Cyclic Hematopoiesis in Grey Collie Dogs: 
A Stem-Cell Problem

By Harvey M. Patt, John E. Lund, and Mary A. Maloney

Studies were made to ascertain the possible basis of cyclic hematopoiesis in the grey collie. The results of $^3$H-thymidine and $^{59}$Fe labeling in conjunction with other marrow parameters and peripheral counts suggest that the regular periodicity of blood neutrophil and reticulocyte levels is caused by a defect at the stem-cell–marrow interface. It is postulated that the ebb and flow of hemic cell production in the grey collie reflects competition for a limiting number of pluripotential stem cells with the alternating competitive pressure provided by activation and deactivation of a neutrophil feedback circuit from periphery to stem cell. This mechanism can account for the characteristic 12-day periodicity and contributes to the phase difference in the cycling pattern of neutrophils relative to reticulocytes.

The evolution of stem cell to blood cell in bone marrow is subject to a hierarchy of controls. These controls are concerned with stem cell maintenance and commitment, as well as with blood cell development and delivery, and apparently involve a series of feedback circuits from periphery to marrow and within the marrow complex. Because of their operant characteristics, it has been suggested that hematopoiesis is intrinsically rhythmic and borders on oscillation even if regular periodicity is not obvious. Cycling of peripheral blood counts, particularly of neutrophils, has been detected from time to time in both animals and man. A dramatic example is seen in grey collies. Lund et al. were the first to note the profound neutrophil oscillation in these dogs and to view this genetically determined behavior as a possible model for human cyclic neutropenia. Subsequent work pointed to the involvement of erythropoiesis as well and hence to the likelihood of a defect at the stem-cell level. These findings were extended by Dale et al., who observed an identical periodicity for all blood cells, with, however, a characteristic phase difference. Significantly, their studies also indicated that cycling was not a result simply of an exaggerated peripheral feedback mechanism. In this paper we present the results of an investigation which was begun several years ago and provides further insight into the phenomenon of cyclic hematopoiesis in the grey collie.

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MATERIALS AND METHODS

This study was carried out on 11 grey collies and five normal litter mates, ages 3 mo to 3 yr. Peripheral blood counts were made in all dogs for at least 33 consecutive days, and this was repeated in several of the dogs after different intervals. Other procedures, e.g., radioisotope labeling and bone marrow analysis, were also performed in some animals, as described below. For peripheral blood counts venous blood samples anticoagulated with EDTA were obtained daily between 9:00 and 11:00 a.m. Total leukocyte and red blood cell counts were made on a Coulter Counter. Differential counts of 100 leukocytes were determined on Wright's-stained smears. Reticulocytes were stained with new methylene blue, and the number per 1000 erythrocytes was scored by two observers. Platelets were enumerated by phase-contrast microscopy. The blood count pattern in each dog was determined by computing the moving average for three consecutive daily points. To facilitate interanimal comparison, the moving averages were related to the mean of all counts for a given blood element. Periodicity was considered to occur if the resulting blood count curve revealed a regular oscillatory pattern upon visual inspection with nadir to apogee variation of at least 50%. The presence or absence of periodicity in normal and grey collies was further evaluated by a $\chi^2$ test based on expected and observed runs as described by Wallis-Moore. Cycle duration was determined by the mean interval between consecutive minima and consecutive maxima over two cycles.

In normal and grey collies selected for tritiated thymidine ($^{3}$H-TdR) labeling, 0.3-0.5 $\mu$Ci/g (specific activity, 0.36 Ci/mM) was injected intravenously. Bone marrow aspirates were collected 0.5 hr later under local anesthesia. Smears were fixed in methyl alcohol; dipped in NTB, NTB$_3$, or Ilford L4; developed with D19; and stained with a modified Giemsa. Exposure times were established so that there was no increase in per cent of labeled cells with a doubling of time. The granulocytic and erythrocytic distribution of 2000 labeled cells was determined, and a differential count was performed on 1000 nucleated marrow cells. Labeled mitosis curves were also determined in two grey collies and two litter mates by scoring at least 50 mitoses in marrow samples obtained at 1- to 2-hr intervals during an 18-hr period. To study the chronology of neutrophil appearance in blood, hourly samples were obtained from 48 to 110 hr after $^{3}$H-TdR injection. Total leukocyte and differential counts were made, and autoradiograms of blood smears were prepared with NTB$_3$. At least 1000 neutrophils were examined at each sampling time, and labeled cells were classified by grain count. A cell with four grains over the nucleus was taken to represent a definitely labeled cell ($p < 0.01$).

