Studies on the Regulation of Granulopoiesis. I.
The Response to Neutropenia

By Alec Morley and Frederick Stohlman, Jr.

It is now well established that erythropoiesis is actively regulated in response to demand and evidence is accumulating that thrombopoiesis is likewise. A priori reasoning and analogy suggest that granulopoiesis also is actively regulated but evidence to support this is scant. Reports by several groups of workers suggest that depletion of circulating neutrophils by means of leucapheresis or injection of antiserum leads to proliferation of marrow precursors. Furthermore, the presence of a feedback loop from blood neutrophil to stem cell provides a satisfying explanation for the periodicity of the neutrophil count occurring in cyclical neutropenia and in some normal individuals.

Although there is some evidence to indicate that granulopoiesis is actively regulated, there is virtually no evidence to indicate on which proliferative cell compartment the primary regulation is exerted or the means by which such regulation is accomplished. Analogy with erythropoiesis and consideration of the conventional morphological sequence for granulocyte development suggest that regulation might be exerted by controlling the rate at which stem cells differentiate into myeloblasts. However, several groups of workers, while not excluding this possibility, have suggested that regulation might also take place at the myelocyte level.

Total body irradiation is a convenient method for producing depletion of circulating neutrophils but has the disadvantage of damaging or destroying the very tissue, the bone marrow, which responds to the neutropenia. This disadvantage can, however, be overcome by shielding a small portion of marrow and studying its response to neutropenia. Such an experimental design has the advantage that changes in the shielded marrow must be the result of either a cellular or humoral alteration in the blood, and may therefore give some indication as to the nature of that alteration.

For these reasons we studied the changes in granulopoiesis occurring in the shielded hindlimb of the mouse in response to irradiation of the rest of the body.

Methods

Virgin female CF1 mice aged 10–12 weeks were used. The right leg was shielded by a cylinder of lead 3-inch thick over which was laid a strip of lead also 3-inch thick. Both control and irradiated mice were anesthetized with intraperitoneal pentobarbital 70 mg/Kg. The
conditions for irradiation were dose rate 48 R./min., 250 kV., 15 mA., and 1.0 mm. Cu and 1.0 mm. Al filtration, and the dose was 800 R. unless otherwise stated. In any one experiment, groups of 4–5 mice were sacrificed on various days after irradiation. Peripheral blood was obtained by heart puncture, marrow smears were made from the shielded femur and marrow suspensions from the shielded tibia. Blood leukocyte counts were performed visually, tibial nucleated cell counts by using a Coulter model B counter. Differential leukocyte counts were done by scoring 50 or 100 cells, the number depending on the total leukocyte count. Marrow differential counts were done by scoring at least 250 cells; they were done at the end of the experiment without foreknowledge as to which animal the smear originated from. Myeloblasts were defined as cells with fine nuclear chromatin but without a nuclear opening or cytoplasmic granules, promyelocytes as cells with azurophil granules with or without a nuclear opening, myelocytes as cells with neutrophilic granulation and with a nuclear–cytoplasmic ratio of greater than one third. Metamyelocytes were defined as cells with a nuclear–cytoplasmic ratio of less than one third, but without well-developed chromatin condensation of their nuclei, band neutrophils as cells showing well-clumped nuclear chromatin but not nuclear segmentation and segmented neutrophils as cells showing nuclear segmentation. Since it was sometimes difficult to classify primitive cells with certainty, the myeloblasts were grouped together with promyelocytes into one compartment in analyzing most of the data. Determination of the number of colony-forming units in the tibia was carried out by a modification of the method of Till and McCulloch.10

RESULTS

Figures 1–4 show data pooled from all experiments. Figure 1 shows the changes in the neutrophil count which in most experiments began to fall on days two–four, reached a nadir on days four–six, rose slowly until day 12 and then increased more rapidly to overshoot the normal range between days 16–20. There were no changes in the blood or marrow of the control mice which had been anesthetized but not irradiated. Granulopoietic hyperplasia developed
in the shielded leg of irradiated mice and Figs. 2A and 2B show the serial changes observed in the various morphological compartments. There was a slight increase in granulopoiesis on day two which seemed to return towards normal on day three. Following this the changes in the various compartments closely followed the development of neutropenia. The earliest changes to appear were an increase in myeloblasts and promyelocytes and a decrease in
segmented marrow neutrophils, but it could not be determined for certain which of these three changes was the first to develop. However, the increases in the myeloblast and promyelocyte compartments appeared to precede the increase in the myelocyte compartment. This change became most apparent when the promyelocyte/myelocyte ratio for each animal was considered. If the promyelocyte and myelocyte compartments had increased in size concomitantly, then the ratio should have remained unchanged but, as shown in Fig. 3, it rose above the control value, indicating that expansion of the promyelocyte compartment preceded expansion of the myelocyte compartment. Because of the small number of myeloblasts and the uncertainty of positive identification, it was not felt worthwhile to determine serial changes in the myeloblast/promyelocyte ratio.
In one experiment the quantitative relationship between the circulating neutrophil level and the response of the shielded bone marrow was evaluated. Neutropenia of various degrees was produced by irradiating leg-shielded mice with 200, 400, 600, 800 or 1000R. Figure 4 shows the significant \((p < 0.01)\) relationship on the fourth post-irradiation day between the number of segmented marrow neutrophils and the circulating neutrophil count. Figure 5 shows the size of the myeloblast-promyelocyte compartment plotted against the circulating neutrophil count (left panel) and the number of segmented marrow neutrophils (right panel). Although it is significantly related to both \((p < 0.01)\), the size of the myeloblast-promelocyte compartment appears to bear a closer relationship to the circulating neutrophil count than to the number of marrow neutrophils.

