Changes in Proliferative Activity of Marrow Leukemic Cells During and After Extracorporeal Irradiation of Blood

By B. W. B. Chan and F. G. J. Hayhoe

Proliferation activity of bone marrow cells was studied in six patients with acute myeloid leukemia treated by extracorporeal irradiation of the blood (ECIB). One patient with acute lymphoblastic leukemia in full remission was also studied and one patient received splenic irradiation in addition to ECIB. The observations support the hypothesis that ECIB may alter feedback mechanisms of growth control within the leukemic tissue, resulting in an increased proliferative fraction. It is suggested that the non-proliferative leukemic blast cell is not an end cell but is capable of being recalled into cell cycle by an appropriate stimulus such as ECIB.

EXTRACORPOREAL IRRADIATION OF THE BLOOD either alone or in combination with chemotherapy, has been used in the therapy of acute leukemia. The basic theoretical argument has been that the leukemic cells in the marrow exchange or “turnover” with the cells in the peripheral blood, or may be induced to do so by ECIB, so that by irradiating the peripheral blood a significant proportion of the leukemic tissue (in an ideal case all the cells) might be destroyed. In a preliminary communication we reported that the proliferative fraction of the leukemic blast cells in the marrow is increased following ECIB and we postulated that some of the resting cells may have been induced to resume proliferative activity as a result of ECIB, possibly via a feedback mechanism. Since most drugs used in acute leukemia act on the proliferative cell cycle either in the phase of deoxyribonucleic acid (DNA) replication or in the preparation for mitosis, the combination of ECIB followed by chemotherapy may have therapeutic advantages.

PATIENTS, MATERIALS AND METHODS

Six patients with acute myeloid leukemia (AML) were studied. One patient (No. 3) received two courses of ECIB and was also given splenic irradiation (SI) on a third occasion. All AML patients received either cytosine arabinoside (Ara-C), or a combination of Ara-C, methotrexate, 6-MP, and prednisone, following ECIB. Patient 1 had two previous complete remissions. Patients 2 and 4 had received chemotherapy for 3 months without response prior to ECIB. All the patients showed rapid fall in the peripheral blood.
Table 1.—Summary of Clinical and Experimental Details in Six Patients Given ECIB *

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical Status</th>
<th>Previous Treatment</th>
<th>Blood Count at Start of ECIB (Cells per cu mm)</th>
<th>Average ECIB Dose to Whole Blood (Rads)</th>
<th>Duration of ECIB (hr)</th>
<th>Timing of Post-ECIB Sample (hr after start)</th>
<th>Labeling Index</th>
<th>x²</th>
<th>Mitotic Index</th>
<th>x²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R</td>
<td>VCR, 6MP, MTX, PRED</td>
<td>8,950</td>
<td>6,400</td>
<td>22,000</td>
<td>24</td>
<td>3</td>
<td>19.6</td>
<td>256.27 †</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>U</td>
<td>VCR, PRED</td>
<td>11,000</td>
<td>5,000</td>
<td>12,000</td>
<td>6</td>
<td>12</td>
<td>17.0</td>
<td>52.2</td>
<td>544.64 †</td>
</tr>
<tr>
<td>3(a)</td>
<td>N</td>
<td>None</td>
<td>33,500</td>
<td>26,800</td>
<td>9,200</td>
<td>17</td>
<td>12</td>
<td>18.4</td>
<td>29.5</td>
<td>67.04 †</td>
</tr>
<tr>
<td>3(b)</td>
<td>U</td>
<td>ECIB, ARA-C</td>
<td>23,500</td>
<td>11,050</td>
<td>16,000</td>
<td>30</td>
<td>18</td>
<td>16.6</td>
<td>24.0</td>
<td>32.97 †</td>
</tr>
<tr>
<td>4</td>
<td>U</td>
<td>6MP, PRED</td>
<td>8,000</td>
<td>0</td>
<td>16,700</td>
<td>25</td>
<td>20</td>
<td>3.1</td>
<td>3.6</td>
<td>0.63 †</td>
</tr>
<tr>
<td>5</td>
<td>N</td>
<td>None</td>
<td>45,900</td>
<td>43,150</td>
<td>17,500</td>
<td>30</td>
<td>24</td>
<td>8.2</td>
<td>18.1</td>
<td>85.96 †</td>
</tr>
<tr>
<td>6</td>
<td>N</td>
<td>None</td>
<td>55,600</td>
<td>41,150</td>
<td>19,300</td>
<td>23</td>
<td>24</td>
<td>6.1</td>
<td>5.5</td>
<td>0.17 †</td>
</tr>
</tbody>
</table>

* Patient 3 had two courses of ECIB. Clinical status: N, new case; R, relapse; U, unresponsive to previous therapy. ARA-C, cytosine arabinoside; PRED, prednisone; MTX, methotrexate; VCR, vincristine.
† p < 0.001.
‡ Not statistically significant.
blast cell count following ECIB and chemotherapy and our impression is that the fall was more rapid and more sustained than if chemotherapy was used alone, although the group was too heterogeneous for a definite conclusion on this point to be drawn. Patients 3 and 4 achieved a partial remission and patient 2 showed a transient recovery of normal granulopoietic activity. None achieved full remission.

