Quantitation of Femoral Bone Marrow Cellularity of Rats with Acute Chloroleukemia

By Evelyn E. Varsa, Eugene S. Handler and Albert S. Gordon

While indirect attempts to quantitate granulocyte reserves in leukemic human subjects1-3 and laboratory rats4 have been reported, direct approaches toward quantitation have been limited by the availability of experimental animals and the facility of obtaining homogeneous samples. Shay et al.5 developed a myelogenous leukemia in Wistar rats following gastric instillation of 20-methylcholanthrene and Harris et al.6 described the pathogenesis of the leukemia after intraperitoneal transfer of cells in pups less than 7 days old. Rosin and Zajicek7 were successful in serially transferring the leukemia into young adult rats (80-160 Gm.) via intravenous injections of leukemic cell suspensions. The pathogenesis of the disease differs depending on the route of administration. Thus, a chronic myelogenous leukemia is evident after intraperitoneal injections, while an acute leukemia appears following intravenous inoculation. Moloney et al.8 have related the morphology and histochemistry of the chloroma cell with the characteristics of cells found in human myelogenous leukemias. A constant aberrant chromosome number of 43 in serially transplanted chloroma cells has been demonstrated.9 Similarities to certain human leukemias and a stability displayed throughout the course of numerous transplant generations make the Shay chloroleukemic rat an ideal experimental animal. The present investigation was designed to quantitate femoral bone marrow cellularity during the progress of this acute leukemia in rats and to develop a uniform leukemic animal for future experimentation.

Materials and Methods

Counting Procedures and Technics

All rats used were of a modified Long-Evans strain maintained on a diet of Purina Laboratory Chow and tap water ad libitum. Counts of total nucleated cells in femoral marrow, including both normal and leukemic elements, were made by standard hemato-
logic technics using the Spencer Brite-Line hemocytometer and 1 per cent acetic acid as diluting fluid. Differential femoral bone marrow cell counts were determined on smeared preparations treated with May-Grunwald stain; percentages were based on 1,000 cells. The technic used for marrow quantitation was essentially that of Fruhman and Gordon. This method determines the total nucleated cellularity of the shaft femoral marrow, estimated to comprise approximately 55 per cent of the total femoral marrow.

Transfer of the Shay Chloroleukemia

The Shay chloroleukemia has been serially transferred in our laboratory by means of intraperitoneal injections of chloroma mass cell suspensions. Donor rats were anesthetized with ether and the tumors removed aseptically. The cell suspension was made in sterile pyrogen-free saline using a loose fitting tissue grinder.* The diluted suspension contained approximately $100 \times 10^6$ chloroma cells per ml. of saline. Recipient rats weighing between 80–120 Gm. were injected intraperitoneally with $20 \times 10^6$ cells prepared as indicated above. Tumors were harvested every three weeks for the next transplant generation.

Pathogenesis in Femoral Marrows

1. Progression in mature rats. Fifty male rats ranging in weight from 200–250 Gm. were injected intravenously (right jugular) with $10 \times 10^6$ chloroma cells. Animals were sacrificed in groups of 5 on days 1, 3, 5, 7, 9, 11, 13 and 17–20 following implantation. Both femurs were removed and femoral marrow hemic cell quantitation, smears, and differential analyses made.

2. Progression in immature rats. Ten male rats weighing between 80–120 Gm. were injected intravenously with $5 \times 10^6$ chloroma cells. Seven survivors were autopsied between day 12 and day 14 after tumor implantation. Right and left femurs were removed and femoral bone marrow cell quantitation and differential analyses were conducted. Eight normal male rats weighing between 80–120 Gm. were also sacrificed. The right femurs were amputated and femoral bone marrow quantitation, smears and differential analyses carried out as indicated above.

