Hematopoietic Stem Cell Regulation.
II. Chronic Effects of Hypoxic-Hypoxia on CFU Kinetics

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Hypobaric-hypoxia, an established stimulator of erythropoiesis, was used to perturb normal steady-state hematopoiesis and thereby to facilitate an analysis of stem cell kinetics. BDF1 male mice were exposed to a simulated altitude of 22,000 ft for 1–15 days. The following parameters were assessed at daily intervals: packed red cell volumes (PCV), femoral and splenic nucleated cellularity, the relative concentration 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. By 10 days of hypoxia the PCV had risen to 65% and by a fortnight was elevated to, and stabilized at, 70%–75%. After an evanescent increase, the femoral cellularity became slightly hypocellular but returned to normal by 3 days, thence becoming hypercellular from days 5 to 15. The spleen was markedly depleted of cells for 48 hr and recovered to hypercellular levels between 4 and 6 days. It then oscillated from hypercellular to normal levels with a period interval of 5 days. Both femoral and splenic CFU demonstrated oscillations, as did the estimations made on the state of CFU turnover. The femoral and splenic CFU did not decrease in number during the 15 days of hypoxia; indeed, they both revealed cyclic increases. These results are discussed in terms of CFU kinetics and humoral mediation.

HOMEOSTASIS as enunciated by Claude Bernard,1 or what more currently has become known as steady-state physiology, comprises the intricate interactions between a stimulative demand and a responsive supply. The interrelationships of stimulation and its physiologic response is enmeshed in a network of feedback loops, in which the product of a stimulus dampens the rate of requisition of the product, hence "negative feedback."

The hematopoietic system is particularly complex in this regard. It is complex because the stimulatory demands impinging on it affect the widely divergent formed elements of the blood. Since these cells are regarded as the progeny of a pluripotent hematopoietic stem cell, it is a reasonable assumption that all such physiologic demands will ultimately have an effect on this progenitor pool of cells. The temporal sequence of events from the distal stimulation to the proximal stem cell stimulation necessitates an analysis of cell population kinetics.

In a previous report, we have examined the acute effects that hypoxic

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erythropoietic stimulus has on stem cell kinetics. The purpose of this presentation is to report the effects that chronic hypoxic exposure has on the kinetics of the hematopoietic precursor cell.

MATERIALS AND METHODS

The details of all procedures are exactly those already described. Briefly, BDF1 (C57Bl \( \times \) DBA2) F1 male mice, 9–12 wk of age were used throughout. They were exposed to a reduced pressure of 0.42 atmospheres (i.e., a simulated altitude of 22,000 ft), and in groups of four, their splenic and femoral marrow cellularities were pooled for assay. The hypoxic environment was continuous, except that every 48 hr the animals were returned to ambient pressure (i.e., 1 atmosphere) where they remained for about an hour during which time food and water was replenished and fresh bedding was supplied.

Groups of not less than ten assay mice were irradiated with 800 rad x-rays (300 kVp, 12 mA, HVL of 2.0 mm Cu, dose rate of 30 rads/min), were injected intravenously with the test cell suspension, and were killed 9 days later when the spleens were removed for colony counting. Stem cells (colony-forming units, CFU) were assessed by their ability to form colonies on the spleens of irradiated mice.

Blood was collected from the retroorbital sinus with a heparinized 75 mm microcapillary tube (Clay-Adams, New York) and the tube was heat sealed; after centrifugation at 12,000 rpm for 3 min, the per cent packed red cell volume was measured.

The "\( ^{3} \)H-thymidine-killing" technique was used to assess the state of turnover in the stem cell (CFU) population. Two aliquots of cells, suspended in Fischer’s medium at a concentration of \( 5 \times 10^{6} \) ml, were incubated at 37°C for 30 min, either in the presence of 200 \( \mu \)Ci/ml high specific activity (e.g., approximately 25 CI/mM) tritiated thymidine or in its absence. Statistical analyses were carried out using standard analyses of variance techniques.

