Flow Cytometry of DNA Content in Human Bone Marrow: A Critical Reappraisal

By Gary M. Dosik, Barthol Barlogie, Wolfgang Göhde, Dennis Johnston, Jan L. Tekell, and Benjamin Drewinko

Because cytokinetic studies of the human bone marrow aspirate as a prognostic factor and as a monitor of drug perturbation are frequently inconsistent, we investigated reproducibility of DNA distribution measured by flow cytometry of DNA content in patients with morphologically normal bone marrow. In 15 patients, correlation was noted between DNA distributions simultaneously obtained on right and left iliac crest bone marrow aspirates (r = .588), although considerable variation in individuals was encountered. Much better reproducibility (r = .879) was achieved using bilateral core biopsy of bone marrow in these same patients. In 60 samples, comparison of DNA distribution between bone marrow aspirate and simultaneously obtained biopsy revealed higher relative proportions of S and G2 + M phase cells in biopsies (p < 0.001), suggesting peripheral blood contamination of aspirate material. Brisk shaking of biopsy specimens in saline expelled a representative sample in the supernatant that could be subjected to simultaneous cytomorphological and cytokinetic analysis. To improve reproducibility of DNA content determinations in normal human bone marrow, bone marrow biopsy should be utilized.

EXAMINATION of human bone marrow ante-mortem was made possible through the introduction of needle aspiration1-2 and core biopsy.4-10 Thus, primary and secondary malignant and nonmalignant disorders of the hematopoietic system could be evaluated. Although the techniques are most frequently utilized qualitatively for histologic diagnosis, there has been a need for more quantitative evaluation to appreciate the efficacy of cytotoxic chemotherapy in reducing leukemic cell mass. Hart et al., for example, have determined that the information on changes in the percentage of morphologically identifiable blast cells is further augmented by a quantitative determination of the cellularity from the bone marrow biopsy or clot section.11

Similarly, other quantitative bone marrow examinations involve cytokinetic parameters such as the tritiated thymidine labeling and mitotic index, resting phase populations, and more recently, automated cytology for cell cycle compartment distribution analysis by DNA flow cytometry.12-20 These parameters are meaningful only if the small amount of material sampled is representative of the entire population under investigation. While this is obviously not the case for certain patchy disorders of the bone marrow, such as multiple myeloma, metastatic carcinoma, and malignant lymphomas, relative morphological and functional homogeneity has been assumed in patients with normal bone marrow, acute leukemia, and other diffuse diseases such as anemias, etc.

Recently, we have sought to document, by serial bone marrow sampling, the effect of in vivo perturbation produced by antineoplastic agents on the cell cycle distribution of normal and leukemic human bone marrow, utilizing DNA flow cytometry. In spite of considerable variation, intrapatient trends such as increase in the S-phase compartment with cytosine arabinoside21 and the G2 compartment with ifosfamide22 were apparent. In other cell kinetics studies addressing the prognostic significance of pretreatment cytokinetic parameters, such as the tritiated thymidine labeling index in patients with acute leukemia, however, variability encountered was so large as to make conclusions of studies in these presumably diffuse bone marrow disorders divergent.23-27

We, therefore, wish to consider the problem of intrinsic variability in the bone marrow aspiration and address the question of whether more precise sampling methods are available. Because DNA flow cytometry provides a very accurate and objective determination of DNA distribution of cellular suspensions, we have utilized this technique to make comparisons. The present study was confined to analysis of normal bone marrows as a first attempt to evaluate the determinants of variability in a setting of presumably homogeneous cellular distribution. Specifically, the reproducibility of needle aspirations were compared to results obtained on bone marrow biopsy determinations. To obtain representative cytokinetic information on bone marrow biopsy material, we utilized an adaptation of the pepsination technique developed for the preparation of solid tumor specimens for flow cytometry.28,29 since pepsinization of solid tissue routinely provides >97% cellular monodispersion of the entire

From the Departments of Developmental Therapeutics, Biostatistics, and Laboratory Medicine, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, Texas.