Results

Pathogenesis in Femoral Marrow

A. Mature rats. Mature male Long-Evans rats proved to be receptive to the intravenous administration of $10 \times 10^6$ chloroma mass cells. Approximately 60 to 80 per cent of the inoculated animals developed signs of acute myeloblastic leukemia 10 to 14 days following the administration of such leukemic cell suspensions. Studies of total and differential cellularity of the right and left femoral marrows of 45 rats are summarized in table 1. A decrease was evident in the numbers of total nucleated cells per mg. of marrow despite an increase in the chloroma cell content of both right and left femoral bone marrows during progression of the disease. No significant differences were noted between right and left femurs throughout the period studied. The data reveal a gradual decrease in total marrow cellularity with little or no change in the chloroma cell content up to day 9 postimplantation. A precipitous decline in mean total marrow cellularity (from $2.82 \times 10^6$ to $1.46 \times 10^6$ cells per mg.

*Arthur H. Thomas, Philadelphia, Pennsylvania, Catalogue #4288B. The pestle is ground down with emory cloth and a handle of stainless steel attached to it in order to facilitate hand homogenization.
of right femoral bone marrow) was noted on day 11 of the leukemic process. A further decrease in mean marrow cellularity of the right femurs (1.13 x 10^6 cells per mg. bone marrow) was observed on day 20. At this time the mean chloroma cell content was 0.957 x 10^6 (84.7 per cent) cells per mg. of right femoral bone marrow. Comparable data for the left femoral marrow are given in table 1 and fig. 1. A sharp decrease in total bone marrow hemic cell numbers is clearly indicated. At the time of complete infiltration of the marrow compartment by leukemic cells, the total marrow cellularity was reduced to a fraction of its control value (fig. 1). During the preparation of marrow samples for analyses, it appeared that those obtained from leukemic animals had a more fatty consistency than those from normal rats. Marrow color changed from a normal red to a pale pink (9 days) to a white-green in the terminal stages preceding death.

B. Immature rats. Mean total cell numbers in femoral bone marrow of 7 male rats (80–120 Gm.) sacrificed 12–14 days after intravenous chloroma cell inoculation are given in table 2. There was no appreciable difference between right and left mean total marrow cellularity (1.11 x 10^6 cells per mg. of right femoral marrow and 1.07 x 10^6 cells per mg. of left femoral marrow). The mean chloroma cell content for right and left femoral marrows was 51.3 and 38.4 per cent respectively. Mean control values for 8 normal male rats (80–120 Gm.) are summarized in table 3. These animals exhibited a mean right total femoral marrow cellularity of 2.11 x 10^6 cells per mg. The decrease of total marrow hemic cell numbers during the pathogenesis of the leukemia

<table>
<thead>
<tr>
<th>Days Postimplantation</th>
<th># Animals</th>
<th>Right Femur Mean TNC/ mg. x 10^4</th>
<th>Left Femur Mean TNC/ mg. x 10^4</th>
<th>Right Femur Mean Chloro./ mg. x 10^4</th>
<th>Mean % Chloro.</th>
<th>Left Femur Mean Chloro./ mg. x 10^4</th>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2.82 (2.00–3.10)</td>
<td>2.75 (2.26–3.23)</td>
<td>.015 (0.1–1.1)</td>
<td>.014 (0.0–1.4)</td>
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<td>3</td>
<td>5</td>
<td>2.45 (1.83–2.99)</td>
<td>2.65 (1.46–3.11)</td>
<td>.013 (0.0–1.1)</td>
<td>.006 (0.0–0.4)</td>
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<td>2.30 (0.79–3.05)</td>
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<td>7</td>
<td>5</td>
<td>2.39 (1.49–3.34)</td>
<td>2.81 (2.32–3.35)</td>
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<td>9</td>
<td>5</td>
<td>2.13 (1.86–2.52)</td>
<td>2.77 (2.29–3.17)</td>
<td>.012 (0.2–1.1)</td>
<td>.019 (0.0–0.8)</td>
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<td>11</td>
<td>5</td>
<td>1.46 (0.98–1.94)</td>
<td>1.81 (0.98–2.65)</td>
<td>.206 (0.6–65.5)</td>
<td>.266 (0.6–67.1)</td>
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<td>1.98 (1.77–2.51)</td>
<td>2.02 (1.58–2.17)</td>
<td>.591 (1.3–83.5)</td>
<td>.584 (1.4–84.7)</td>
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<td>17–20</td>
<td>10</td>
<td>1.13 (0.42–2.10)</td>
<td>1.02 (0.26–2.20)</td>
<td>.957 (0.79–7.1)</td>
<td>.693 (20.1–94.1)</td>
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* TNC—Total nucleated cells (normal and leukemic elements).
† Chloro—Chloroleukemic myeloblasts.