In addition, one patient with acute lymphoblastic leukemia in full clinical and hematological remission consented to have a course of ECIB prior to receiving chemotherapy for the maintenance of his remission. He is still in remission, 18 months after ECIB, at the time of writing. Table 1 summarizes the clinical and experimental details. Calculation of the ECIB dose was as described by Lajtha et al.2

ECIB was carried out using a beta-ray extracorporeal irradiator similar to that described by Gilbert and Lajtha.6 Essentially the arrangement consists of (1) a coil of nylon tubing (2.7 mm bore, 3.26 mm overall diameter) through which the blood circulates, wound on an elongated coil holder, and (b) four 90Sr/90Yt beta ray sources (total amount of 90Sr in 1966: 26.3 Ci). The sources were prepared and loaded into the source holder by Radiochemical Centre, Amersham. They were arranged in pairs and each arm of the elongated nylon coil slides between a pair.

Arterial blood from a Scribner type A/V shunt flowed through a short length of Teflon tubing containing a junctional T or Y piece, the side arm of which was connected to a 500-ml bottle of normal saline containing 10,000 units of heparin. The amount of heparin given was 10,000–30,000 units in 24 hr. The blood then flowed through the nylon coil in the irradiator and back via a short length of Teflon tubing to the venous side of the shunt.

Marrow samples were aspirated from the sternum and/or iliac crest. Part of each sample was smeared on glass slides and stained by the May-Grünwald-Giemsa method for the determination of the mitotic index. Another part of each sample was incubated with 3H-thymidine 1 μCi/ml, specific activity 5 Ci/mM (Radiochemical Centre, Amersham) in a culture medium containing TC 199 (Claxo) 70% and AB rhesus positive serum 30% for 1 hr. Autoradiographs were then prepared using Ilford G5 emulsion with an exposure time of 6 days. Mitotic index and 3H-thymidine labeling index were determined by counting 2000 cells from each sample. Cells with 5 or more grains were counted as labeled. The majority of labeled cells had 20 grains or more.

The timing of the “post-ECIB sample” is given in Table 1. In some cases ECIB was continued for some hours after the “post-ECIB sample,” the sample being taken just before the patients were given intensive chemotherapy.

RESULTS

Data are available on seven courses of ECIB treatment on patients with full relapse status (Table 1). In five patients the labeling index was significantly increased in the post-ECIB sample (p < 0.001). The mitotic index was significantly increased in two cases only (in the other cases the changes were so small that no statistical test was attempted).

In two patients additional marrow samples were taken at different sites during ECIB (Table 2). In one case the labeling index remained stable for the first 8 hr of study and showed a significant increase in the 12th-hr sample. In the other case a significant increase was found 4 hr after ECIB. There was little change in the mitotic index.

Patient 3 was given a single dose of 250 rads to the spleen on an occasion subsequent to his second ECIB course. It was noted that on the day after irradiation the spleen size was approximately halved, so destruction of leukemic tissue in the spleen probably had occurred. Marrow samples (outside the irradiation field) were taken before SI, and at 80 min and 24 hr after
Table 2.—Changes in Labeling and Mitotic Indices in Marrow Blast Cells During ECIB in Patients 3 and 5

<table>
<thead>
<tr>
<th>Patient</th>
<th>Hours After Start of ECIB</th>
<th>Labeling Index Per Cent</th>
<th>Mitotic Index Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>18.4</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>(first course of ECIB)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>17.6</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>19.8</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>29.5</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>8.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>18.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>19.7</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>18.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

SI. No significant changes in the labeling index (12.8–13.9%) or the mitotic index (0.8–1.2%) were observed.

In the patient in full remission, marrow samples were obtained at 0, 4, 8, and 12 hr after the start of ECIB (17,500 rads in 25 hr). The labeling and mitotic indices were not significantly changed during ECIB. (Labeling and mitotic indices for myeloblasts + promyelocytes, 49.6–54.2% and 2.7–3.3% respectively; for normoblasts, 36.2–38.6% and 1.6–2.1%, respectively.) These values are within the range for normal marrow precursor cells studied in our laboratory.

**DISCUSSION**

In five of the seven studies, the observations on the labeling index clearly indicated a change in the proliferative behavior of the marrow blast cells after ECIB. Of the two cases in which there was no significant change, one (patient 4) had no demonstrable circulating leukemic cells at the time of ECIB. A careful search of blood smears taken just before the start of ECIB showed no blast cells in 500 cells seen. Two days previously, the patient had 50/cu mm circulating blasts and the decision was taken to include him in the ECIB series. Since there were hardly any leukemic cells to be destroyed by ECIB, the absence of any observable marrow change was not surprising and did not contradict the general trend of increased proliferation observed in the other cases. Indeed, had a significantly increased labeling index been found in this case the question would have been raised whether the observed change might be due to some nonspecific effect of ECIB, rather than a response to destruction of the peripheral leukemic cell mass.

