Bone Marrow Cell Count per Cubic Millimeter Bone Marrow: A New Parameter for Quantitating Therapy-Induced Cytoreduction in Acute Leukemia

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A new technique is introduced for determining the number of bone marrow cells per cubic millimeter marrow, providing an accurate and objective means for quantitating therapy-induced cytoreduction. The method requires a correction for admixed peripheral blood in bone marrow aspirates to measure the fraction of remaining pure marrow. While cell kinetic differences between blood, aspirates, and biopsy identify the proportion of contaminating blood cells, the ratio of red cell hematocrits in blood and aspirate gives the volume of trapped blood. By combining both procedures, bone marrow cell counts per unit volume pure marrow result (BMC/cu mm BM), which were found highly reproducible. Blast cell counts (BMBC/cu mm BM) were obtained by additional morphological differentiation. BMC and BMBC/cu mm BM were monitored in 16 patients with acute nonlymphoblastic leukemia treated with daunorubicin, cytosine arabinoside, and 6-thioguanine in combination and in 4 patients with end-stage acute leukemias and non-Hodgkin’s lymphomas during high-dose thymidine therapy. Total and daily therapy-induced cytoreduction rates were significantly greater (p < 0.01) in responders than nonresponders to either regimen. Changes in BMC/cu mm BM were also found representative for changes in BMBC/cu mm BM, since the majority of bone marrow cells were blasts. In acute leukemia, BMC/cu mm BM thus provides accurate and objective measurements of treatment efficacy in vivo and after short periods of drug exposure. Differences in cytoreduction rates within the group of responders also suggest possible prognostic implications.

Materials and Methods

The technique described here provides the means to obtain the number of pure bone marrow cells per unit volume of marrow. This is achieved by correcting bone marrow aspirates for contaminating peripheral blood. The correction is made independently for blood cells and blood volume.

Bone Marrow Cell Count Determination

Cell kinetic differences between corresponding blood, bone marrow aspirate, and biopsy specimens provide the means to identify the proportion of peripheral blood cells mixed with marrow in the aspirate. This approach is based on previous cell kinetic studies of our group, indicating that differences in S-phase index between biopsies and aspirates are exclusively due to the contamination of aspirates with blood cells. The differences in S-phase index are proportional to the number of admixed blood cells.

Using biopsy material as a cell kinetic reference for pure marrow, the proportion of nucleated blood cells in the aspirate is determined according to the formula:

Percent nucleated blood cells = \( \frac{SI_{\text{Bop}} - SI_{\text{Ap}}}{SI_{\text{Bop}} - SI_{\text{Blood}}} \times 100 \)

Where SI is the S-phase index. Subtracting this percentage from the total nucleated cell count in the aspirate reveals the number of pure marrow cells (Fig. 1).
QUANTITATION OF CELL KILL IN ACUTE LEUKEMIA

Cell Kinetic Analysis (Flow Cytometry)

DNA Histogram

BM Biopsy + Blood ➔ BM Aspirate

X x 8_Biop + (100-X) x 8_Blood = 8_Asp x 100

X = % BM Cells/mm³ Aspirate

Processing of samples. Bone marrow aspirates and Jamshidi biopsies were taken from the same posterior iliac crest under local anesthesia. A quantity of 0.5–1 ml of marrow was aspirated into a 10-ml syringe containing 0.1 ml preservative-free heparin (1000 U/ml) for anticoagulation. Biopsies were taken from an immediately adjacent site and placed in 3–5 ml of Hank’s balanced salt solution (HBSS, Gibco, Grand Island, N.Y.). After mechanical dispersion, the cell suspension was drained through a nylon filter to remove residual bone chips. Two milliliters of venous blood were taken simultaneously with the marrow specimens using heparin as above for anticoagulation. Nucleated cell counts in aspirate and blood were determined in a Clay Adams cell counter (Hematology Analyzer 5).