Range.

Table 1.—Mean Total Femoral Marrow Cellularity of Rats (200–250 Gm.) Sacrificed Serially after the Intravenous Administration of 10 x 10^6 Chloroma Cells

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<th>Days Postimplantation</th>
<th># Animals</th>
<th>Right Femur Mean TNC/ mg. x 10^4</th>
<th>Left Femur Mean TNC/ mg. x 10^4</th>
<th>Right Femur Mean Chloro./ mg. x 10^4</th>
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was evident, therefore, in the young as well as the mature rat (fig. 2). A fatty consistency and changes in marrow color were also observed. Some animals in the terminal stages exhibited femoral bone marrows almost completely devoid of both normal and leukemic hemic elements. The only cells detectable were small mononuclear and fibroblast-like elements.

**DISCUSSION**

The present study indicates that the femoral bone marrow becomes hypocellular during the development of the acute Shay Chloroleukemia. This condition is most likely a reflection of the alterations in the marrow in other regions of the body as well. Other investigations on the pathogenesis of the Shay Chloroleukemia have reported leukemic infiltration of the marrow. In previous studies, however, no attempt was made at marrow quantitation. It is worth noting that reference to marrow “hypercellularity” in human leukemias may be related to increased leukemic cell numbers within a particular cell line and not necessarily to an augmented total marrow cellular-

**Table 2.—Mean Total Femoral Marrow Cellularity of Rats (80–120 Gm.) Sacrificed 12–14 Days after the Intravenous Administration of 5 x 10<sup>6</sup> Chloroma Cells**

<table>
<thead>
<tr>
<th># Animals</th>
<th>Right Femur Mean TNC* / mg. x 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Left Femur Mean TNC* / mg. x 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Right Femur Mean Chloro./ mg. x 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Mean % Chloro.</th>
<th>Left Femur Mean Chloro./ mg. x 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Mean % Chloro.</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1.11 (?0.210-1.57)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1.07 (?0.311-1.70)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.569 (?0.0-2.03)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>51.26 (?8.4-71.5)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.411 (?0.0-1.50)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>38.431 (?21.0-66.6)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* TNC—Total nucleated cells (normal and leukemic elements).
† Chloro—Chloroleukemic myeloblasts.
‡ Two animals exhibited marrows devoid of hemic elements.
§ Range.
NORMAL LEUKEMIC NORMAL LEUKEMIC

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Table 3.—Mean Femoral Marrow Cellularity of Normal Rats (80–120 Gm.)

<table>
<thead>
<tr>
<th>Animals</th>
<th>Right Femur Mean TNC/mg. x 10^-6</th>
<th>Mean Blast/mg. x 10^-6</th>
<th>Mean % Blast</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.11 (1.52–2.89)</td>
<td>0.007</td>
<td>0.35</td>
</tr>
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</table>

*TNC = Total nucleated cells.
†Range.

ity. Studies in humans, for the most part, have not included marrow quantitation. Reduced total marrow cellularity appears to have considerable significance in view of the diminished total leukocyte release noted from isolated chloroleukemic hind legs.14 The possibility of reduced marrow granulocyte numbers in human acute leukemic conditions may well contribute to the increased frequency of bacterial infections15,16 and to the atypical leukocytic inflammatory responses characteristic of the leukemias.17-20