Supported in part by Grants CA11520, CA14528, and CA5831 from the National Cancer Institute, National Institutes of Health, Bethesda, Md. B. B. is a Junior Faculty Fellow of the American Cancer Society.

Submitted July 20, 1979; accepted January 2, 1980.

Address reprint requests to Gary M. Dosik, M.D., Hematology-Oncology Medical Group of the San Fernando Valley, 16055 Ventura Boulevard, Suite 535, Encino, Calif. 91436.

© 1980 by Grune & Stratton, Inc.

0006-4971/80/5505-0004$01.00/0

734  Blood, Vol. 55, No. 5 (May), 1980
tissue sample. After establishing the relationship of pepsinized biopsy specimens to aspirates, we showed that the pepsinization technique was not necessary for representative sampling of bone marrow biopsies. Obtaining cells from biopsies by brisk agitation in saline, we demonstrated the reproducibility of morphological and cytokinetic measurements on biopsy samples and the superiority of this sampling method for cytometric studies relying on minimal variation secondary to sampling.

MATERIALS AND METHODS

Sequential patients undergoing bone marrow evaluation at the University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute for various nonleukemic malignancies were utilized in the study.

Processing of Bone Marrow Aspirates

After obtaining informed consent, 2 ml of bone marrow was aspirated from the posterior iliac crest with an Illinois needle into a 10-cc syringe. Half of the sample was utilized for routine smears, while the remainder was placed into a tube containing 0.5 ml of preservative-free heparin (1000 U/ml). The sample was immediately subjected to Ficoll-Hypaque separation (density = 1.078/cc, 1000 g for 15 min at 4°C), and interphase cells were collected, washed, and resuspended in 0.9% NaCl, fixed in 70% ethanol, and stored at 4°C.

Processing of Bone Marrow Biopsies

Bone marrow biopsies were performed with a Jamshidi needle utilizing routine preparation and xylocaine anesthesia. The proximal 3 mm of the biopsy was removed with a scalpel and immediately placed in 0.9% NaCl 2 cc with 250 U/ml of preservative-free heparin. After mincing of the bone fragment, the specimen and supernatant were centrifuged at 1000 g for 2 min and the resulting supernatant removed. A single cell suspension was generated by the addition of 2 ml of 0.5% pepsin in HCI (Serva, Heidelberg, Germany). Routinely, more than 30,000 cells were measured and 128-channel histogram generated. Distribution of DNA content was evaluated utilizing a modification of Fried’s model.

Morphological Analysis

Routine bone marrow aspirate and cytocentrifuge preparations from saline biopsy expellates were stained with Wright’s Giemsa stain. Differential counts were performed on 500 cells of the bone marrow aspirates by a medical technologist in the clinical bone marrow laboratory. Differential counts on the cytocentrifuge preparations from saline biopsy expellates, also on 500 cells, were performed separately, by a different technologist, who had no knowledge of the bone marrow aspirate results.

Statistical Method

For comparison of techniques involving two analyses per patient, the matched-pair Student’s t test was utilized. Correlations were made utilizing the linear regression analysis method.

RESULTS

During the months of March and April 1979, bone marrow examinations were obtained from 49 sequential patients. In six cases, the morphological diagnosis of bone marrow infiltration with malignancy was made. Three of these were solid tumor malignancies and three cases had leukemia. Of the remaining 43 patients, 25 had unilateral bone marrow aspiration and biopsy, while 18 patients underwent bilateral simultaneous aspirations and biopsies. In one of these cases the right iliac crest biopsy specimen was inadequate. In 15 to 25 patients with unilateral bone marrow studies, biopsy samples were split so that the pepsinized whole biopsy could be compared to the saline expellate procedure.

