Nucleic Acid Analysis of Human Bone Marrow Particles in Malignancy*

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In the nucleic acid analyses of human bone marrow aspirate reported by others (2, 3, 6, 11), one is impressed by the wide range of values and high standard error for each of the clinical entities investigated. Because of this limitation, few clear-cut biochemical differences have been apparent in the various malignant and hematologic disorders which have been studied. The present work was undertaken with the thought that a more satisfactory preparation of the sample taken for analysis might yield more uniform results and possibly disclose biochemical differences which remained masked with other technics. Bone marrow particles are those bits of tissue found in a specimen aspirated from the bone marrow cavity. If a reasonably complete recovery of the particles from the aspirated sample could be achieved and the peripheral blood removed from them, the samples subjected to analysis should be more uniform. This report describes an approach involving the use of a special filtering device which was designed to realize these goals. The technic developed was applied to 50 bone marrow specimens from 43 clinical cases, and the results are presented below. A preliminary report of this investigation has already appeared (5).

MATERIALS AND METHODS

To separate the bone marrow particles from the peripheral blood, it seemed advisable to employ a filtration technic. Schleicher (9) applied this principle to obtain bone marrow tissue for histological study. His device made use of a rubber valve type of arrangement, and the filtration rate was controlled by the rate of flow of fluid in the column under the rubber valve. He felt that most of the particles were retained by the valve. If the smallest particles are 200 μ (8), it should be possible to trap all particles with a sufficiently small filtering space. To accomplish this and to achieve a simpler procedure, more consistent performance, and an apparatus that would not be attacked by chemical reagents, we designed an all-glass apparatus with three strips of platinum foil (60 mm. × 2 mm. × .025 mm.) as spacers to obtain an annular space of 25 μ. This device and the micro-immersion heater discussed below1 are illustrated in Chart 1. A space of 25 μ was regarded as most suitable to assure maximum retention of the bone marrow particles.

The procedure employed for the isolation of nucleic acids from washed bone marrow particles is essentially a combination of the Schneider technic for removal of non-nucleic acid components (10), and the Ogur-Rosen procedure for the extraction of nucleic acids (7). The fractionation was carried out in the following way. The apparatus was pre-cooled to 4° C., and 2 ml. of ice-cold normal saline was transferred to the glass funnel with the stopcock closed. The ice-cold specimen of bone marrow aspirate, collected in a paraffined tube, was gently swirled, and a 0.2- or 0.4-ml. sample was quickly transferred to the saline in the funnel while the latter was being gently rotated. A small negative pressure, controlled by the stopcock, was applied just before introducing the bone marrow specimen so that the saline started to pass through the annular space just before the introduction of the sample. The retained particles were quickly washed twice more, with swirling of the funnel, with 2-ml. portions of ice-cold saline. This was sufficient to remove visible signs of peripheral blood. The peripheral blood and washings were collected, and cell counts were done. Cell counts were also performed on a separate aliquot of the original sample after 10 minutes of mechanical agitation. The number of cells retained by the filter was determined by difference. In some instances the nucleic acid content per cell obtained in this way agreed closely with literature values, but in other instances the values were markedly different. Despite the application of various technics in an attempt to break down the bone marrow particles into free-floating single cells so that accurate ini-

1 This apparatus may be obtained from Western Scientific Supply Co., Berkeley, California.
tial cell counts could be obtained, no procedure was found which yielded consistent results. This problem is still under investigation. As an alternative it may be of value in future studies to relate the nucleic acid content of the particles to the residual nitrogen remaining on the filter after the extraction of nucleic acids.

To extract the acid-soluble phosphorus fraction, the funnel was again precooled to 4°C, and 2 ml. of ice-cold 8 per cent trichloroacetic acid (TCA) was added. The funnel was left at 4°C. for 1 hour with occasional agitation, after which interval the TCA was drawn off. The residue was washed twice with 0.5-ml. portions of cold 8 per cent TCA. Although the non-nucleic acid fractions were discarded in this investigation, it would have been a simple matter to collect these fractions for phosphorus analysis.

Lipid phosphorus was extracted by treating the residue successively with 2-ml. portions of 80 per cent ethanol, 95 per cent ethanol, 3:1 methanol-chloroform twice, and finally with ether. Two-minute periods with agitation, at room temperature, were allowed for each extraction. These conditions were considered adequate in view of the large surface exposed.

