci/mM) and in some cases also with \(^3\)H-UDR (\(^3\)H-deoxyuridine [specific activity 5.0 Ci/mM]). After radioautograms had been processed (Kodak NTB-2 dipping emulsion, exposure time 7–25 days according to the labeling of appropriate test slides), the labeling index (\(I_L\), percentage of cells labeled with nucleoside) was determined. \(I_L\) and \(I_M\) of the basophilic erythroid precursors were in most cases determined from a count of 300 cells. In the myeloblasts, these parameters were based on counts of 1000 and 3000 cells, respectively. The mean grain count (MGC) was determined from 25 labeled cells. In one case (patient 3), measurements of nuclear DNA content were performed by the Zeiss microspectrophotometer UMSP-I after Feulgen staining. In the study where the DNA content of labeled erythroblasts was determined, cells were plotted on a photomap of the radioautograph and after the Feulgen staining was performed, the cells were relocated and measured.

**RESULTS**

The results on \(I_L\), \(I_M\), peripheral reticulocyte count, and hemoglobin concentration are shown in Table 2. It is seen that in erythroid cells, the \(I_M\) was found in the range usually reported for normal basophilic erythroid precursors. In contrast to this, in five of the cases (patients 1, 2, 3, 5, and 6)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Previous Treatment</th>
<th>Treatment During Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>F</td>
<td>CML</td>
<td>None</td>
<td>VCR, 0.04 mg/kg body weight i.v. after first bone marrow</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>F</td>
<td>CML</td>
<td>Bulsulfan, 4 mg/day, D/C 14 days before study</td>
<td>VCR, 0.04 mg/kg body weight i.v. after first bone marrow</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>M</td>
<td>AML</td>
<td>None</td>
<td>MTX, 0.5 mg/kg body weight i.v. after first bone marrow</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>M</td>
<td>EL/AML</td>
<td>None</td>
<td>ARA-C, 2.0 mg/kg body weight i.v. after first bone marrow</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>M</td>
<td>AML</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>F</td>
<td>EL/AML</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*AML, acute myeloid leukemia; CML, chronic myeloid leukemia; EL/AML, erythroleukemia; VCR, vincristine; MTX, methotrexate; ARA-C, cytosine-arabinoside.
Table 2. Data on \(^3\)H-TDR and \(^3\)H-UDR Labeling Indices (I\(_L\)), Mitotic Indices (I\(_M\)), and Mean Grain Counts (MGC) in Leukemic Myeloblasts and Basophilic Erythroblasts

<table>
<thead>
<tr>
<th>Patient</th>
<th>Myeloblasts</th>
<th>Basophilic Erythroblasts</th>
<th>Reticulocytes (%)</th>
<th>Hemoglobin (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^3)H-TDR I(_L)</td>
<td>(^3)H-UDR I(_L)</td>
<td>MGC</td>
<td>I(_M)</td>
</tr>
<tr>
<td>1</td>
<td>19.0</td>
<td>—</td>
<td>30.0</td>
<td>1.91</td>
</tr>
<tr>
<td>2</td>
<td>24.5</td>
<td>—</td>
<td>28.3</td>
<td>1.61</td>
</tr>
<tr>
<td>3 Before MTX</td>
<td>8.5</td>
<td>10.8</td>
<td>20.6 (TDR)</td>
<td>0.80</td>
</tr>
<tr>
<td>2 Hr after MTX</td>
<td>10.6</td>
<td>0</td>
<td>—</td>
<td>0.07</td>
</tr>
<tr>
<td>6 Hr after MTX</td>
<td>14.4</td>
<td>0.5</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>16 Hr after MTX</td>
<td>15.7</td>
<td>7.9</td>
<td>4.3 (TDR)</td>
<td>0.07</td>
</tr>
<tr>
<td>24 Hr after MTX</td>
<td>16.4</td>
<td>17.5</td>
<td>—</td>
<td>0.20</td>
</tr>
<tr>
<td>In remission</td>
<td>27.0</td>
<td>25.0</td>
<td>&gt;100 (TDR)</td>
<td>0.40</td>
</tr>
<tr>
<td>&gt;100 (UDR)</td>
<td>13.5</td>
<td>—</td>
<td>61.2</td>
<td>0.95</td>
</tr>
<tr>
<td>4 Before ARA-C</td>
<td>14.6</td>
<td>—</td>
<td>52.8</td>
<td>0.39</td>
</tr>
<tr>
<td>16 Hr after ARA-C</td>
<td>17.6</td>
<td>—</td>
<td>36.0</td>
<td>0.53</td>
</tr>
<tr>
<td>5</td>
<td>7.0</td>
<td>—</td>
<td>35.4</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. Some of the observations in case 3 have been reported before.\(^{15}\) Unless otherwise mentioned, MGC refers to \(^3\)H-TDR labeling.
the IL was disproportionally low and on the average was lower than the IM. In the sixth patient (patient 4, before therapy), the IL was not much less than normal, but the MGC was exceedingly low. No such discrepancy was seen between the IL and IM in the myeloblasts counted in the same slides. Similarly, the MGC of myeloblasts was high without exception. In case 3, a marked increase in the IL was observed after methotrexate (MTX); at the same time, the MGC increased somewhat (Table 2). In case 4, the IL of the erythroid cells temporarily fell after cytosine arabinoside (ARA-C) but subsequently, the IL rose considerably above the pretreatment value, and the MGC increased tenfold. Figure 1 shows the stathmokinetic effect of a single intravenous injection of vincristine (VCR) on the erythroid cells in cases 1 and 2. As seen from the figure, a definite accumulation of mitoses was found, demonstrating a good flux of cells into mitosis. Calculating the mitotic duration of the erythroid cells from the increment in prophase + metaphase index, as related to the initial prophase + metaphase index (these data are shown in Fig. 1), mitotic duration was about 70 min in case 2, and 130 min in case 1. In Fig. 2, the DNA content of basophilic erythroblasts in case 3 before treatment is presented. Approximately 80% of the unlabeled early erythroid cells contained a DNA amount higher than the amount that corresponds to the G1-phase. The histogram thus demonstrated that the large majority of the cells were in DNA synthesis phase. Figure 3 gives the distribution of 3H-UDR-labeled basophilic erythroblasts 16 hr after MTX was given. It is seen that there is a relative lack of cells that incorporate the precursor in the early phase of DNA synthesis.

