A Semi-Quantitative Study of Bone Marrow in Rats Following Total Body X-Irradiation

By ROBERT L. ROSENTHAL, M.D., BRYANT L. PICKERING AND LEONTINE GOLDSCHMIDT, Ph.D.

ALTHOUGH THE NEED for a method for the quantitative enumeration of bone marrow cells in experimental hematology, radiobiology and other fields is self-evident, a review of the literature reveals few attempts at quantitative evaluation of bone marrow. In theory, this evaluation entails adjustment of the differential count of the nucleated cells to the number of cells per unit volume of bone marrow in order to compensate for variations in its total cellularity. However, quantitations of cellularity present great difficulty, since bone marrow, unlike blood, is a relatively non-homogeneous, semi-solid substance. In previous methods, the total cellularity has been determined either by the ratio of the area of active marrow to the total marrow space, or by the number of nucleated cells per unit area of marrow on a slide. Both technics have required the use of fixed sections of bone marrow. A method of quantitative measurement of the bone marrow cells, developed in this laboratory, makes possible rapid determination of the cellularity from marrow imprints prepared in a uniform manner. It is the purpose of this paper to describe this method and to demonstrate its validity and use in a serial study of the bone marrow and blood changes following 700 r total body x-irradiation in the rat.

Since Heineke's initial demonstration of the extensive effects of radiation on the hematopoietic system, a large number of studies on various aspects of this subject have appeared. With few exceptions, however, these studies have been qualitative in nature, and as emphasized in the reviews of Denstad and Dunlap, except for general agreement on the marked radiosensitivity of the marrow, the interpretation of detailed changes is steeped in controversy. This situation is largely the result of variables in the type of radiation and dosage measurement, species reaction, and especially in the different technics of bone marrow evaluation.

The depressant effects of radiation upon the myeloid and lymphoid cells in both blood and bone marrow have received much attention. Recognition of the marked destruction of erythroid elements in the bone marrow has been relatively recent. The megakaryocytes, reticulum cells and plasma cells show less extensive changes after radiation exposure and have received considerably less attention. Some authors indicate the possibility of absolute increases in the number of reticulum cells and plasma cells following irradiation.

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METHOD OF BONE MARROW STUDY

The left femur was completely removed from rats anesthetized with ether. The marrow was uncovered by the careful removal of a longitudinal splinter of bone. Preparations were made from marrow of the middle third; a thin smear was made in the center portion of the slide, and several imprints then made on each side of the smear along the length of the slide. The imprints were made in the following, uniform manner. A wooden applicator stick was cut at one end to give a smooth, slanted surface. This end was gently brought into contact with the marrow and then lightly touched to the slide to make a thin imprint, oval to round in shape, and 3 mm. to 4 mm. in the greatest diameter. To assure a representative sampling, fresh marrow was used for every 5 or 6 imprints. The number of imprints on each slide varied between 15 and 25. Slides were then fixed in methyl alcohol and stained with Giemsa.

The bone marrow was evaluated in the following manner. Under high dry magnification the smear portion and several imprints were examined in order that areas of representative cell distribution could be chosen for study. Differential counts of 250 to 500 cells were then performed under oil immersion, with cells classified as shown in table 1. This classification is a modification of that presented by Vogel, Erf and Rosenthal.14 The myeloid cells with ring nuclei were divided into the neutrophilic and eosinophilic. No attempt was made to classify them according to the degree of maturity as recommended by Brecher et al.7 Differential counts made from various portions of the smear and the outer portion of the im-