The funnel containing the lipid-free residue was again precooled to 4°C., the stopcock was closed, and 1 ml. of ice-cold 1 N perchloric acid (PCA) was added to the residue for the extraction of ribonucleic acid (RNA). The funnel and contents were left at 4°C. overnight, 17-19 hours, with a fitted rubber stopper covering the funnel. The perchloric acid extract was drawn off with gentle negative pressure into a tube under the funnel stem. The residue was then washed with two 0.5-ml. portions of ice-cold 1 N PCA. The combined extract and washings were made to 3 ml. with 1 N PCA, and the spectral absorption curve was obtained with a model DU Beckman spectrophotometer. Appropriate dilutions were made with 1 N PCA when necessary.

To the remaining residue in the funnel were added 1-2 ml. of 1 N PCA. The micro-immersion heater (Chart 1) was passed through a flat one-hole rubber stopper so that the stopper would cover the funnel when the heater was in place. The heater was designed to pass over the stem of the glass plug with its base resting at the bottom of the funnel cone. Control tests showed that a very small current regulated with a variable transformer maintained the temperature at 80 ± 2°C. The micro-immersion heater was put in place over the glass plug, and the PCA solution was kept at 80°C. for 80 minutes. The hot PCA extract, containing deoxyribonucleic acid (DNA), was then drawn into a tube, as above. The residue was washed with two 0.5-ml. portions of 1 N PCA. The combined extract and washings were made to 3 ml., diluted when necessary, and the spectral absorption curve was obtained, as above.

For the purposes of this report the relative ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) values are considered, in addition to qualitative information derived from the nature of the nucleic acid spectral
absorption curve. Taking the figures of Ogur and Rosen (7) for the atomic phosphorus extinction coefficient (1), the RNAP:DNAP ratio was calculated as follows: \( E_{260} - E_{260}/1.2 = \) relative RNAP value; \( E_{260} - E_{260} = \) relative DNAP value. Any protein associated with the nucleic acids would not be expected to have an appreciable effect on the extinction at these wave lengths. Appropriate factors were introduced when the dilution of the RNA and DNA fractions differed. In virtually every instance the maximum of the RNA curve fell at 260 m\( \mu \), and the maximum of the DNA curve fell at 268 m\( \mu \); the RNA minima fell at 293 m\( \mu \) with a few exceptions, and the DNA minima were more variable between 235 and 245 m\( \mu \).

Of the 50 fractionations performed, 37 were

**RESULTS AND DISCUSSION**

Fadem and Berlin (4) have shown by means of radioactive tracer technic that bone marrow aspirate may be 50-90 per cent contaminated with peripheral blood. In those analyses performed on the whole aspirate (6) one might expect quite variable results. When the analysis is performed on the marrow particles adhering to the walls of the tube after the blood is drained away, three sources of error should be considered: (a) There is a question as to the percentage of total marrow particles recovered in this manner; (b) the adherent particles may represent a selected sample; and (c) the contaminating peripheral blood may be appreciable in relation to the amount of marrow tissue which is present.

**TABLE 1**

**ULTRAVIOLET ABSORPTION DATA ON NUCLEIC ACIDS FROM HUMAN BONE MARROW PARTICLES**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No.</th>
<th>RNAP</th>
<th>DNA</th>
<th>E(<em>{260}) : E(</em>{260}) Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>1</td>
<td>.34</td>
<td>22.8</td>
<td>18.2</td>
</tr>
<tr>
<td>Assorted malignancies</td>
<td>4</td>
<td>.37±.01</td>
<td>2.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Acute lymphocytic leukemia</td>
<td>14*</td>
<td>.39±.03</td>
<td>5.3±.87</td>
<td>2.8±.36</td>
</tr>
<tr>
<td>Acute mononuclear leukemia</td>
<td>1</td>
<td>.45</td>
<td>2.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td>2</td>
<td>.49</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Myeloma</td>
<td>4</td>
<td>.53±.06</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Reticulum-cell sarcoma</td>
<td>3</td>
<td>.66</td>
<td>4.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Chronic granulocytic leukemia</td>
<td>1</td>
<td>.66</td>
<td>5.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Anemias</td>
<td>6</td>
<td>.66±.08</td>
<td>4.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Normals (hospitalized cases)</td>
<td>10</td>
<td>.79±.10</td>
<td>2.9±.37</td>
<td>2.1±.25</td>
</tr>
<tr>
<td>Polycythemia</td>
<td>2</td>
<td>.80</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Hyperplastic marrow</td>
<td>1</td>
<td>.88</td>
<td>1</td>
<td>6.6</td>
</tr>
<tr>
<td>Mononucleosis</td>
<td>1</td>
<td>1.08</td>
<td>3.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* This series includes four specimens on one patient, and two on each of two other patients.