Cell kinetic analysis. Cell kinetic analysis was carried out by flow cytometry (FCM) using the metachromatic fluorochrome acridine orange for simultaneous staining of cellular DNA and RNA. Aliquots of aspiration, biopsy, and blood samples were subjected to Ficoll-Hypaque gradient separation (density 1.078 g/ml, 1000 g for 20 min at room temperature), and interphase cells were collected, washed, and resuspended in HBSS. A quantity of 0.2 ml of cell suspension, containing approximately 0.2–0.4 x 10⁶ cells, was mixed with 0.4 ml of a solution containing 0.08 N HCl, 0.15 N NaCl, and 0.1% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). After 30 sec, 1.2 ml of acridine orange (6 µg/ml) (Polysciences Inc., Warrington, Pa., chromatographically purified) in 10⁻³ M EDTA, 0.15 N NaCl, 0.1 M phosphate-citrate buffer, pH 6.0, was added, resulting in a final dye concentration of 1.3 x 10⁻⁴ M.

For each histogram, 5–10,000 cells were measured in a computer interfaced research cytofluorograph, model FC 200 (Ortho Diagnostic Instruments, Westwood, Mass.) at separate wavelength bands for green (F₂₅-DNA) and red (F₃₆—RNA) fluorescence. Data were stored in computer memory (Nova 1220) and analyzed using computer programs developed by Sharpless in our laboratory. The proportion of cells in S-phase is referred to as FCM S-phase index (FCM-SI).

Bone Marrow Volume Determination

The proportion of blood volume in the aspirate was determined by the ratio of red cell hematocrits in corresponding aspirates and blood, the remaining fraction was pure marrow volume (Fig. 2). Red
Fig. 2. Using the ratio of red cell hematocrits in corresponding blood and aspirate, the proportion of contaminating blood volume and thus the fraction of pure marrow volume is determined.

Clinical Application

In order to measure treatment-induced cytoreduction in the bone marrow, BMC and BMBC/cu mm BM were monitored in patients with acute nonlymphocytic leukemia (ANLL) who were previously untreated or had relapsed. They received chemotherapy with daunorubicin (DNR), cytosine arabinoside (ARA-C), and 6-thioguanine (6-TG) in combination (DAT). Other patients with end-stage acute leukemias and non-Hodgkin’s lymphomas with bone marrow involvement were followed during phase I and II studies of high-dose thymidine (TdR).

High-dose thymidine therapy. In 4 patients TdR was administered by continuous infusion over a period of 18–28 days at a dose of 140–240 g/sq m/day by which mM serum levels were maintained. In BMC and BMBC/cu mm BM were determined before, at least twice during, and at the end of the TdR infusion.

DAT therapy. Eleven previously untreated patients and 5 others who had prior chemotherapy or were in relapse of ANLL received a 5-day course of combination chemotherapy consisting of: DNR 60 mg/sq m/day on days 1, 2, and 3; ARA-C 200 mg/sq m/day from day 1 to 5 by continuous infusion after an initial loading dose of 25 mg iv.; and 6-TG 200 mg/sq m/day from day 1 to 5. In BMC and BMBC/cu mm BM were measured before treatment and on the second and fifth day of therapy.

In 9 patients treated with either DAT or TdR, aspirates were performed in duplicate to test the reproducibility of the quantitation procedure. The two specimens differed in aspirated volume by a factor of 2 or more and were obtained from different sites within the same anesthetized area.

RESULTS

The described quantitation procedure was applied to a total of 65 sets of blood, aspiration, and biopsy specimens. In Fig. 4, the S-phase indices of corresponding biopsies, aspirates, and blood are summarized. Based on these results, a mean blood cell contamination of 39.7% of the total nucleated cell count in aspirates was determined according to the formula given in Fig. 1, indicating an average cell dilution factor of pure marrow cells of 1.67. According to the ratio of red cell hematocrits in aspirates and blood (Figs. 2 and 5), blood volume admixture accounted for 75% of the total aspirated volume, which means that pure marrow was diluted by blood by a mean factor of 4.