<table>
<thead>
<tr>
<th>Cells</th>
<th>Controls 15 animals</th>
<th>One day after irradiation 5 animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent of control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean ± S.D. (Cells/S.V.)*</td>
<td>Range (Cells/S.V.)</td>
</tr>
<tr>
<td>Myeloid series</td>
<td>56.2 4.4 50.6-64.0</td>
<td>73.2 27.1 (23.7-31.5)</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>0.6 0.6 0 - 2.2</td>
<td>0.2 0.1</td>
</tr>
<tr>
<td>Pro-myelocytes</td>
<td>1.7 1.0 0.7-4.8</td>
<td>0.8 0.3</td>
</tr>
<tr>
<td>Myelocytes, neut</td>
<td>11.3 5.5 3.8-19.2</td>
<td>2.7 1.0</td>
</tr>
<tr>
<td>Myelocytes, eos,</td>
<td>0.4 0.5 0 - 1.7</td>
<td>0.1 0</td>
</tr>
<tr>
<td>Non-seg., neut</td>
<td>16.9 4.3 11.0-25.9</td>
<td>21.9 7.9</td>
</tr>
<tr>
<td>Non-seg., eos,</td>
<td>0.8 0.6 0 - 1.9</td>
<td>0.8 0.3</td>
</tr>
<tr>
<td>Seg., neut</td>
<td>9.1 4.6 4.5-17.5</td>
<td>24.9 9.3</td>
</tr>
<tr>
<td>Seg., eos</td>
<td>0.1 0.1 0 - 0.9</td>
<td>0 0</td>
</tr>
<tr>
<td>Ring nucleus, neut</td>
<td>13.7 5.2 7.1-23.9</td>
<td>19.2 7.2</td>
</tr>
<tr>
<td>Ring nucleus, eos</td>
<td>1.6 0.8 0.7-3.8</td>
<td>2.6 1.0</td>
</tr>
<tr>
<td>Erythroid series</td>
<td>31.0 4.9 20.0-38.2</td>
<td>11.2 4.2 (2.4-5.4)</td>
</tr>
<tr>
<td>Erythroblasts</td>
<td>1.8 0.8 0.4-3.3</td>
<td>0.3 0.1</td>
</tr>
<tr>
<td>Normoblasts, baso</td>
<td>3.6 2.1 0.8-9.1</td>
<td>0.5 0.2</td>
</tr>
<tr>
<td>Normoblasts, poly. &amp; ortho</td>
<td>25.6 5.1 13.3-24.1</td>
<td>10.4 3.9</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>11.5 3.4 3.2-18.4</td>
<td>4.1 1.6</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>0.3 0.3 0 - 1.2</td>
<td>2.3 0.8</td>
</tr>
<tr>
<td>Reticulum cells</td>
<td>1.1 1.0 0 - 3.2</td>
<td>8.7 3.3</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>0.1 0.2 0 - 0.5</td>
<td>0.3 0.1</td>
</tr>
<tr>
<td>Mast cells</td>
<td>0.3 0.5 0 - 1.6</td>
<td>0.4 0.1</td>
</tr>
</tbody>
</table>

* S.V. = Standard volume.
prints showed no significant differences. The smear tended to show more damaged cells and more reticulum cells than did the imprint.

The degree of cellularity, which was used for adjusting the percentage of each cell in the differential count to a standard scale, was obtained from the outer portion of the imprints. The average number of intact, nucleated cells per oil immersion microscopic field, 0.16 mm. in diameter, was determined from 10 imprints on each slide. So far as possible, fields of representative cellularity were used, and the values were checked on duplicate slides.

The cellularity of the marrow of experimental animals was then expressed in terms of per cent of that in the control animals. Fifteen control animals showed an average of 180 nucleated cells per oil immersion field and this was taken arbitrarily as 100 per cent cellularity. Animals at one day after irradiation averaged 67 cells per oil immersion field which was a total cellularity of 37 per cent of the average value in control animals. The per cent of each cell in the differential count was then converted to the number of cells per standard volume in accordance with the equation:

\[
\text{Number of cells per standard volume (S.V.)} = \frac{A \times B}{100}
\]

where \(A\) = Per cent of cell in differential count, and \(B\) = Total cellularity (per cent of control).

The standard volume is thus equivalent to the volume occupied by 100 nucleated cells in the bone marrow of control animals. This assumes the area occupied by the nucleated cells in the imprint preparation is equivalent to their relative volume in the bone marrow of both experimental and control animals.