RESULTS

Figure 1 demonstrates that BDF1 male mice respond to chronic hypoxia by increasing their packed red blood cell volume (PCV). It should be noted that after 10 days of exposure the hematocrit readings were above 65%, and by a fortnight of hypoxia, they had attained a level of between 70% and 75% where they became stabilized.

Figures 2 and 3 depict the response of the total nucleated cellularity in femur and spleen to prolonged hypoxia. The abrupt increase (i.e., about 50%) in femoral cellularity (Fig. 2) was short lived and returned to normal or slightly hypocellular levels by 2–3 days. Thereafter, the total femoral cellularity gradually increased to a hypercellular level by 6–7 days, where it remained for the duration of the observations.

The changes in splenic cellularity (Fig. 3) were more pronounced than in femoral cellularity. In contrast to the femur’s ephemeral cellular increase, the spleen decreased its cellularity by more than 50% during the first 48 hr of hypoxia. An equally rapid recovery resulted in a hypercellular spleen by day 4. There was evidence of some oscillation, since the cellularity returned to normal levels at 7–8 days only to become hypercellular again on day 10. A periodicity of about 5 days is suggested. It should be noted that the nucleated cell numbers in the hypercellular spleens are a minimum estimation. This is so because the grossly enlarged spleens become difficult to handle. The spongy texture of the parenchyma and delicate nature of the capsule made it quite impossible to extract all of the cells.
Fig. 1. Effect of hypoxia on packed red cell volume.

Figures 4 and 5 represent the total number of CFU in femur and spleen as a function of the duration of hypoxia. In the femur (Fig. 4), there was neither a pronounced nor a sustained decline in femoral CFU; in fact on four occasions their numbers were enhanced. There was some indication of oscillation in the numbers of stem cells.

The spleen followed the same pattern as the femur (Fig. 5). Aside from a moderate decrease (i.e., about 33%) in CFU during the first 48 hr, the number of splenic CFU did not decrease, and, if anything, were elevated over the 15-day period of examination. Furthermore, there was again evidence for oscillation in numbers of splenic CFU.

Supportive of both of these observations regarding the absolute numbers of splenic and femoral CFU undergoing oscillatory elevations is evidence accrued from an analysis of the CFU population turnover (Fig. 6). Due to the limitation imposed by the technique on the results, we can justifiably regard them only in relative terms. Hence, we arbitrarily regard a killing of 20% or more of the total CFU as “marked killing” and anything less than 20% as “moderate killing.” Using this demarcation, the spleen and especially the femur display dramatic shifts in their cell cycle dynamics.
Fig. 2. Effect of hypoxia on nucleated cellularity of femur. Control, nonhypoxic levels are given by the symbol on the ordinate with its standard error.

Fig. 3. Effect of hypoxia on nucleated cellularity of spleen. Solid line intimates general trend. Control, nonhypoxic levels are given by the symbol on the ordinate with its standard error.
Fig. 4. Effect of hypoxia on absolute numbers of CFU in femoral shaft. Solid line intimates general trend, while broken line denotes oscillatory pattern. Control, nonhypoxia levels are given on the ordinate. Each point is presented with its standard error.

After two fleeting accelerations of splenic CFU proliferation occurring during the first 48 hr, the population turnover rate of CFU remained markedly elevated (Fig. 6).

The femoral CFU kinetics are more varied (Fig. 6). There are multiple accelerations in the turnover rate of femoral CFU and again the possibility of oscillatory mechanics is apparent.

Fig. 5. Effect of hypoxia on absolute numbers of CFU in the whole spleen. Solid line intimates general trend, while broken line denotes oscillatory pattern. Control, non-hypoxic levels are given on the ordinate. Each point is presented with its standard error.
Fig. 6. Effect of hypoxia on turnover of CFU population in femur and spleen determined by percentage of CFU killed after 30-min incubation with ³H-thymidine. Control, nonhypoxic levels are given on the ordinate. Each point is presented with its standard error.
DISCUSSION

It is clear that BDF\(_1\) mice respond to severe and prolonged hypoxia by elevating the numbers of circulating erythrocytes (i.e., PCV) and at the same time by increasing the size and turnover rate of the pluripotent progenitor compartment of CFU. Inherent within these increases, there appear to be oscillations in CFU turnover rate and in the size of this cell population.