Comparison of All Aspirates Versus All Pepsinized Biopsies

In 60 cases, comparison of DNA distributions obtained on bone marrow aspirate with that of pepsinized bone marrow biopsy was possible (Table 1). In bone marrow biopsies, a significantly higher proportion of cells in the S-phase compartment at the expense of G1 (p < 0.001) was found. In addition, significant correlation between the two G1 and S-phase populations, measured by both techniques, was noted. Figure 1 illustrates the correlation between S-phase fractions measured by the two techniques, showing considerable scatter present about the regression line. No correlation was seen between the G2 + M phases of both techniques.
Comparison of Bilateral Pepsinized Bone Marrow Biopsies

In 17 of the above 18 patients, bilateral pepsinized bone marrow biopsies were also available. Again, there was no significant difference in the DNA distributions or variation coefficient in the bilateral studies, with highly significant correlation of the G110 and S-phase compartments. As shown in Fig. 3 and compared to Fig. 2, the degree of correlation for bone marrow

Comparison of Bilateral Bone Marrow Aspirates

As demonstrated in Table 1, there was a significant correlation between the G1,0, S, and G2 + M compartments, as well as the coefficient of variation between the right and left bone marrow aspirates in 18 patients. However, considerable variability about the regression line was again noted (Fig. 2).

Table 1. Comparability of Bone Marrow Aspirate and Biopsy DNA Distributions

<table>
<thead>
<tr>
<th>No. of Cases</th>
<th>Percent G10 (SEM)</th>
<th>Percent S (SEM)</th>
<th>Percent G2 + M (SEM)</th>
<th>Percent CV (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All biopsies</td>
<td>60</td>
<td>76.0 (0.9)</td>
<td>18.2 (0.8)</td>
<td>6.1 (0.3)</td>
</tr>
<tr>
<td>All aspirates</td>
<td>60</td>
<td>80.0 (1.0)</td>
<td>14.3 (0.9)</td>
<td>5.5 (0.5)</td>
</tr>
<tr>
<td>p value*</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>r†</td>
<td>0.410</td>
<td>0.514</td>
<td>-0.125</td>
<td>-0.16</td>
</tr>
<tr>
<td>p†</td>
<td>&lt;0.005</td>
<td>&lt;0.001</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Left aspirate</td>
<td>18</td>
<td>83.6 (1.8)</td>
<td>11.3 (1.5)</td>
<td>5.0 (0.8)</td>
</tr>
<tr>
<td>Right aspirate</td>
<td>18</td>
<td>81.3 (2.0)</td>
<td>13.9 (1.9)</td>
<td>4.6 (0.6)</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>0.2</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>r</td>
<td>0.611</td>
<td>0.588</td>
<td>0.75</td>
<td>0.7</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Left biopsy</td>
<td>17</td>
<td>76.1 (2.4)</td>
<td>19.1 (0.9)</td>
<td>5.1 (0.6)</td>
</tr>
<tr>
<td>Right biopsy</td>
<td>17</td>
<td>74.1 (2.3)</td>
<td>20.2 (0.8)</td>
<td>5.5 (0.4)</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>r</td>
<td>0.871</td>
<td>0.879</td>
<td>0.316</td>
<td>0.528</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.04</td>
<td>&lt;0.001</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*p Value refers to matched sample Student's t test.
†r and p Refer to linear correlation coefficient and corresponding p value, respectively.

Fig. 1. Comparison of DNA flow cytometry determined S-phase compartments of simultaneously obtained normal bone marrow aspirates and biopsies.