**Experimental Procedure**

Male rats of the Sprague-Dawley strain, weighing between 200 and 300 Gm., were exposed to single doses of 700 r* total body x-irradiation. The radiation factors used were 250 kv., 0.6 mm. copper filter, and a dose rate of approximately 25 r per minute as measured in air by means of a Victoreen ionization chamber, and a target to skin distance of 102 cm. Exposure to this dose was found to produce a 27 per cent mortality in thirty days in a group of rats irradiated at the same time and observed for mortality alone. At various intervals up to fifteen days after exposure, groups of 5 rats were sacrificed. Bone marrow studies were performed as described above, and cell counts were done on blood obtained by cardiac puncture. These included red cell, white cell and differential counts, which were performed according to standard methods, and platelet counts which were done by the direct method, using freshly prepared 3.8 per cent sodium citrate as the diluting fluid. Unirradiated, control animals were similarly studied at each time of sacrifice and since no unusual trends or discrepancies were noted, the results of all the control animals were averaged together.

**Results**

**Normal Bone Marrow**

The average values, standard deviations and range of values for bone marrow cells of 15 control animals are shown in table 1. These results are similar to those obtained by other observers, who have presented extensive descriptions of the cell morphology for normal rats.4 15-17

**Total Cellularity of Bone Marrow**

Figure 1 shows the pattern of change of the total number of nucleated cells expressed as per cent of that in the control animals. One day post-irradiation

* This is an approximate LD50 dose for the rat and was selected in this study in order to provide a hematologic effect of radiation midway between overwhelming or massive destruction and minimal destruction. The LD50 dose for man is approximately 400 r of total body x-irradiation.
the cellularity dropped from 100 per cent to 37 per cent and remained between 4 per cent and 7 per cent from the fifth to the ninth day. At twelve and fifteen days, the cellularity showed individual variations with a wide range of values from 3 per cent to 53 per cent; about one-half of the animals showed definite increases in cellularity, while the other half remained below 9 per cent. Typical bone marrow fields representing the outer portion of the imprint preparations before and at various intervals after radiation are shown in figures 2 to 7.

**Initial Changes in Cell Counts in Bone Marrow**

An indication of the early changes in the number of the various bone marrow cells is obtained by comparison of the percentage change between the values obtained in the control animals and the values one day after irradiation. The erythroid cells and lymphocytes both showed decreases of 86 per cent, while the myeloid cells decreased 52 per cent by the first day (table 2). As the numbers of mast cells, megakaryocytes, plasma cells and reticulum cells are relatively small, the per cent changes indicated for these cells are subject to greater variation. Evaluation of the factors involved in these changes is discussed below.

**Table 2.**—Changes in the Bone Marrow of Rats One Day Following 700 r Total Body X-Irradiation

<table>
<thead>
<tr>
<th>Cell</th>
<th>Change from control value (percent)</th>
<th>Estimated relative importance of factors in change after radiation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Destruction</td>
</tr>
<tr>
<td>Erythroid series</td>
<td>-86</td>
<td>++++</td>
</tr>
<tr>
<td>Erythroblasts</td>
<td>-94</td>
<td>++++</td>
</tr>
<tr>
<td>Normoblasts, baso.</td>
<td>-94</td>
<td>++++</td>
</tr>
<tr>
<td>Normoblasts, poly. and orth.</td>
<td>-85</td>
<td>++++</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>-86</td>
<td>+</td>
</tr>
<tr>
<td>Mast cells</td>
<td>-67</td>
<td>0</td>
</tr>
<tr>
<td>Myeloid series</td>
<td>-52</td>
<td>0 to +</td>
</tr>
<tr>
<td>Myelocytes, neut.</td>
<td>-91</td>
<td>0</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>-83</td>
<td>+</td>
</tr>
<tr>
<td>Pro-myelocytes</td>
<td>-82</td>
<td>+</td>
</tr>
<tr>
<td>Non-seg., neut.</td>
<td>-53</td>
<td>0</td>
</tr>
<tr>
<td>Ring nucleus, neut.</td>
<td>-47</td>
<td>0</td>
</tr>
<tr>
<td>Seg., neut.</td>
<td>+ 2</td>
<td>0</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>+167</td>
<td>0</td>
</tr>
<tr>
<td>Reticulum cells</td>
<td>+200</td>
<td>0</td>
</tr>
</tbody>
</table>

* 0 none, + slight, ++ moderate, +++ marked importance.