Plasma iron turnover was evaluated in several normal and grey collies. The dogs were given three weekly injections intramuscularly of 37.5 mg repository testosterone in preparation for these studies. One hour before intravenous injection of transferrin-bound $^{59}$Fe (5 $\mu$Ci; specific activity, 6-14 Ci/g) the collies also received an intramuscular injection of 150 mg iron dextran and an oral dose of 150 mg ferric chloride. Plasma samples were collected at 0, 5, 15, 30, 45, 90, 120, and 180 min. The radioactivity in 0.5-1.0 ml was determined in a scintillation counter, and samples were measured until 1 x 10$^5$ counts had accumulated or 100 min had elapsed. Serum iron was determined on 0- and 3-hr samples.

The possibility of a generalized periodicity of cell production was explored in four grey collies by examining regenerating hair follicles early and late in the cycle. For this purpose, hair was plucked from the dorsal thorax near the center of the back. Four or five days later $^{3}$H-TdR was injected as described above; after 1 hr, four skin biopsies were taken with a 4-mm punch. Tissue was fixed in neutral buffered formalin, embedded in paraffin, prepared for autoradiography with NTB$_3$ or Ilford L4 and stained with Mayer's hematoxylin eosin or Giemsa. The labeling index and the total number of cells per mitotic zone of the secondary hair follicle were determined. The cell count was made only on hair follicles which, on microscopic examination, were sagitally sectioned so that the cells were approximately equally distributed around the follicle.

RESULTS

At least two consecutive cycles were observed in each of the 11 grey collies studied, the mean neutrophil and reticulocyte periodicities being 12.0 $\pm$ 0.4 and 12.9 $\pm$ 0.6 days, respectively. Platelet counts, made in only four of the grey collies, were suggestive of a similar periodicity. Although the periods were the
same, the reticulocyte (and platelet) levels, as described by Dale et al.,7 were usually elevated when the neutrophil count was depressed, and vice versa (Fig. 1). The normal litter mates did not manifest a periodicity during the course of 33–38 daily observations (Fig. 2). Despite the profound oscillation of peripheral neutrophils and reticulocytes, total production during the cycle was about the same as in a normal dog. Thus, the average neutrophil count derived from the mean count for each of the 11 cyclic dogs was 6438 ± 770/cu mm, compared to 6741 ± 583 for the five normal litter mates. For reticulocytes, the average counts were 35,800 ± 4200 and 37,000 ± 5700 in the cyclic and normal collies, respectively. Although the average counts were not significantly different, the distribution of individual counts in the two types of dogs differed markedly, as shown for the neutrophils in Fig. 3.

Analysis of marrow punch biopsies and aspirates in four of the grey collies indicated that, in general, marrow cellularity began to decrease at the height of neutrophilia and then to increase as neutropenia became severe. During the cellular phase the marrow was mainly granulocytic and during the hypocellular phase predominantly erythrocytic. Restoration of cellularity was initiated by a wave of proliferating granulocytes; the granulocyte composition then shifted to increasing maturity as the peripheral neutrophil count increased. Qualitatively similar events were seen in relation to changes in the blood reticulocyte level.
Fig. 2. Peripheral blood counts in a normal collie. Relative values and curves were determined as in Fig. 1.

The nadir of the blood neutrophil count, which corresponds approximately to the apogee of the reticulocyte count, was taken to represent the first day of the cycle. Accordingly, the neutrophil count increased and the reticulocyte count usually decreased during the first half of the cycle, and this sequence was reversed during the second half of the cycle. The ratio of presumptive

Fig. 3. Distribution of relative neutrophil counts in normal and cyclic collies. Each neutrophil count was related to the mean of all neutrophil counts in a given dog. The mean relative count of 10 corresponds to 6700 cells/cu mm for the normal and 6400 for the cyclic collies.
proliferating granulocyte to erythrocyte precursors averaged 1.7 during days 3–6 and 0.6 during days 8–11, the normal ratio being 1.2. This difference corresponds closely to the difference in distribution of cells that were flash labeled with $^3$H-TdR. The ratio of initially labeled granulocytes and erythroid cells, which provides a measure of relative rates of cell production, decreased from a mean of 2.0 to 0.7 (normal, 1.1) from the first to the second half of the cycle. The temporal pattern of labeled mitoses was the same for progenitors of granulocytes and erythrocytes and did not differ with position in the cycle. The myeloid to erythroid (M:E) ratio of bone marrow aspirates increased from 2.1 on day 3 to 13.8 on day 6 and then decreased to 1.2 on day 8 and 0.5 on day 11. Decrease of the M:E ratio with cycle progression was a consistent finding in the four grey collies studied. In three normal litter mates this ratio varied from 1.9 to 3.4 with a mean of 2.9.