Fig. 2. Comparison of DNA flow cytometry determined S-phase compartments of simultaneously obtained normal bilateral bone marrow aspirates.
distribution histograms of cells expelled into saline from vigorous shaking were compared with pepsinized biopsy material (Table 2). There was no significant difference between DNA distribution and coefficients of variation measured by the two techniques. In fact, a high degree of correlation was seen in the quantitative DNA distribution, as evident from the comparison of S-phase values measured by the two techniques \( r = 0.923 \) (Fig. 4). In eight additional patients the bone marrow biopsy specimen was not large enough to allow sample splitting. The biopsy in these patients was subjected to the expellate procedure only. Thus, expellate samples were available in a total of 15 patients. In these specimens, the residual bone chip was pepsinized after removal of the cell containing saline expellate suspension, in order to determine whether cells in a specific DNA compartment were preferentially left behind after vigorous shaking. As shown in Table 2, a high degree of correlation between the \( G_{10} \) and S-phase compartments of the expellate and pepsinized biopsy residual was noted, with no significant difference in these values measured by the two techniques.

The DNA distribution of bone marrow aspirates versus saline expellates from the biopsies in these 15 patients show again higher proportions of cells with S and \( G_2 + M \) DNA contents for expellates when compared to aspirates. As in the case of pepsinized biopsies versus aspirates, some correlation was seen \( r = 0.392 \) for \( G_1 \), .266 for S, and .311 for \( G_2 + M \).

**Morphological Studies**

In 15 patients, cytologic examinations of 500 cells for bone marrow aspirate smears were compared with

---

**Table 2. Comparability of Bone Marrow Biopsy Saline Expellate With Pepsinized Residual and Pepsinized Biopsy**

<table>
<thead>
<tr>
<th>No. of Cases (Patients)</th>
<th>Percent ( G_{10} ) (SEM)</th>
<th>Percent S (SEM)</th>
<th>Percent ( G_2 + M ) (SEM)</th>
<th>Percent CV (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy saline expellate</td>
<td>7</td>
<td>76.0 (2.7)</td>
<td>16.8 (2.0)</td>
<td>7.1 (1.4)</td>
</tr>
<tr>
<td>Pepsinized biopsy</td>
<td>7</td>
<td>74.4 (2.9)</td>
<td>17.1 (2.1)</td>
<td>8.4 (1.5)</td>
</tr>
<tr>
<td>( p ) value*</td>
<td></td>
<td>0.2</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>( r )†</td>
<td></td>
<td>.934</td>
<td>.923</td>
<td>.655</td>
</tr>
<tr>
<td>( \rho )†</td>
<td></td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Biopsy saline expellate</td>
<td>15</td>
<td>76.1 (2.3)</td>
<td>19.5 (2.3)</td>
<td>4.3 (0.3)</td>
</tr>
<tr>
<td>Pepsinized residual‡</td>
<td>15</td>
<td>77.8 (2.4)</td>
<td>17.9 (2.3)</td>
<td>4.1 (0.2)</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>( r )</td>
<td></td>
<td>.931</td>
<td>.961</td>
<td>.062</td>
</tr>
<tr>
<td>( \rho )</td>
<td></td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.08</td>
</tr>
<tr>
<td>Aspirate</td>
<td>15</td>
<td>83.2 (1.3)</td>
<td>13.4 (1.2)</td>
<td>3.2 (0.3)</td>
</tr>
<tr>
<td>Biopsy saline expellate</td>
<td>15</td>
<td>75.7 (1.3)</td>
<td>19.1 (1.2)</td>
<td>5.0 (0.4)</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>( r )</td>
<td></td>
<td>.392</td>
<td>.266</td>
<td>.311</td>
</tr>
<tr>
<td>( \rho )</td>
<td></td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*\( p \) Value refers to matched sample Student’s t test.
†\( r \) and \( \rho \) Refer to linear correlation coefficient and corresponding \( p \) value, respectively.
‡Pepsinized residual refers to any cells left in biopsy sample after saline expellate is removed.
saline biopsy expellate cytocentrifuge preparations (Table 3). Excellent correlation between the percentage of myeloblasts, myeloblasts plus promyelocytes, all myeloid precursors (including myeloblasts, promyelocytes, myelocytes, metamyelocytes), erythroid precursor cells, and megakaryocytes was seen using the two techniques. A trend suggesting higher percentages of proliferating cells was noted in bone marrow biopsy specimens. A sample cytocentrifuge preparation obtained from a saline biopsy expellate showing myeloid and erythroid precursors as well as an early megakaryocyte is demonstrated in Fig. 5. Cell morphology of all three lineages is well preserved so that cytologic differentiation can be performed with ease.