Between days two–eight after irradiation, the degree of granulocytic hyperplasia appeared to be fairly closely related to the circulating neutrophil count. However, between days eight–twelve the number of myeloblasts, promyelocytes and myelocytes and the promyelocyte/myelocyte ratio seemed to show a
Fig. 5.—Relationship on day four between size of myeloblast–promyelocyte compartment and circulating neutrophil count (left panel), and number of segmented marrow neutrophils (right panel). Open circles indicate unirradiated animals and closed circles indicate animals irradiated at various doses. Correlation coefficients \( r \), and regression lines calculated from all observations and are significant at \( p < 0.01 \).

fall which was then followed by a secondary rise. This pattern suggests that during this time influx into the myeloblast–promyelocyte compartment temporarily declined.

Table 1 shows the number of colony-forming units in the shielded tibia on various days after irradiation. It indicates that the number of transplantable stem cells did not change markedly as granulopoietic hyperplasia developed.

**DISCUSSION**

We have made two main assumptions in interpreting our data. They are:

1) The changes observed in the shielded limb were the direct or indirect result of neutropenia and not of nonspecific tissue damage due to irradiation. This assumption seems reasonable on theoretical grounds since the changes in granulopoiesis were such as to correct the neutropenia. Furthermore, with the exception of day two after irradiation, the time course of the increase in granulopoiesis and its magnitude on any one day were closely related to the number of circulating neutrophils. The reason for the abortive rise in granulopoiesis

Table 1.—Colony forming units in tibias of control mice and in shielded tibias of leg-shielded mice irradiated on various days prior to transplantation. The data were derived from several different experiments. Each value is the mean ± 1 S.E. of a group of 8–10 mice.

<table>
<thead>
<tr>
<th>Day</th>
<th>Irradiated leg-shielded</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3460 ± 400</td>
<td>3110 ± 480</td>
</tr>
<tr>
<td>8</td>
<td>4010 ± 250</td>
<td>3110 ± 480</td>
</tr>
<tr>
<td>12</td>
<td>2950 ± 290</td>
<td>2970 ± 370</td>
</tr>
<tr>
<td>16</td>
<td>4490 ± 470</td>
<td>3660 ± 380</td>
</tr>
<tr>
<td>23</td>
<td>2930 ± 600</td>
<td>2750 ± 500</td>
</tr>
</tbody>
</table>
on day two is uncertain but tissue damage due to irradiation may have been responsible.

2) Increases in size of the various morphological compartments were due to increases in flux through them. This again is a reasonable *a priori* assumption, since increased flux would tend to correct the neutropenia. The assumption is most critical for the myeloblast–promyelocyte compartment since changes in succeeding compartments seemed to follow and be the result of changes in this compartment. It seems unlikely that increased intracompartmenental birth or decreased death could have been of sufficient magnitude to have expanded the number of myeloblasts and promyelocytes fourfold within two–three days, and at the same time produced increased efflux from the compartment. Rather, it seems likely that at least the principal factor in expansion of the compartment was increased differentiation of morphologically unidentifiable precursor cells. Stem cells which are capable of granulocytic differentiation but which are unrecognizable morphologically are known to exist in small numbers in normal marrow. We have found that induction of polycythemia even further increases the degree of granulocytic hyperplasia in irradiated leg-shielded mice, and other workers using different experimental models have likewise found that varying the demand for erythropoiesis results in reciprocal variation in the rate of granulopoiesis. Since an alteration in the stimulus to erythropoietic differentiation can only affect granulopoiesis by acting through the stem cell, it seems very likely that the increase in granulopoiesis observed in irradiated leg-shielded mice is mediated principally through an increase in the rate of differentiation of stem cells into the myeloblast–promyelocyte compartment.