Reproducibility of the Quantitation Procedure

In each of 9 patients, BMC/cu mm BM were calculated from two separate simultaneously taken aspirates differing in volume by a factor of 2 or more. The paired samples differed in red cell hematocrits and nucleated cell counts by an average of 87% and 116%, respectively. Yet, the BMC/cu mm BM correlated significantly ($r = 0.91$) in corresponding bone marrow samples (Fig. 6).

Clinical Results

Bone marrow blasts counts per cubic millimeter bone marrow were monitored during chemotherapy of
ANLL with DAT and high-dose thymidine in end-stage acute leukemias and non-Hodgkin's lymphomas. The measured cytoreduction rates were related to the percentage of the residual leukemic blasts in the marrow on morphological evaluation 1 wk after completion of therapy. Less than 5% of residual blasts at this timepoint were defined as response and more than 5% as nonresponse. Additional data are given on the subsequent achievement of complete remission, death in aplasia, or resistance to therapy.

High-dose thymidine therapy. Sequential determinations of BMBC/cu mm BM during continuous TdR infusion revealed a progressive decrease of 0.17, 0.13, and 0.13 log^{10}/day in 3 of 4 patients. The total reduction in BMBC/cu mm BM varied from 2.2 to 3.6 log^{10}, due to different durations of TdR treatment. All three patients achieved bone marrow aplasia with less than 5% residual blasts. While in two patients the leukemic cell population came back, the one patient receiving TdR over the longest period of time (28 days) achieved the largest total blast cell kill (3.6 log^{10}) and subsequently went into complete remission.
Fig. 4. FCM S-phase indices in corresponding biopsies, aspirates, and blood that provide the means to determine the percentage of contaminating blood cells in aspirates according to the formula in Fig. 1.

Fig. 5. Red cell hematocrits in corresponding aspirates and blood that provide the means to determine the proportion of contaminating blood volume in aspirates according to Fig. 2.
In the fourth patient, a total reduction in BMBC/cu mm BM of 0.9 log\(^{10}\) was measured during 18 days of TdR therapy, indicating a daily reduction rate of 0.05 log\(^{10}\). Morphological assessment of bone marrow aspirates and biopsies revealed unchanged blast infiltration and cellularity (Fig. 7, Table 1).

**DAT combination therapy.** For all 16 patients treated with the combination of DNR, ARA-C, and 6-TG, the total reduction of BMBC/cu mm BM during the 5-day treatment period ranged from 1.0 to 3.3 log\(^{10}\) (Table 2). Seven patients, 4 of whom had previously received major therapy or had relapsed, were clinically resistant to DAT and had more than 5% residual blasts on bone marrow examination 1 wk after completion of therapy. Blast cell kill in these cases was significantly lower (\(p < 0.01\)) than in the 9 responding patients who had less than 5% blasts on posttreatment marrow evaluation (Figs. 8 and 9). Seven of the 9 responders subsequently went into complete remission. One patient died due to an infection 10 days after the end of the induction course, and one patient who had a total reduction in BMBC/cu mm BM of 2.7 log\(^{10}\) developed an early relapse 4 wk after DAT therapy. This patient was the only one of the nine responders in whom the leukemic cell population was found to be aneuploid by flow cytometry with a hyperdiploid DNA stemline.

**BMC Versus BMBC/cu mm BM for Monitoring of Treatment Effect**

Since in ANLL the majority of bone marrow cells are leukemic blasts, we anticipated that therapy-induced changes in the total bone marrow cell count (BMBC/cu mm BM) also represent the effect of treatment on the leukemic blast count/cu mm BM. All 20 patients in our study were eligible for this evaluation, and a highly significant correlation was found (\(r = 0.93\)) between changes in total BMC/cu mm BM and BMBC/cu mm BM (Fig. 10).