**DISCUSSION**

Thymidine monophosphate, which after a sequence of phosphorylation steps is utilized for DNA synthesis, is produced either by de novo synthesis from carbamyl-aspartic acid or by phosphorylation of exogenous TDR by
ABNORMAL DNA SYNTHESIS IN CML

Fig. 2. Distribution of basophilic interphase erythroblasts with respect to nuclear DNA content. 2n indicates DNA content of granulocytes = G1 phase; 4n indicates DNA content of mitoses equal to G2 phase. Note that although labeling indices were low in this situation, a considerable number of cells were found with a DNA content corresponding to cells in DNA synthesis phase. VK = case 3.

means of the TDR kinase (salvage pathway).6 Due to end product inhibition by thymidine triphosphate, the activity of the TDR kinase and, in this way, the ability of the cell to take up exogenous TDR is regulated.7 The negative feedback control is highly specific; thus, thymidine triphosphate does not influence the thymidylate kinase.7 Most cell systems seem to be able to utilize the de novo synthesis, as well as the salvage pathway, but cell systems with TDR kinase deficiency have been reported.8,9 In the present study, erythroblasts were found to incorporate only small amounts of exogenous TDR into DNA, giving the illusion that no DNA synthesis took place. At the same time, however, mitoses were observed in the erythroblasts in a number equal to that reported for normal erythroid cells. Conceivably, this high hi could have been due to a very long mitotic duration or to a definite arrest of cells in mitosis. This can be ruled out for two reasons. (1) In all patients the peripheral reticulocyte count was normal or elevated, which is evidence of a rather effective red cell production (Table 2). (2) The studies in which VCR was given (Fig. 1) demonstrated a steep increase in the number of metaphases;

Fig. 3. 3H-UDR labeled basophilic erythroblasts: distribution with respect to nuclear DNA content. Note relative lack of cells in first part of S phase 16 hr after MTX. VK = case 3.
the calculated mitotic duration was found to be equal to or only slightly longer
than that observed in normal erythroid precursors. This observation shows
that erythroid cells progressed through the cell cycle at an almost normal
speed. In a proliferating steady-state cell system:

\[
\frac{N_s}{N_c} = \frac{T_s}{T_c}
\]

where \(N_c\) is the total cell number, \(N_s\) is cells in DNA synthesis, \(T_c\) is cell
cycle time, and \(T_s\) is DNA synthesis time. If DNA synthesis time were very
short in comparison to the total cell cycle time, \(I_i\) would be slow. This explana-
tion for the observed low labeling indices is ruled out by the study where the
DNA content of individual basophilic erythroblasts was measured. As seen
from Fig. 2, a considerable proportion of cells was found in S phase, indicating
that S phase was far from being a negligible part of the total cell cycle time.

