Hematopoietic Stem Cell Regulation.
I. Acute Effects of Hypoxic-Hypoxia on CFU Kinetics

By Martin J. Murphy, Jr., and Brian I. Lord

This paper, the first of a series on the regulation of the hematopoietic stem cell, examines the effects of stem cell kinetics during an acute exposure to hypoxic-hypoxia. This physiologic perturbation was chosen because of the well-established role it plays in influencing erythropoiesis. BDF₁ male mice were exposed to a simulated altitude of 22,000 ft for periods of time ranging from 3 to 48 hr. The following parameters were assessed: packed red cell volume, number of nucleated cells per femur and spleen, relative and total number of colony-forming units (CFU, i.e., stem cells) in the femoral shaft and whole spleen, as well as the turnover of this population of cells using the "³H-thymidine-killing" technique. The spleen responds by releasing more than one-half (i.e., approximately 10⁸ cells) of its prehypoxic complement of nucleated cells. On the other hand, the femoral cellularity did not decrease but showed a moderate increase. Within 12 hr of hypoxia exposure the total femoral CFU had markedly risen, in some cases between 50% and 75% above control levels. There were, nevertheless, great fluctuations over a short period of time. The population turnover kinetics revealed that after 3 hr of hypoxia an average of 25% of the CFU in both marrow and spleen were in the DNA synthetic phase. It is clear that stem cells are capable of responding rapidly to hypoxia. The possible regulatory mechanisms are discussed in terms of what is known about the kinetics of erythropoietin generation.

THE PHYSIOLOGIC REGULATION of blood cell production revolves about the influence of external stimuli and inhibitors on the undifferentiated blood cells and their progeny. In normal steady-state hematopoiesis, the pluripotential compartment of cells is characterized by its ability to self-perpetuate and its propensity to differentiate into appropriate numbers of mature and, hence, utile elements of the blood. Perturbation of this orderly and highly complex sequence of events, whether exogenously induced by experimentation or endogenously by naturally occurring mutation or disease, is the basis of experimental and pathologic hematology.

Confining our attention to erythropoiesis, we find that the oxygen supply to the body tissues is the most potent regulator of red cell production.¹ The humoral mediator in question is erythropoietin, which induces the differentiation of erythropoietin-responsive cells to enter the erythroid cell series.¹⁻⁴

The purpose of the present report is to describe the effect that acute hypoxic-
hypoxia has on the number of hematopoietic stem cells and to advance some details about their cellular kinetics.

MATERIALS AND METHODS

BDF$_1$ (C$_{57}$ x DBA$_2$) male mice, 9–12 wk of age, were used throughout. At frequent intervals throughout these studies, groups of four control donor mice were assayed to obtain normal data for all the parameters measured.

Hypoxia

Those mice serving as hematopoietic donors were maintained in an altitude chamber at a reduced atmospheric pressure of 320 mm Hg (i.e., 0.42 atmospheres or a simulated altitude of 22,000 ft) for intervals of 3–48 hr. During the regime the animals were supplied with food and water ad libitum. Groups of four donor mice were used per assay point.

Irradiation

The recipient assay mice were irradiated with a Siemens x-ray machine operating at 300 kVp, 12 mA, HVL 2.0 mm Cu. The total dose of 800 rads (30 rad/min) represents a LD$_{100}$/10 and reduces endogenous colony formation to less than 0.2 colonies/spleen.

Cell Suspensions

The animals were killed within 15 min after having been returned to normal atmospheric pressure (i.e., 1 atmos), and their femoral bone marrow and spleen cell suspensions were prepared in ice-cold Fischer's medium, according to the techniques of Schofield and Cole. The cell concentration, determined by hemocytometric counting, was adjusted to render countable spleen colonies in recipient assay mice at 9 days. Bone marrow cells, 3.3 X 10$^5$, (giving about ten colonies per spleen in controls) and 5 X 10$^5$ spleen cells (giving about nine colonies per spleen) were inoculated. The suspended cells were kept for no more than ½ hr at ice-bath temperature before incubation and were injected within an hour after incubation.

Incubation

To assess the turnover state of the stem cell pool (i.e., the per cent of the cells undergoing DNA synthesis), the "3H-thymidine-killing" technique was modified as follows. Two aliquots of the cell suspension under test, containing 5 X 10$^6$ cells/ml, were prewarmed for 10 min in ventilated 5-ml bijou bottles at 37°C in a constantly shaking water bath. One of the paired samples then received 200 µCi/ml of cell suspension of high specific activity (about 25 Ci/mM) tritiated thymidine (1 mCi/ml) in Fischer's medium. The paired control received the same volume of Fischer's medium. After 30 min of incubation the samples were suitably diluted with ice-cold Fischer's medium and injected via the lateral tail vein into groups of not less than ten mice. They were housed, two or three to a cage, and had free access to food and water.