**DISCUSSION**

The present study was undertaken in order to determine the reproducibility of DNA distribution measurements on bone marrow aspirates, which in turn influence the value of cytokinetic evaluation for prognosis and drug effects on cycle progression in leukemia. The result of our analysis of bilateral bone marrow aspirates shows this procedure, in general, provides reproducible cytokinetic data. However, considerable variation is encountered in certain individuals. Pepsinization of bone marrow biopsies yielded considerably higher reproducibility when bilateral analyses were studied. In addition, bone marrow biopsy material harbored a significantly higher proportion of cells with S-phase DNA content and a trend toward greater numbers of morphologically identifiable proliferative cells. This suggests contamination of bone marrow aspirates with nonproliferating peripheral blood mononuclear cells, which are almost invariably in resting stage.\(^{13,36}\) Such contamination of varying degree has been shown with the use of \(^{51}\text{Cr}\-
labeled red blood cells. Our results have recently been confirmed by Hiddemann et al. who have similarly demonstrated higher S-phase fractions in bone marrow biopsies as compared to simultaneously obtained aspirates in patients with acute leukemia.

The trend for higher DNA flow cytometry determined S-phase compartments in biopsies when compared to aspirates was more consistent and significant than that suggested by morphological analysis of myeloid and erythroid precursors in the same specimen. This observation may be explained by several circumstances. First, morphological evaluation of 500 cells is considerably less precise than automated cytology on 30,000 cells. In addition, variation inherent in microscopic assessment more readily obscures fine discrimination than completely objective DNA distribution analysis. Thus, the appearance of significantly larger S-phase proportions in biopsy washings provided by a highly accurate technique reveals true differences better than a method fraught with observer subjectivity.

The high degree of correlation between the DNA distribution of cells expelled from biopsy material by vigorous shaking with pepsinized biopsies suggest that such preparations can be used interchangeably. This is important because it allows the use of biopsy material for simultaneous cytomorphological and cytokinetic studies.

Evidence from analysis of the residual bone chips after the expellate procedure suggests that the technique samples a representative population from the biopsy specimen. Thus, for studies requiring simultaneous morphology and DNA distribution analysis, the saline expellate method is indicated; while more rapid direct biopsy pepsinization may be utilized whenever cytokinetic study alone is required. For any quantitative analysis relying on serial bone marrow examinations, it is crucial to utilize the most reproducible procedure possible. With exceedingly increasing accuracy of flow cytometric methods, contamination of bone marrow aspirations by resting phase cells with a predominance of cells having G$_{1}$/G$_{0}$ DNA content from peripheral blood becomes the major error-factor in cytokinetic studies of human bone marrow. Bone marrow biopsies show a high degree of reproducibility. Since they can be utilized for simultaneous morphology and cytokinetic determinations, they should be used routinely on normal bone marrow when analysis of drug perturbation by flow cytometry of DNA content is sought. Studies of bone marrow biopsy in acute leukemia and other diffuse disorders of the bone marrow are underway to determine whether bone marrow biopsies can be utilized interchangeably for aspirates in this disorder. Similarly, in circumstances of "dry tap" the use of saline biopsy expellates for cytomorphology and cell kinetics should be superior. Finally, since one parameter DNA content analysis does not routinely distinguish between different cell types, procedures supplying the most representative cell populations should be preferred to minimize variability in cytokinetic analyses of human bone marrow.

REFERENCES

19. Dosik GM, Barlogie B, Smith TL, Gehan EA, Keating MJ,


Flow cytometry of DNA content in human bone marrow: a critical reappraisal

GM Dosik, B Barlogie, W Gohde, D Johnston, JL Tekell and B Drewinko