Although absolute estimates of marrow cellularity parameters were not feasible in these studies, changes in plasma iron clearance and turnover and in the blood reticulocyte level indicate that erythroid as well as myeloid marrow components are affected in the grey collie. As shown in Table 1, the plasma iron $T_{1/2}$ decreased and the turnover increased from days 3–4 to days 9–10 of the cycle. There was a 70\% decrease in plasma iron turnover relative to that in the normal collie early in the cycle (M:E ratio normal or above normal) and a 130\% increase later in the cycle (M:E ratio below normal).

The chronology of neutrophil appearance in blood as revealed by $^3$H-TdR labeling is presented in Figs. 4 and 5. In one grey collie, $^3$H-TdR was given on day 6 of the cycle, i.e., a few days before the anticipated onset of severe neutropenia (Fig. 4), and in another on day 11, i.e., a few days before the anticipated onset of blood neutrophil recovery (Fig. 5). Labeled cells appeared in blood at about the same time in each dog. In the first dog, labeled as well as unlabeled neutrophils disappeared exponentially with a normal $T_{1/2}$ of about 6 hr. Because of the limited observation period it was not possible to estimate a disappearance rate when $^3$H-TdR was given early in the marrow recovery phase. It is noteworthy, however, that the initial increase of labeled neutrophils was about twice as great when $^3$H-TdR was given on day 11 instead of day 6. The correlation coefficients of the linear regressions derived from the first 12 hr of the labeling

<table>
<thead>
<tr>
<th>Dog</th>
<th>Cycle Day</th>
<th>Plasma Iron</th>
<th>$T_{1/2}$ (min)</th>
<th>Turnover (mg/100 ml blood/day)</th>
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<tbody>
<tr>
<td>G-1</td>
<td>4</td>
<td>158</td>
<td>90</td>
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<tr>
<td></td>
<td>9</td>
<td>241</td>
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<td>5.2</td>
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<tr>
<td>G-2</td>
<td>3</td>
<td>188</td>
<td>184</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>223</td>
<td>50</td>
<td>5.6</td>
</tr>
<tr>
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<td>0</td>
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<td>123</td>
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<td>444</td>
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<td>2.1</td>
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<td>N-3</td>
<td>0</td>
<td>382</td>
<td>104</td>
<td>1.9</td>
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Table 1. Plasma Iron Kinetics in Grey (G) and Normal (N) Collies
Fig. 4. Time course of labeled neutrophils in peripheral blood after injection of \(^{3}\text{H-TdR}\) during the apogee of the neutrophil count. Curves were derived from the moving average for ten consecutive hourly points.

curves (i.e., from 52 to 64 hr and from 55 to 67 hr after \(^{3}\text{H-TdR}\)) were 0.98 and 0.97, respectively, and the difference between the slopes was significant \((p < 0.01)\). There was a similarly rapid increase of unlabeled as well as labeled neutrophils at the onset of recovery with a progressive deceleration to an asymptote, which was reached first for the labeled cells, presumably because of label dilution with successive mitoses.

In order to evaluate the cumulative production of cells during the cycle, daily production was estimated by correcting the blood count on a given day for residual cells based on a neutrophil T\(_{1/2}\) of 6 hr and an assumed mean reticulocyte time of 30 hr. As shown in Fig. 6, the cumulative distribution of cell production is sigmoidal for the grey collies, whereas it is linear for the normal collies with an identical slope (correlation coefficient, 0.99) for neutrophils and reticulocytes. The sigmoidal curve delineates the oscillation from low to high cell production rates. Since there is an obligatory time delay for maturation, oscillation of the peripheral blood elements must reflect a similar oscillation occurring a few days earlier in marrow.

Significantly, periodicity of cell production does not seem to be a general phenomenon. The stimulated hair follicle responds in an equivalent fashion
Fig. 5. Time course of labeled neutrophils in peripheral blood after injection of $^3$H-TdR during the nadir of the neutrophil count. Curves were determined as in Fig. 4.