Spleen Colony Counting

Nine days after inoculation the recipients were killed; their spleens were removed, freed of fatty adventitia, and placed flat on filter paper in a plastic Petri dish containing Bouin's solution. After at least 24 hr of fixation, the spleen colonies were counted using a stereoscopic dissecting microscope at X 10 magnification. Tritiated thymidine killing was calculated by the difference in colony yield from the paired samples of cell suspensions.

RESULTS

Figure 1 shows the over-all cellular response of femoral marrow and spleen to acute hypoxia. Most notable is the consistent decline in the numbers of splenic nucleated elements. Within 24 hr of hypoxic exposure, the spleen lost
more than one-half of its nucleated cells (i.e., about $10^8$ cells) and on gross examination was reduced in size. In contrast to this decline in splenic cellularity, the femoral marrow cellularity did not decrease, rather there was evidence of a slight increase at 6 and 12 hr of hypoxia.

The concentrations of colony-forming units (CFU) in femoral marrow and spleen during hypoxia are given in Fig. 2. Neither the spleen nor the marrow experienced a decline in CFU concentration; in fact, the concentration in the femur remained substantially unchanged while that in the spleen increased significantly.

Converting this data to the absolute numbers of CFU in femur and spleen, the dynamics of this population become more obvious (Fig. 3). Whereas the spleen was more prominent in terms of cellularity changes (Fig. 1), the femoral marrow shows the greater response to acute hypoxia in terms of its total content of CFU (Fig. 3). During the first 12 hr of lowered barometric pressure

Fig. 1. Effects of hypoxia on nucleated cellularity in femur and spleen. Each point represents the mean of four femora (black circles) and four spleens (open circles). Control, nonhypoxic values are hatched areas off the ordinate.

Fig. 2. Effects of hypoxia on concentration of CFU in femur (black circles) and spleen (open circles), each with standard error of mean. Control, nonhypoxic values are hatched areas off the ordinate.

Fig. 3. Effects of hypoxia on absolute numbers of CFU in femur (black circles) and spleen (open circles), each with standard error of mean. Control, nonhypoxic values are hatched areas off the ordinate.
there was an increase in CFU; this was observed as early as 3 hr. The definition of the CFU changes during this period is not clear, since rapid fluctuation of this nature (i.e., small time differences in exposure to hypoxia, in sampling after removal from the altitude chamber, etc.) can make significant differences to the CFU content measured. However, it is clear that a rapid increase in CFU content has taken place during these short exposures to hypoxia. From 12 to 24 hr the femoral CFU returned to control levels and were again elevated at 48 hr.

The splenic CFU showed a marginal increase at 3 and 6 hr of hypoxia, but this was short lived and returned to control levels by 12 hr. They remained at or just below control levels for the rest of the observations.

The percentage of CFU killed by $^3$H-thymidine, a measure of the percentage of CFU in DNA synthesis, is given in Fig. 4. Arbitrarily setting 20% as a discriminatory line and ascribing "marked kill" for 20% or over and "moderate kill" to everything below 20%, we can assess cell cycle activity during acute hypoxia. Two peaks of activity were noted.

An average of 25% of both marrow and splenic CFU were in S phase at 3 hr of hypoxia. The next marked killing of stem cells appeared in splenic CFU after 18 hr. At all other times of observation the percentage of CFU in DNA synthesis was either indistinguishable from controls, or the comparison between individual experiments yielded a spread of points that made an analysis futile.

**DISCUSSION**

The acute application of a physiologic stress on an organism may afford insight into the animal's sensitivity and manner of responsivity to the stimulus. The experiments just described demonstrate that the BDFi mouse responds on a cellular level within 3 hr of an acute hypoxic stress.

The decrease in spleen cellularity was equivalent to losing about $7.4 \times 10^6$ nucleated cells/hr during the first 6 hr of hypoxia (Fig. 1). The rate of loss during the subsequent 12-hr period slowed to about $3.5 \times 10^6$ nucleated cells/hr. By 18 hr of hypoxia, the spleen had lost approximately $5 \times 10^7$ nucleated cells. This cellular attrition gradually continued over the following 30 hr and reached a nadir at $70 \times 10^6$ nucleated cells/spleen.

Contrariwise, the marrow experienced no sudden depopulation and, if anything, showed an increase in nucleated cells during the first 12 hr of hypoxic exposure (Fig. 1).