Based on these assumptions, the changes in granulopoiesis observed in the shielded marrow were interpreted as follows. Fluctuations with time in the number of circulating neutrophils were closely followed by corresponding fluctuations in the number of segmented neutrophils in the marrow (Figs. 1 and 2B). When different degrees of neutropenia were produced by different doses of irradiation, the size of the marrow store of segmented neutrophils on day four also correlated with the degree of neutropenia (Fig. 5). During the first four–five days after irradiation, as neutropenia and depletion of the marrow store developed, the number of metamyelocytes and band neutrophils did not decline. This indicates that during this time the rate of flux into the segmented neutrophil compartment did not decrease and that depletion of the compartment was therefore due to increased efflux. Thus, the observations suggest that the number of circulating neutrophils affected the size of the marrow store by regulating the rate of release of mature neutrophils into the blood. The mechanism for regulation must have been rapidly acting since the size of the marrow store closely followed the blood neutrophil count. Other workers have also produced evidence suggesting that the rate of release of marrow neutrophils is regulated by the number of circulating neutrophils. Several have suggested that a humoral factor is involved.

In addition to its effect on release of mature cells, neutropenia also appeared to increase the rate of production of neutrophil precursors. Although myelo-
blasts were possibly the first cells to increase in number, they have generally been grouped into one compartment with promyelocytes since they were sometimes difficult to distinguish from other primitive cells with certainty. The myeloblast-promyelocyte compartment was the first compartment to increase in size; the reasons for ascribing this to increase in the rate of differentiation of stem cells have already been discussed. The size of this compartment on day four was found to be linearly related to the logarithm of the neutrophil count. This suggests that the rate of stem-cell differentiation and hence, ultimately, the granulocyte production rate are approximately linear functions of the logarithm of the neutrophil count. However, it seems likely that such functions only obtain within certain limits since there is probably a fixed upper limit beyond which the rate of granulopoiesis cannot be increased. All of the changes in the distal morphological compartments could be attributed to cells leaving the myeloblast-promyelocyte compartment and traversing succeeding compartments as they proliferated and matured. From kinetic studies of normal granulopoiesis, Patt and Malony proposed the existence of a myelocyte “sink,” and Boggs et al. suggested that myelocytes have stem-cell potentiality. However, in the present study, in which shielded normal marrow was exposed to an increased demand for neutrophils, there was no evidence that a decrease in myelocyte death rate or an increase in myelocyte proliferative rate played a substantial role in accelerating production in response to demand. If a response to neutropenia at the myelocyte level had been important, one would have expected to observe an initial expansion of the myelocyte compartment, but this was not seen. However, it is possible, although unlikely, that under conditions of normal demand one mechanism predominates and controls neutrophil production at the myelocyte level, but when demand is increased a second mechanism supervenes and accelerates production at the stem-cell level.

When different doses of irradiation were used to produce different degrees of neutropenia, the size of the myeloblast-promyelocyte compartment seemed to correlate better with the number of circulating neutrophils than with the number of segmented marrow neutrophils. This is evidence, admittedly not strong, that the number of circulating neutrophils directly controls influx into this compartment through a humoral mechanism, rather than indirectly through regulation of the marrow segmented pool. The maximum size reached by the myeloblast-promyelocyte compartment was four-five times normal. That this might be the limit to which granulopoiesis can be increased in response to demand is also suggested by the DF32P studies of Athens et al. These workers found that the granulocyte turnover rate in patients with various infections was increased up to five times the mean determined for normal subjects.

The identity of the stem cell responding to neutropenia and the mechanism by which this is effected is speculative. Available evidence indicates that for erythropoiesis and thrombopoiesis there are committed stem-cell compartments distal to the pluripotent or transplantable compartment. For the case of erythropoiesis it is the committed rather than the pluripotent stem cell that responds to erythropoietin. By analogy it might be suggested that there also exists a committed compartment for granulopoiesis, and that the
increased flux into the myeloblast–promyelocyte compartment observed in irradiated leg-shielded animals was the result of an influence, possibly humoral, on this compartment. Quantitative considerations support this possibility since the size of the pluripotential compartment, at least as measured by transplantation, is quite insufficient to account for both the normal rate of influx into the myeloblast–promyelocyte compartment and the increased rate of influx observed in the irradiated leg-shielded mouse. The various committed stem-cell compartments must, however, be closely linked to the pluripotential one by feedback loops in order to account for the interrelationship and competition between erythropoiesis and granulopoiesis observed in various perturbed states.11-14

SUMMARY

Mice were irradiated with one leg shielded in order to study the response of the protected marrow to neutropenia. The findings suggested that neutropenia increases the rate of release of segmented neutrophils from the marrow and also increases the rate of production of neutrophil precursors, possibly by increasing the rate of flux from a committed granulocytic stem cell compartment into the myeloblast–promyelocyte compartment. A humoral mechanism may be involved.

REFERENCES

STUDIES ON THE REGULATION OF GRANULOPOIESIS


Studies on the Regulation of Granulopoiesis. I. The Response to Neutropenia

ALEC MORLEY and FREDERICK STOHLMAN, JR.