**Myeloid Cells**

Figure 1 shows the changes in the number of myeloid cells of the bone marrow and in the blood neutrophils. Almost a threefold increase occurred in the blood neutrophils one day after irradiation, while the bone marrow showed a decrease of 52 per cent in myeloid cells. Further analysis revealed that the reduction was
most marked for the younger cells: myelocytes 91 per cent, myeloblasts 83 per cent, and pro-myelocytes 82 per cent. The cells at more mature stages of development showed less depression in their count. From the third to the ninth day, typical values for the various myeloid cells per standard volume were: myelo-

![Diagram of cell counts over time](image)

**Fig. 1:** Changes in the cells of the bone marrow and blood in rats exposed to 700 r of total body x-irradiation.

blasts and pro-myelocytes 0, myelocytes 0.1, non-segmented neutrophils 0.3 to 0.6, and segmented neutrophils 0.1 to 0.9.

At twelve and fifteen days after irradiation, one-half of the animals showed slight increases in all stages of myeloid cell development. Values at twelve and
fifteen days, respectively, averaged: myeloblasts 0.1, 0; pro-myelocytes 0.1, 0.1; myelocytes 1.0, 0.3; non-segmented neutrophils 1.3, 1.2; segmented neutrophils 0.8, 0.4. Despite the slight increases in the marrow myeloid cell numbers, blood neutrophil counts still remained at low levels at twelve and fifteen days following irradiation.

**Fig. 2.**—Bone marrow before irradiation, normal distribution of myeloid and erythroid cells (X 500).

**Fig. 3.**—Bone marrow one day after irradiation, hypocellular, consists mostly of the more mature myeloid cells, erythroid cells almost completely disappeared (X 500).
Erythroid Cells

The red blood cell count showed no significant change until twelve days following irradiation, when a slight decrease became apparent. In the bone marrow

![Image of bone marrow three days after irradiation, markedly hypocellular with few myeloid, plasma and reticulum cells present (X 500).](image1)

![Image of bone marrow seven days after irradiation, markedly hypocellular (X 500).](image2)

The erythroid elements were markedly decreased in number one day after irradiation (figure 1). They were almost completely absent until the ninth day, when an average of 0.3 basophilic and 0.6 polychromatic normoblasts per standard
volume appeared. At twelve and fifteen days, animals showing increased cellularity and an increase in myeloid cells also showed modest increases in the number of erythroid cells.

Fig. 6.—Bone marrow twelve days after irradiation, early stage of regeneration with small foci of erythroid elements, fewer myeloid elements (X 500).

Fig. 7.—Bone marrow fifteen days after irradiation, regeneration progressing with more foci of erythroid and of myeloid cells (X 500).

Lymphocytes

Figure 1 shows the similar patterns of change observed in blood and bone marrow lymphocytes. Twelve and fifteen days after irradiation the bone marrow lymphocyte counts showed greater increases than did those of the blood.
Megakaryocytes

One day following irradiation, the number of megakaryocytes appeared to show no change (figure 1). Gradual decreases were seen at three and five days. From seven to fifteen days, there was a complete absence of megakaryocytes, although at fifteen days a few megakaryocytes appeared in the bone marrow of animals showing the increased total cellularity. The changes in the platelet counts followed those of the megakaryocytes.

Mast Cells

The mast cells gradually decreased and disappeared by five days, and then reappeared in all animals by twelve and fifteen days (figure 1).

Reticulum Cells and Plasma Cells

These cells are considered together because they showed no apparent post-irradiation change and became the most prominent cells in the post-irradiation bone marrow. Table 3 lists both the unadjusted and adjusted differential count values for these cells at various intervals after irradiation. For both cells, the unadjusted per cent in the differential counts showed marked increases. However, in terms of the number of cells per standard volume, no significant absolute increases above the control range were apparent at any time after irradiation.