In the remaining case (patient 6) although the peripheral blood blast cell count was high at the time of ECIB, there was no significant change in the labeling index. The behavior of the leukemic cell population in this case thus appeared to have been different from the others, although a transient change in the labeling index could have been missed.

The changes in mitotic index were less consistent than the changes in the labeling index. However, this is not a surprising result, since the duration of mitosis may be as short as 1/20 of that of the synthesis of DNA; that is, the time taken to undergo mitosis may be less than 1 hr. Thus, under conditions...
of increasing cell proliferation, the mitotic index may increase little if there is much spread in the duration of the phases of the cell cycle. Alternatively, if there is little spread in these time parameters and a high degree of synchrony is achieved by an inducing stimulus, the mitotic index will show a transient rise, which may be observed only if samples are obtained at the right times. Furthermore, the timing of samples might not have allowed cells recalled into cycle to have completed $S$ and $G_2$ phases. Thus the mitotic index is a less reliable indicator of cellular proliferation compared with the labeling index, and especially so under nonsteady-state conditions.

It is known that leukemic cells in the blood have a lower proliferative rate than the ones in the marrow,\textsuperscript{8,9} probably due to a selective release of nonproliferative cells from the marrow. When the leukemic cells in the blood are destroyed by ECIB, there may be increased release of nonproliferative cells from the marrow. This will have the effect of temporarily nonincreasing the proliferative fraction in the marrow. However, the selective release phenomenon is very unlikely to account for all the observed changes in the labeling index. If this phenomenon alone operates, there would be no rebound in the peripheral blood leukemic cell count after ECIB, whereas in one patient treated in this center in whom ECIB was not followed by cytotoxic drugs there was a very sharp rebound each time ECIB was stopped.\textsuperscript{10} This rebound phenomenon was also reported by other investigators.\textsuperscript{2-4} The finding of rebound of the leukemic cell count to very high levels after ECIB supplements the marrow labeling index findings and suggests that increased proliferation probably occurred at some stage.

In the one study with SI it appeared that the effects of SI were different from those of ECIB. However, no definitive conclusions could be drawn from this single case.

The patient in full clinical and hematological remission, with no circulating leukemic cells at the time of ECIB, acted as control and made possible the study of normal marrow precursor cells during and after ECIB. The finding of no significant changes in the marrow in this case suggests that the increase in marrow proliferative activity observed in most of the leukemic cases following ECIB was in some way dependent on the destruction of leukemic cells by ECIB.

The principal conclusion to emerge from this work is that at least part of the nonproliferative leukemic cell population consists not of end cells but of cells temporarily out of cell cycle that may be recalled into proliferative cycle by an appropriate stimulus, in this instance the acute depletion of the peripheral leukemic cell mass by ECIB.

There is now increasing evidence that proliferative activity in a cell consists of an orderly sequence of synthetic events, which may be considered to be gene-directed (see Baserga\textsuperscript{11}). Cell proliferation is, therefore, a special example of the expression of genetic information. The question whether the nonproliferative leukemic cell is an "end cell" can now be posed more precisely: Does the genetic information for cell proliferation continue to exist in the nonproliferative cell? Viewed in this way, the only truly irreversible
changes in a cell are those of a genetic nature—that is, involving a change in the nucleotide sequence of DNA. All other changes are at least potentially reversible. There are in the intact animal many cell types that, though normally quiescent, may be induced to resume DNA synthesis by an appropriate stimulus.11

Recent research suggests that mitotic homeostasis may be mediated by feedback mechanisms relating to tissue mass and function, and effected by humoral agents or chalones.12,13 Evidence for such feedback mechanisms have been found in a variety of both normal and malignant tissue,14,15 including normal granulocytic tissue,16,17 and a form of experimental leukemia.18 The conditions in ECIB are such that a part of leukemic tissue is destroyed, whereas another part, containing proliferative cells (that is, the marrow), is not subject to the same injury. We believe that this may operate a feedback mechanism of growth control within the leukemic tissue, resulting in an increased proliferative fraction. Further evidence that nonproliferative leukemic cells may reenter the cell cycle is also provided in a recent detailed investigation of the kinetics of small and large blast cells following in vivo $^3$H-thymidine labeling.19 Studies on the effect of cytosine arabinoside, which specifically inhibits DNA synthesis and thus selectively acts on part of the leukemic cell population, have shown that, following the initial effect of the drug in depressing the labeling index, a rebound may occur, thus indicating an increase in the proliferative fraction.20 These studies support the hypothesis that a feedback mechanism exists. Knowledge of the effect of drugs or ECIB on tissue control mechanisms will clearly be of great importance in guiding the proper choice of therapy.

ACKNOWLEDGMENT

We are grateful to Dr. J. A. Bulimore, Dr. G. Stone, and Dr. T. W. Wheeler for carrying out the irradiation procedures, and to Prof. R. Y. Calne, Dr. H. White, and Dr. R. Sells for performing the surgical insertion of A/V shunts.

REFERENCES

9. Gavosto, F., Pileri, A., Gabutti, V.,


Changes in Proliferative Activity of Marrow Leukemic Cells During and After Extracorporeal Irradiation of Blood

B. W. B. CHAN and F. G. J. HAYHOE