The most likely explanation for the present observations is that exogenously
supplied \(^3\)H-TDR was not taken up by the cells, or at least was not incor-
porated into macromolecular compounds, and hence was invalid as a marker
of DNA synthesis. In the cases reported, typical values of \(I_i\) and MGC of
myeloblasts were found. The fact that myeloid cells were adequately labeled
rules out simple technical factors (e.g., degradation of \(^3\)H-TDR, poor film
emulsion, etc.) as an explanation of the extremely low labeling of the eryth-
roid precursors. It is notable that the MGC of the erythroid cells was very
low. The MGC of erythroblasts normally approximates 50% of the MGC of
myeloid cells.\(^{11}\) Geometric factors may explain this phenomenon.\(^{12}\) The present
data show that the MGC of erythroid cells was far lower than one-half of the
MGC of myeloid cells. The studies where drugs were administered support the
hypothesis that a biochemical abnormality is operative. In the cases where
MTX and ARA-C were given, a dramatic rise in the \(I_i\) and MGC of the
erythroblasts took place. This rules out kinase deficiency as a reason for the
low incorporation, unless enzyme induction took place during cytostatic
treatment, which does not seem likely within the short observation period of
the present study. Therefore, the most likely explanation of the present data
is that a biochemical disturbance in DNA formation in erythroid cells was
present that led to a higher intracellular pool of nucleoside triphosphates than
normal. A high intracellular concentration of nucleoside triphosphates would
lead to a dilution of the labeled precursors and to depression of the kinase
activity from end product inhibition by the triphosphates. We believe that
when MTX or ARA-C was given, both of which interfere with the formation
of thymidine monophosphate,\(^{13,14}\) the de novo synthesis of DNA was
inhibited, and the nucleoside triphosphate pool was diminished, either by
polymerization to DNA or by degradation to low molecular weight metabolites.

Anyhow, the size of the nucleoside triphosphate pool was not sufficient to
keep up the synthesis of DNA when the de novo synthesis of nucleoside
triphosphates was blocked by MTX or ARA-C, as determined from the decline
in the \(I_i\). As the size of triphosphate pool diminished, the end product
inhibition of the kinase decreased, and this led to a higher incorporation rate
of TDR and UDR. Concerning the study where MTX was given, TDR was
readily incorporated into DNA because the cells were now supplied with the
precursor that they were not able to synthesize themselves. The problem of UDR incorporation is more complex. By blocking the de novo synthesis of DNA, one might expect an accelerated incorporation of UDR. However, the ability of the cell to utilize this precursor would at the same time be hampered because of inhibition of the folate reductase, so that finally no incorporation into DNA would take place. We have presented evidence that MTX does not affect cells in G1 phase. The data of Table 2 (case 3) show that the supposedly high thymidine triphosphate pool is soon exhausted, as evidenced by the rapid rise in 3H-TDR IL. However, there is a lag with respect to the rise in the UDR IL that must be due to the MTX action on folate reductase. Between 6 and 16 hr after MTX, these erythroblasts began to incorporate 3H-UDR. According to the hypothesis, these cells should be cells that had had their thymidine monophosphate synthesis blocked, i.e., cells that were in S phase at the time MTX was available but which between 6 and 16 hr after MTX had recovered from the deoxyuridine/thymidine monophosphate block, having a normal intracellular concentration of triphosphates and having resumed their progression through the S phase. Exactly where in S phase this cohort would be would depend on when the metabolic block became ineffective. Determination of single cell DNA content of 3H-UDR-labeled basophilic erythroblasts, although for technical reasons only a few cells could be measured, showed a tendency toward accumulation in late S phase 16 hr after MTX (Fig. 3).

So far, the reason for the suggested biochemical abnormality remains a matter of speculation. Cells with a DNA content corresponding to cells in S phase but that do not incorporate TDR ("U cells") have been demonstrated among late erythroblasts in conditions like megaloblastic and sideroblastic anemia but rarely in leukemic blast cells. However, the number of such cells is generally small, and in megaloblastic erythropoiesis there is evidence that such cells are dying. In contrast to this, the reticulocyte counts of the present patients do not suggest any appreciable erythroid death function. The phenomenon of asynchronous DNA replication might explain the occurrence of occasional "U cells." However, if this were the explanation of the very large fraction of U cells found here, one would have to make the highly unlikely assumption that the flashes of DNA synthesis were extremely short and quick, keeping in mind the high flux of cells into mitosis.

Evidence that erythroid cells in AML are derived from a common leukemic stem cell is accumulating and is convincing in CML: it is thus possible that the observed phenomenon reflects a biochemical aspect of "leukemicness."

The data reported here imply that cytokinetic studies of erythroid cells in the myeloid leukemias using nucleoside tracers should, as far as possible, be checked with mitotic index determination. In cases of discrepancies between the data, stathmokinetik techniques and single cell DNA content determinations are necessary to obtain interpretable results.

REFERENCES


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