**DISCUSSION**

In the present study we report on a new technique, recently introduced by us,\(^{17}\) to quantify the number of bone marrow cells per cubic millimeter bone marrow (BMBC/cu mm BM). The method is based on the correction of bone marrow aspirates for contaminating peripheral blood using two separate procedures: blood

| Table 1. Cytoreduction Rate During High-Dose Thymidine Therapy |
|------------------|------------------|------------------|------------------|
| Patients | Diagnosis | Cell Kill in Log\(^{10}\) | Response |
| T.P. | ALL relapse | 2.2 | 0.17 | BM aplasia |
| B.J. | ALL blast crisis | 2.5 | 0.13 | BM aplasia |
| M.V. | ALL relapse | 3.6 | 0.13 | BM aplasia |
| G.P. | ALL relapse | 0.9 | 0.05 | CR |

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Table 2. Cytoreduction Rate During DNR-ARA-C-6-TG Combination Therapy

<table>
<thead>
<tr>
<th>Patients</th>
<th>Diagnosis</th>
<th>Cell Kill in $\log_{10}$ Total per Day</th>
<th>Response (% Blasts After 1st Course)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.M.</td>
<td>APL</td>
<td>1.6 0.33</td>
<td>&lt;5% CR</td>
</tr>
<tr>
<td>A.G.</td>
<td>RAEB−AML</td>
<td>2.0 0.40</td>
<td>&lt;5% CR</td>
</tr>
<tr>
<td>G.M.</td>
<td>AML</td>
<td>2.1 0.40</td>
<td>&lt;5% CR</td>
</tr>
<tr>
<td>C.W.</td>
<td>AML</td>
<td>2.2 0.44</td>
<td>&lt;5% CR</td>
</tr>
<tr>
<td>L.G.</td>
<td>AML</td>
<td>2.3 0.46</td>
<td>&lt;5% CR</td>
</tr>
<tr>
<td>P.O.</td>
<td>APL</td>
<td>2.4 0.48</td>
<td>&lt;5% CR</td>
</tr>
<tr>
<td>C.A.</td>
<td>AML</td>
<td>2.7 0.54</td>
<td>&gt;5% NR</td>
</tr>
<tr>
<td>T.J.</td>
<td>AML</td>
<td>3.2 0.64</td>
<td>&gt;5% NR</td>
</tr>
<tr>
<td>F.A.</td>
<td>AMML</td>
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<td>&gt;5% NR</td>
</tr>
<tr>
<td>B.O.</td>
<td>RAEB−AML</td>
<td>1.4 0.28</td>
<td>&gt;5% NR</td>
</tr>
<tr>
<td>P.R.</td>
<td>RAEB−AML</td>
<td>1.8 0.36</td>
<td>&gt;5% NR</td>
</tr>
<tr>
<td>H.L.</td>
<td>AMOL relapse</td>
<td>3.3 0.65</td>
<td>&lt;5% CR</td>
</tr>
<tr>
<td>B.O.</td>
<td>AML relapse</td>
<td>1.7 0.34</td>
<td>&gt;5% PPR</td>
</tr>
<tr>
<td>T.C.</td>
<td>AML relapse</td>
<td>1.7 0.34</td>
<td>&gt;5% NR</td>
</tr>
<tr>
<td>K.P.</td>
<td>AML relapse</td>
<td>1.0 0.20</td>
<td>&gt;5% NR</td>
</tr>
<tr>
<td>R.N.</td>
<td>AML relapse</td>
<td>1.1 0.20</td>
<td>&gt;5% NR</td>
</tr>
</tbody>
</table>

cell admixture is quantitated by the comparative analysis of the S-phase indices in corresponding blood, aspirate, and biopsy specimens. This approach is based on the finding that the fraction of cells in S-phase is constantly lower in the blood than in the marrow. The admixture of blood cells during the aspiration process therefore results in a relative increase in $G_0/G_1$ and a concurrent decrease in the S-phase index that are proportional to the number of contaminating blood cells.