Histologic examination of femora from chloroleukemic rats reveals the marrow to be homogeneous, consisting of predominantly leukemic cells.14 The major vasculature is intact, however, the numerous venous sinusoids which characterize normal marrow parenchyma are not apparent. This finding strongly suggests that there is a diminished blood supply to the chloroleukemic femoral marrow. Evidence for an impaired marrow vascularity is derived from perfusions of isolated femora of chloroleukemic rats.21 Abnormally high perfusion pressures are required to initiate blood flow through these preparations. Histologic examination of these perfused femora reveal that, in all instances, effluents traverse the marrow via artificially induced pathways. No normal vascular pathway was evident. Further studies on the blood supply to normal and leukemic marrows are now in progress. The initial consequences

Fig. 2.—Comparison of mean total femoral bone marrow cellularity of immature and mature, normal and leukemic rats 14 days after intravenous administration of chloroma cells. Blast denotes chloroleukemic myeloblast.
of reduced vascularity are readily seen on the normal cell population which is first to succumb. As indicated in table 1, it is not until there has been a considerable decline in the total marrow cellularity that the chloroma cell content increases. This renders it unlikely that the reduced total marrow cellularity results from large leukemic cells replacing smaller normal marrow elements. Altered vascularity may be due to infiltration of chloroleukemic cells or to actions of their metabolic products. This, in turn, may induce a microenvironment which favors further proliferation of chloroleukemic cells. In the final stages of the disease, not even these cells are capable of survival. It is conceivable that at this time the vascular pathways have completely broken down and the marrow has become deprived of essential nutrients.

No correlation was observed between the chloroleukemic cell content of the marrow and the peripheral circulating leukemic cell levels. This may be due to a random entry of these cells from the bone marrow into circulatory pathways and/or to the contribution of extramedullary sites of production. Both of these possibilities have been discussed by Killmann et al. in their consideration of sources of tritiated thymidine-labeled cells in human leukemias. The presence of normal or elevated neutrophil numbers in the peripheral blood of the acute Shay Chloroleukemic rat concomitant with a decrease in marrow neutrophils suggests possible sites of myelopoiesis other than the bone marrow. The possibility that the spleen participates actively in hematopoiesis in the Shay Chloroleukemic rat is currently under investigation.

SUMMARY

Procedures of bone marrow quantitation have been applied to the study of the pathogenesis of a leukemia in rats. Mature Long-Evans rats developed an acute form of the Shay Chloroleukemia after intravenous administration of leukemic cells. Assessment of total nucleated cell numbers (normal and leukemic) per mg. of femoral bone marrow was made during the course of the pathogenesis (20 days). Reductions in the numbers of normal marrow elements were observed prior to significant increases in chloroleukemic cells. A progressive decrease in total marrow cellularity was noted in all subsequent stages. In animals surviving 17–20 days, the total number of hemic cells in the femoral marrow was found to be approximately 40 per cent of that seen in normal animals. The leukemia developed more rapidly in young than in adult animals. Using total and differential bone marrow cellularity as a criterion for the stage of pathogenesis, standardized leukemic rats can be prepared for experimentation.

SUMMARIO IN INTERLINGUA

Technicas de quantification del cellularitate de medulla ossee esseva utilisate in studiar le pathogenese de un leucemia in rattos. Rattos matur del linea Long-Evans disveloppava un forma acute de chloroleucemia Shay post le administracion intravenose de cellulas leucemic. Le valutationes del numeros total de cellulas nucleate (normal e leucemic) per mg de femoral
BONE MARROW QUANTITATION AND CHLOROLEUKEMIA

medulla ossee esseva effectuate durante le curso del pathogenese (20 dies). Reductiones del numeros de normal elementos medullari esseva observate ante augmentos significative del cellulas chloroleucemic. Un progressive declino in le total cellularitate medullari esseva notate in omne le subsequente stadios. In animales supervivente 17 a 20 dies, le numero total de cellulas hemic in le medulla femoral amontava a approximativemente 40 pro cento de lo que es vidite in animales normal. Le leucemia se disveloppava plus rapidemente in juvène animales que in adultos. Con le uso del cellularitate total e differential del medulla ossee como critero del stadio pathogenetic attingite, standardisate rattos leucemic pote esser preparate pro objectivos de experimentation.

REFERENCES

22. Unpublished observations.
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