Degenerated Cells

An attempt was made to enumerate the number of degenerated cells at each interval following irradiation. The results were as follows: one day, 1.1 cells per standard volume; three days, 2.2 cells; five days, 3.3 cells; seven days, 1.3 cells; and nine days, 1.9 cells. These values are only approximate owing to the difficulty of distinguishing between cells degenerated in vivo and those damaged in the course of the marrow preparation.
Morphologic Studies

In view of the extensive literature on the effects of irradiation of animals upon their bone marrow morphology, a few observations, some of which have thus far received little or no attention, will be emphasized below.

The erythroid cells showed extensive degenerative changes, especially one day after irradiation. A few cells recognizable as normoblasts had nuclei of bizarre shapes and there was no evidence of any post-irradiation maturation. At twelve and fifteen days, regenerating erythroid cells showed no abnormalities.

In the myeloid series, evidence of degeneration was relatively infrequent and chiefly confined to the myeloblasts and pro-myelocytes. A moderate number of myeloeytes and non-segmented and segmented neutrophils were of very large size with nuclei of bizarre shapes and with hypersegmentation in the mature stages. No unusual forms were seen in the recovery period at twelve and fifteen days following irradiation.

The megakaryocytes showed no morphologic alterations in the early post-irradiation period but gradually lost their cytoplasm. A fair number of typical megakaryocytic nuclei without cytoplasm were seen at three, five and seven days after irradiation.

The reticulum cells appeared to show no unusual alterations for the first nine days after irradiation, except for evidence of phagocytic activity in about 10 per cent of these cells at one and three days. At twelve and fifteen days after irradiation, transitional stages from the reticulum cell to other bone marrow cells became apparent. The development of mast cells, apparently from the reticulum cells, was observed in all animals twelve and fifteen days following irradiation. Cells in various transitional stages appeared to show transfer of small fragments from the nucleus into the cytoplasm. These fragments then appeared to undergo changes in staining characteristics, thus becoming the metachromatic granules identified with the mast cell. Transition from reticulum cells to megakaryoblasts was suggested by the appearance of fine, reddish-purple staining granules in the cytoplasm in certain reticulum cells. Transition from the reticulum cells to plasma cells sometimes seemed to occur, but transition of the former to the erythroid and myeloid elements was not readily apparent. However, there were suggestions of a reticulum cell to pro-myelocyte transformation.

The plasma cells showed some variation in the degree of coarseness of the nucleus and basophilic staining of the cytoplasm. Sometimes these cells closely resembled basophilic normoblasts.

Discussion

Critique of Technic

The weaknesses in any method for the quantitative enumeration of bone marrow cells are readily apparent. A small sampling from a limited number of areas is considered as representative of the entire marrow; also, evaluation of the degree of cellularity and identification of the cells in the differential count

* A similar genesis of the mast cell granules has been observed in mast cells of the peritoneal cavity of rats and mice.18
BONE MARROW IN RATS FOLLOWING X-IRRADIATION

may be highly subjective. In the present work, determination of the cellularity from the number of cells per oil immersion field from imprint preparations was found to provide consistent and reproducible data. It is felt that the results of the present study indicate the validity of this method for adjusting differential counts of bone marrow to a standard scale. The use of imprints is not only simple and rapid, but also avoids the necessity for preparing fixed sections of marrow.

It is not implied that the method described in this paper can be used for marrow obtained by bone puncture and aspiration. Marrow obtained in this manner may show various degrees of dilution with blood and a lower number of fixed cells, such as reticulum cells and megaloblasts.

Effects of Radiation on Bone Marrow

The literature contains numerous conflicting opinions concerning the responses of the bone marrow to total body irradiation. These conflicting opinions may be resolved to some degree by the recognition that in addition to destruction, radiation may cause many less drastic effects, as, for example, inhibition of mitosis and formation of new cells from the reticulum or stem cell, acceleration or inhibition of maturation, and shortened or prolonged life span of the cell. In the present study, an attempt is made to relate the numerical changes in the bone marrow cells with observations on the morphologic changes, in order to evaluate the relative importance of the various effects of radiation on each type of cell. This evaluation is summarized in table 2 and forms the basis for the following discussion. In this evaluation, it is essential to state that with the possible exception of the plasma cells, no evidence of formation of new cells from the reticulum cells was observed within the first nine days following irradiation.