This dramatic cellular release is, however, not a random one, for certain cells, notably stem cells (CFU), decreased neither in relative concentration (Fig. 2) nor in absolute numbers (Fig. 3). The increase in the concentration of CFU in the spleen can be ascribed to the relative decline in the other nucleated cells. This argument is not applicable in attempting to explain the increased concentration and number of CFU in the femur. During the first 12 hr of hypoxia, when relative and absolute numbers of marrow CFU were enhanced, the nucleated cell content was not diminished and, if anything, had increased.
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There are at least two explanations that could account for this: either CFU of nonfemoral origin migrating via the blood settled in the marrow, or the endogenous femoral CFU population was triggered to undergo increased proliferation, thus resulting in the elevated numbers of femoral CFU.

The use of the $^3$H-thymidine-killing technique (Fig. 4) is supportive of both of these propositions. At 3 hr after the initiation of hypoxic stimulation there was marked killing of femoral CFU, indicative of stem cell proliferation. This is further supported by the fact that control suspensions of femoral marrow (i.e., not incubated with $^3$H-thymidine) contained elevated numbers of CFU. The suggestion of increased proliferation of the femoral CFU population seems more tenable, however, because if migration from spleen to femur were the cause, one would expect a fall of CFU in the spleen. The reverse is the case.

The possibility that splenic CFU triggered after 3 hr of hypoxia are responsible for the increase in the endogenous splenic stem cell pool can neither be affirmed nor negated due to the kinetic complexity introduced by the rapidly decreasing nucleated cellularity of the spleen. The fact remains, however, that CFU turnover is accelerated at 3 hr and again at 18 hr, and the proclivity with which stem cells migrate from one hematopoietic tissue to another may also contribute to the increase in femoral CFU.$^{9,10}$

The sequence of events whereby hypoxia stimulates stem cells remains unclear. Two obvious possibilities are (1) that hypoxia directly effects stem cells, or (2) that the stress of lowered barometric pressure stimulates mediating humoral factor(s) that in turn influence CFU kinetics.

In a series of thoughtful investigations, Gordon et al.$^{11-13}$ have studied the appearance and control of one well-known humoral factor, erythropoietin. They have shown that plasma erythropoietin levels increase from just detectable levels (i.e., 0.05 IU/ml) at 1 hr of hypoxia to a peak at 8–10 hr yielding 0.9 IU/ml. There then follows a decline in the circulating titer to approximately 0.05 IU/ml at 16 hr of hypoxia.$^{13}$ Camiscoli and Gordon$^{14}$ conclude that the fluctuation in erythropoietin levels during hypoxia is due to variation in the production and/or availability of the hormone’s substrate. They deduce this

Fig. 4. Effects of hypoxia on turnover of CFU populations in femur (black circles) and spleen (open circles) determined by percentage of CFU killed after 30-min incubation with $^3$H-thymidine. Standard error of means are given.
from studies on hypobaric-induced hypoxic-hypoxia and hemorrhagic-induced hypoxia.\textsuperscript{15} They propose: "This mechanism apparently operates through an ability of the ESF (erythropoietin) to decrease the production and/or the activity of plasma substrate."\textsuperscript{16}

The actual role that erythropoietin plays in stem cell regulation is not clear.\textsuperscript{23} Exogenously administered erythropoietin to both normal\textsuperscript{17,18} and exhypoxic polycythemic mice\textsuperscript{19,20} results in an increase in CFU. Furthermore, the endogenous generation of erythropoietin by hypoxic exposure results in increased numbers of CFU, as demonstrated by this present report and by others.\textsuperscript{21,22} Guzman and Lajtha\textsuperscript{23} demonstrated that administration of 10 U of erythropoietin to hypertransfused plethoric mice resulted in an increase in the turnover rate of both femoral and splenic CFU and an absolute increase in the numbers of CFU in the femur but not in the spleen. All of these studies agree with Fogh’s statement "... that an increase in erythropoietic activity is associated with an increase in the total number of stem cell ..."\textsuperscript{24} These current studies show that femoral marrow, during acute hypoxia, responds with increased stem cells in cycle, an absolute increase in CFU numbers, and possibly by recruitment of splenic CFU.

It is of interest to note that acute hypoxia results in a plasma titer peak of erythropoietin that coincides with an increase in the numbers of splenic and femoral CFU in cycle and precedes by about 6 hr the peak in the absolute number of femoral CFU. While such information may not be causal, it warrants further and serious consideration.

ACKNOWLEDGMENT

We gladly and gratefully acknowledge the excellent technical assistance of Mrs. S. M. Battrick and the thoughtful discussions contributed by Dr. Raymond Schofield. One of us (MJM) would like to thank Professor L. G. Lajtha for his invitation to conduct these studies in his laboratories.

REFERENCES

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