Previous extensive experimental and clinical studies of our group have shown that bone marrow biopsy provides representative cell kinetic material for pure marrow and that the differences in S-phase index between biopsies and aspirates are directly dependent on the number of admixed blood cells in aspirates. Hence, differences in S-phase index between simultaneously taken blood, aspirate, and biopsy specimens are used to correct the total number of nucleated cells in aspirates for the proportion of contaminating blood cells revealing the number of pure bone marrow cells.

Since this cell count has to be related to the appropriate volume of pure marrow, the ratio of red cell hematocrits in blood and aspirate is utilized to correct aspirates for contaminating blood volume applying a modification of a method recently described by Holdrinet et al. Studies with radioactive erythrocytes have shown that mature red cells circulate exclusively intravascularly and are not present in pure bone marrow tissue. The red cell hematocrit in aspirates, therefore, represents admixed blood volume and is used to calculate the proportion of contaminating blood volume.

By combining the two quantitation procedures the corrected bone marrow cell count per cubic millimeter pure bone marrow (BMC/cu mm BM) is obtained, providing for the first time an objective numeric parameter for bone marrow cellularity.
Excellent reproducibility of data was shown when BMC/cu mm BM were determined from separate, simultaneously taken aspirates. Based on the described technique, it was found that blood cells and blood volume in an aspirate account, on average, for 39.7% of the total nucleated cell count and 75% of the total volume, thus confirming previously reported data.

Sequential determinations of BMC/cu mm BM provide the means for an accurate evaluation of therapy-induced cytoreduction in the bone marrow and thus treatment efficacy. Measurements of BMC/cu mm BM during the DAT induction therapy for ANLL and high-dose thymidine in end-state acute leukemias and non-Hodgkin's lymphomas revealed significant differences in the cytoreduction between responding and nonresponding patients within the first 3–5 days of therapy. All patients on either protocol with a total cell kill of 2 log10 or more achieved bone marrow aplasia and blast clearance in marrow and blood. Further clinical outcome, however, seems to depend not only on the total cytoreduction but also on the rapidity of the cell reduction in the marrow, as expressed by the daily cell kill rate. In two patients on thymidine with a total cytoreduction of 2.2 and 2.5 log10 and daily cell kill rates of 0.17 and 0.13 log10, bone marrow aplasia was followed by the recurrence of leukemia while 7 of 8 patients on DAT with a cell kill greater than 2.0 log10 but at least 2.5 times faster cytoreduction rates went into complete remission. These data suggest that the comparably slow cytoreduction during thymidine treatment may allow early recovery and regrowth of leukemic cells after or even during therapy and may result in the development and selection of resistant cell clones. This relative or secondary resistance is obviously prevented by the more intensive DAT therapy, inducing a rapid cell kill. However, even in the group of responders of DAT, differences in the blast cell reduction rates were found ranging from 0.33 to 0.64 log10/day, indicating individual differences in the sensitivity to the applied treatment and also suggesting differences in the remaining leukemic cell burden after induction therapy. Further follow-up will show if the initial cytoreduction is also of significance for the duration of remission in ANLL as was reported for childhood ALL based on morphological examination of bone marrow smears.
Hence, quantitation of treatment-induced cytodestruction not only permits accurate measurements of treatment efficacy in vivo, thus discriminating response and nonresponse at an early stage of therapy, but it may also provide a quantitative subclassification of remission status with prognostic implications.

Finally, by morphological analysis in addition to the determination of BMC/cu mm BM, the differential counts of the specific marrow cell types are revealed. Thus, for example, the reduction of leukemic cells and normal myeloid precursors can be compared for a given treatment protocol. Neglecting the possibility of differentiation into normal cells, a comparison of cell kill rates in leukemic and normal cell populations will add to our knowledge about the action of cytostatic drugs and might have an impact on the design of chemotherapeutic regimens such as consolidation and maintenance therapy in remission of acute nonlymphocytic leukemia.

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