The erythroid cell series showed extensive destruction with no indication of any maturation on the first day following irradiation. However, the observation of significant elevations of the red cell count and reticulocytes within four hours following lethal range doses of total body irradiation indicates a brief period of accelerated maturation of either the polychromat or orthochromat normoblasts to reticulocytes. It is interesting to note that Hennessy and Huff have observed a 90 per cent reduction from normal in the uptake of radioactive iron in rat bone marrow one day after 200 r total body x-irradiation.

While showing some evidence of destruction, the myeloid cells, for the most part, undergo accelerated maturation, as evidenced by the increase in segmented forms in both bone marrow and blood, accompanied by decreases in the younger forms, such as myelocytes, pro-myelocytes and myeloblasts. The large, bizarre forms of myelocytes, non-segmented and segmented forms, described in this paper and often reported in the literature, could probably represent a series of cells derived from accelerated and anomalous maturation of the pro-myelocyte or myeloblast. These unusual types of cells were not seen in the marrow regenerating at twelve and fifteen days post-irradiation.

The lymphocytes, mast cells and megakaryocytes show no indication of destruction or maturation changes. Their disappearance rates apparently depend...
upon their life spans, which in turn may be shortened as a result of the irradiation.

While it is beyond the scope of this discussion to dwell extensively upon the important relationships of bone marrow depression to mortality, infection, hemorrhage and anemia in the syndrome of radiation sickness, it is generally agreed that recovery of the bone marrow is essential to survival after exposure to total body x-irradiation. This recovery involves regeneration of the myeloid, erythroid and megakaryocytic elements, which are most probably derived from the reticulum cells, as is discussed in Downey’s extensive review. Since the reticulum cells are highly radioresistant, and thus theoretically available to produce hematopoietic cells following any dose of total body irradiation that is not large enough to kill the animal in a few days, recovery of bone marrow appears to depend to a great extent upon the factors involved in reticulum transition to other cells.

The present study suggests that there are differences among the conditions or factors required for the development of each of the several series of cells from the reticulum cell. Formation of plasma cells did not appear to be inhibited at any time following irradiation. Transition of the reticulum cell to other cell types was absent until mast cells appeared, in all animals sacrificed twelve and fifteen days following irradiation, while erythroid, myeloid and later megakaryocytes reappeared in only 50 per cent of these animals. It is pertinent to mention some recent observations on patients with polycythemia associated with multiple myeloma. In these cases it appeared that the reticulum cells which at first showed an increased proliferation into the erythroid series, then differentiated into myeloma or plasma cells.

Investigation of the physiologic, metabolic and nutritive factors involved in the transition of cells from the reticulum cell, as well as in the maturation of the various hematopoietic cell series, offers a promising approach to an understanding of, and basis for, therapy in radiation sickness.

**Summary**

1. A method has been presented for the quantitative enumeration of the bone marrow cells by adjusting the differential count for the degree of cellularity, which is derived from imprint preparations of femoral marrow.

2. This method has been employed in a study of bone marrow changes at various intervals following 700 r total body x-irradiation in the rat. The findings have been correlated with the peripheral blood counts.

3. On the basis of numerical changes and morphologic observations, the mechanism of the effects of irradiation on the various cell series of bone marrow is evaluated. The erythroid cells show rapid decreases in number due to their marked destruction. The myeloid cells chiefly undergo accelerated maturation resulting in increased segmented forms in both blood and bone marrow. The decreases in megakaryocytes, mast cells and lymphocytes appear to be related to their life spans in the absence of further production. The reticulum cells and plasma cells show neither an absolute increase nor decrease.

4. Formation of new cells, except possibly for plasma cells, from the reticulum
cells is completely inhibited for the first nine days following total body irradiation of rats with 700 r. Regeneration is first apparent at twelve days after x-ray as evidenced by areas of erythropoiesis, and to a lesser extent, myelopoiesis. Megakaryocytes reappear in small numbers at fifteen days. Although this regeneration is apparent in only 50 per cent of the animals studied at twelve and fifteen days following irradiation, all these animals showed evidence of transition of reticulum cells to mast cells.

REFERENCES

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