Fig. 6. Cumulative production estimates for neutrophils and reticulocytes in cyclic collies and normal litter mates.
Table 2. Proliferative Activity in Regenerating Hair Follicles of Grey (G) and Normal (N) Collies

<table>
<thead>
<tr>
<th>Dog</th>
<th>Cycle Day</th>
<th>Hair Follicle Mitotic Zone</th>
<th>³H-TdR Labeling Index</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Plucking</td>
<td>Assay</td>
<td>No. Cells</td>
</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G-1</td>
<td>2</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10</td>
<td>74</td>
</tr>
<tr>
<td>G-2</td>
<td>4</td>
<td>8</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>G-3</td>
<td>5</td>
<td>10</td>
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<td></td>
<td>11</td>
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<td>80</td>
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<td>G-4</td>
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</tr>
<tr>
<td>N-2</td>
<td>0</td>
<td>4</td>
<td>77</td>
</tr>
</tbody>
</table>

whether hair plucking and subsequent assay of proliferative activity occur early or late in the hematopoietic cycle. The data, summarized in Table 2, failed to disclose a difference in either the ³H-TdR labeling index or the number of cells in the mitotic zone as a function of cycle stage.

DISCUSSION

The concept that human cyclic neutropenia mirrors cyclic changes in bone marrow, which was put forth some years ago, receives strong support from recent work in the grey collie. It was thought from the human experience that erythropoiesis was not affected, and hence that alteration of the marrow differential was due mainly to fluctuation of myeloid elements. But it is now known that this assumption is untenable for the grey collie. The changes in plasma iron turnover reported here, along with the fluctuating reticulocyte count, affirm our earlier suggestion of erythropoietic involvement. An oscillatory behavior has also been reported for platelets, monocytes, and even lymphocytes, our preliminary data, however, failed to disclose a blood lymphocyte periodicity. Apropos the likelihood of a generalized oscillation of proliferative activity in the grey collie, it is noteworthy that the hair follicle response to plucking is independent of the stage of the hematopoietic cycle.

The ³H-TdR labeling curves for blood neutrophils are particularly informative. It is of interest that the earlier-than-normal appearance time of labeled cells occurs when the marrow pool of mature neutrophils is thought to be depressed, as determined by the differential count or by functional assay with a bacterial endotoxin. The minimum transit time of about 55 hr for appearance of labeled circulating neutrophils corresponds closely to the mean time from the midpoint of myelocyte DNA synthesis to the formation of a mature marrow neutrophil. It seems, therefore, that the blood level has little effect on the kinetics of metamyelocyte and band cell development, a conclusion that was also evident from our previous work in irradiated animals. The normal blood neutrophil T₁/₂ of 6 hr provides further evidence that the severe neutopenic phase is not caused by an exaggerated rate of loss from circulation.
other hand, the difference in initial slopes of the two labeling curves is suggestive of a twofold increase in neutrophil production rate from day 6 to day 11. Thus, the composite picture is one of an ebb and flow of production, and this is indeed reflected in the sigmoidal cumulative production curve in Fig. 6.

Turning now to the possible mechanism of the hematopoietic periodicity, it is necessary to inquire whether the phenomenon reflects the inherent lability of a physiologic regulatory process, a regulatory defect, or a stem-cell–marrow imbalance. A satisfactory explanation must account for the characteristic cycle time, for the phase difference in the cycling pattern of neutrophils relative to reticulocytes, and for the essentially normal total production from one cycle to another.

Morley et al. have proposed that the blood-forming system may have a natural oscillatory tendency because of an obligatory time delay in the response of a regulatory feedback loop from periphery to stem cell. Theoretically, this tendency would be less obvious for neutrophils than for reticulocytes because of the damping effect of another control circuit from periphery to marrow neutrophil reserve. Neutrophil oscillation apparently does not occur in normal persons. A reticulocyte cycle with a 2-wk period has been noted in six of 11 normal dogs, but cycling of these blood elements has not been detected in other work or in the present study, where normal collies showed a remarkably constant production of both reticulocytes and neutrophils over an interval equivalent to the cycle time for grey collies. It is possible, as has been suggested, that the failure to detect reticulocyte oscillation in normal dogs reflects a very low amplitude or a borderline tendency between stability and oscillation.

Several lines of evidence indicate that neutrophil cycling in the grey collie does not result from a peripheral mechanism of cell removal or simply from a failure of the damping effect of the marrow reserve. Although it would seem, therefore, that the predisposing mechanism is central rather than peripheral, peripheral feedback mechanisms could, nevertheless, play an essential role in the periodicity. In general, the hematopoietic behavior of the grey collie is consistent with an intermittent failure of production originating at the pluripotential stem-cell–marrow interface. This follows from the cyclic variation of more than one blood element and from the absence of an obvious defect of proliferation or maturation among recognizable marrow elements.