† Minima were very low in these cases, giving very high ratios.

‡ Three specimens on one patient.

Data obtained in the present study are summarized in Table 1. Although only the acute lymphatic leukemia series and the hospitalized patient series are sufficiently large to provide a representative mean value, it is of interest to note that the RNAP:DNAP ratios in all the malignant conditions (except polycythemia) are at the low end of the scale. The highest value in a malignant condition, 0.66 for one case of chronic granulocytic leukemia, coincided with the lowest value for the nonmalignant cases (the anemias). It should also be pointed out that in the four cases of assorted malignancies (two lung cancer, one rectal cancer, and one adenocarcinoma) the RNAP:DNAP ratios were also low. The very low standard error observed for these four cases may be fortuitous. The cases of polycythemia and nonspecific hyperplastic marrow fall in the normal range, and the ratio for the mononucleosis case would appear to
be above normal. The ratio obtained for the hospitalized cases, which we regard as normal, agrees with the normal value obtained by Davidson et al. (3), who analyzed bone marrow particles after the peripheral blood was drained away. Their leukemic specimens from untreated patients showed no significant difference from their normal specimens, 0.90 vs. 0.81, respectively. Our leukemic specimens from treated patients yielded ratios which were appreciably lower than those obtained with normal specimens, 0.39 vs. 0.79, respectively (P = 0.0005). Our standard error for normal bone marrow was one-third as large as theirs, and for leukemic bone marrow one-tenth as large. Since our malignant cases had all been under treatment with chemotherapeutic agents or x-radiation, this might well account for the observed difference. On the other hand, contamination of bone marrow particles with peripheral blood, which occurs when the blood is removed from the particles by drainage, may explain the higher RNAP:DNAP ratio obtained by Davidson et al. in specimens from untreated leukemic patients. Further work is in progress to investigate this point.

Calculations were made for the maximum:minimum ratios of both the RNA and DNA spectral absorption curves to compare possible qualitative differences between the nucleic acid fractions isolated from different cell populations. These ratios are recorded in Table 1. The difference between acute lymphocytic leukemia cases and hospitalized cases in these extinction ratios for RNA and DNA are suggestive of a significant difference. More cases would have to be studied to establish this point. The ratios for chronic lymphocytic leukemia are strikingly high. A specimen of leukocytes isolated from peripheral blood from another case of chronic lymphocytic leukemia yielded ratios of the same order of magnitude (15.6 and 15.8) and a similar RNAP:DNAP ratio (0.39). One possible explanation for these differences in maximum:minimum extinction ratios may be that varying amounts of protein are associated with the extracted nucleic acids. If there is more protein bound to nucleic acid, one would expect more end absorption and consequently a higher extinction minimum. The maximum:minimum ratio would then be lower. Conversely, a high ratio would imply a lower level of associated protein. These differences might be due to inherent dissimilarities in the nucleoprotein from different cells or to dissimilar enzymatic activities within the cells. In any event, such observations may provide additional information concerning the nature of the nucleoprotein or cellular environment of different cell types.

SUMMARY

A filtering device was designed to separate bone marrow particles from bone marrow aspirate. In 43 clinical cases the nucleic acids in the particles were fractionated according to the procedure of Ogor and Rosen, and the RNAP:DNAP ratios were obtained by means of ultraviolet spectral absorption measurements. The ratio for normal bone marrow (hospitalized cases) was 0.79 and for treated cases of acute lymphatic leukemia, 0.89. The standard errors for these mean values were appreciably lower than those hitherto reported. Ratios are also given for other malignant and hematologic disorders, and these were found to be at the lower end of the scale in all of the malignant conditions. Comparison of maximum:minimum extinction ratios for bone marrow RNA and DNA of the cases under study revealed suggestive differences which may be characteristic of different cell types.

REFERENCES

3. ———. Quantitative Studies on the Content of Nucleic Acids in Normal and Leukemic Cells, from Blood and Bone Marrow. Ibid., 63:471-83, 1941.
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