An important feature of the ebb and flow of production is the nearly 180° phase difference of blood neutrophils and reticulocytes, and it is difficult to account for this solely in terms of differences in the respective marrow maturation times, as was inferred by Dale et al. A peripherally determined competition for a limiting number of pluripotential stem cells may provide the simplest unifying mechanism. A functional limitation of stem cells could be due, for example, to a greatly restricted pool of pluripotential elements or to an exaggerated inhibition of the flow of these cells into committed stem-cell compartments. Competition for a restricted pool of pluripotential stem cells has been demonstrated in mice during recovery from x-irradiation. Moreover, competition may exist even in a presumably normal situation, since it is known that selective changes in hematopoietic activity often occur at the expense of the formation of other blood elements during the response to severe specific
stresses. Regular cycling, however, would be unlikely in these cases because of the acute nature of the competing demands and/or the eventual restoration or compensatory response of the stem cell pool. Because of the exceedingly brief time span of blood neutrophils relative to other blood cell types, activation and deactivation of a neutrophil feedback loop from periphery to committed stem cell could provide the alternating competitive pressure and determine the characteristic 12-day periodicity of the grey collie. The greatly increased recruitment of committed stem cells with the rapidly declining blood neutrophil count would divert pluripotential stem cells for a few days from, say, the erythroid pathway, and thus set the scene for the transient depression or turning off of erythropoiesis. The process would be reversed with a rising neutrophil count, and the available pluripotential stem cells would then be diverted from granulopoiesis to erythropoiesis because of the diminished erythroid stem cell pool. The presumed 6-day transit time from stem cell to neutrophil release is consistent with the requisite time delay.

It would be of interest to know whether periodicity can be detected in the W/W mouse with its congenitally smaller-than-normal stem-cell pool and in animals whose stem-cell pools are continuously depressed by daily low-dose irradiation. Neutrophil cycling has been produced in dogs by repeated administration of cyclophosphamide. Although cycling in this instance was apparently associated with the absence of a marrow neutrophil reserve as a result of the sustained moderate marrow depression, the loss of a damping effect may reflect a reduced stem cell pool.

If there is competition for a limiting number of pluripotential cells, one might expect that imposition of a specific hemic stress would selectively modify the peripheral response of the grey collie. However, the evidence thus far is inconclusive. Neither hypertransfusion nor a single phlebotomy seems to alter the underlying periodicity; neutrophil cycling continues under these circumstances in the face of a transient disappearance or elevation of blood reticulocytes. But this is perhaps not unexpected. An acute moderate stimulation of erythropoiesis or its temporary suppression need not alter pluripotential stem-cell requirements sufficiently to overcome a cyclic process driven by a predominant peripheral neutrophil feedback loop. There is suggestive evidence that the phase of neutrophil oscillation can be reversed by an appropriately timed single injection of antineutrophil serum in a cyclophosphamide-treated normal dog. Moreover, our preliminary data reveal that daily administration of endotoxin over a period of several weeks not only depresses the amplitude of neutrophil oscillation in the grey collie but may even tend to eliminate the cyclic behavior. Reticulocyte cycling also appears to be muted with continuous endotoxin treatment, but the mean reticulocyte level is not depressed, contrary to what would be expected if there were a limiting number of stem cells. It is possible, however, that a more sustained endotoxin treatment is necessary to accomplish this.

Although the hematopoietic periodicity of the grey collie can be understood in terms of competitive pressures for a limiting number of pluripotential stem cells, it must be emphasized that direct evidence is not yet at hand and that other factors could be involved. Among these are the possibilities of the cycling of specific marrow stimulators or inhibitors and changes in operating character-
istics of various feedback controls. There are reports that the levels of a presumptive neutrophil regulating factor and erythropoietin change during the cycle. The former is consistent with the postulate of an alternating peripheral feedback acting on a limiting stem-cell pool. The significance of the latter is not obvious, since a fluctuating erythropoietin level could reflect changes in utilization as well as in production. Although there is some depression of erythrocyte mass in the grey collie, the level is fairly stable during a cycle and should not lead pere to a changing erythropoietin production. It is conceivable that the over-all cyclic hematopoiesis in the grey collie reflects a biochemical anomaly leading to cyclic production of erythropoietin and of neutrophil and platelet humoral stimulators. But this does not seem likely in view of the identical periodicity and the phase differences of the several blood elements.

Whatever the ultimate mechanism, it is necessary to account for the normal neutrophil and reticulocyte production integrated over the entire cycle. An important contributing factor here appears to be a local regulatory mechanism whereby proliferative activity is geared to marrow cellularity. We found that this production rate control has an exponential form such that proliferative activity is increased by a factor of 3 or more when marrow cellularity is 5% - 10% of normal. Thus, when the stem-cell flux into one or another hemic cell line is resumed, each stem cell should give rise to a larger progeny of mature cells. This greater-than-normal stem cell amplification could compensate for a reduced total stem-cell input during the cycle.

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