These observations are at some variance with those already reported in the literature. Bruce and McCulloch\(^7\) exposed C\(_{57}\)Bl and (C\(_{3}\)H X C\(_{57}\)Bl) \(_1\) mice to an atmosphere of 10.5 volumes/100 ml oxygen. After 10 days of exposure, the PCV increased to about 56% during which time approximately 90% of splenic CFU were lost. During the same period of hypoxic exposure, BDF\(_1\) mice elevated their PCV to an average of 65%, and their complement of splenic CFU remained equal to or above nonhypoxic controls. Schooley et al.\(^8\) exposed C\(_{3}\)H female mice to 0.5 atmospheres of hypoxia for 5 days, and noted an increase in PCV to only 51.2% and a concomitant decrease in splenic CFU to 42% of nonhypoxic controls. Contrariwise, after 5 days at 0.4 atmospheres, BDF\(_1\) males increase their PCV to about 59% while there was a 62% increase in splenic CFU. It appears, therefore, that strains of mice that are capable of maintaining their splenic CFU number during prolonged exposure to hypoxia are also capable of a significant elevation in the numbers of circulating erythrocytes. On the other hand, strains that are unable to maintain their splenic CFU number cannot maintain a sufficiently high rate of erythropoiesis to sustain an increasing PCV of circulating erythrocytes.

The work of McDonald and Lang,\(^9\) Nohr,\(^10\) Kubanek et al.,\(^11,12\) and Shaduck et al.\(^13,14\) indicates that certain mouse strains (e.g., BALB/c and CAF\(_1\)) have impaired responsivity to hypoxic stimulation, i.e., the production of erythropoietin during a hypoxic situation is suboptimal. Therefore, the mice are unable to elevate their hematocrit level to compensate properly for the reduced oxygen availability. They are, nevertheless, not only capable of a normal response to exogenously administered erythropoietin\(^9,11,13\) but also are eminently capable of responding normally and of endogenously producing significantly elevated levels of erythropoietin in response to anemia, post-hemorrhagic or hemolytic.\(^12,13\) The nature of this differential response to anemia vs. hypoxic-hypoxia remains unresolved but clearly is a fundamental one and of undoubted importance in fully understanding the regulatory mechanics of erythropoietin production and response.

These currently reported studies support the proposition of Kubanek et al.\(^11,12\) that ascribes the decline of splenic CFU in certain mouse strains to the toxic effects of hypoxia caused by their inability to generate sufficient levels of circulating erythropoietin.

The toxicology of hypoxia has unfortunately received little contemporary attention. Talbot, reporting in 1936 on an Andean expedition, noted a maximum erythropoietic response at an altitude of 5340 m (17,500 ft).\(^15\) When the climbers ascended to 6140 m (20,000 ft), however, erythropoiesis was inhibited. Stohlman et al.,\(^16\) describing a patient with polycythemia secondary to regional hypoxia, noted a suppression of erythropoiesis in iliac crest marrow
that was locally hypoxic. They suggested that this regional hypoxia "...was severe enough so that the red cell precursors could not respond to the primary humoral stimulus." Furthermore, Stohlman and Brecher, investigating the effect of various levels of hypobaric hypoxia on rat erythropoiesis, concluded that "...the diminished erythropoietic response at higher altitudes is the consequence of the inability of the marrow to respond to the erythropoietic stimulus rather than a decreased production of erythropoietine [sic]." More recently, Schooley et al. exposed mice to a simulated altitude of 18,000 ft for 5 days with daily injections of antierthropoietin. In so doing, they reduced the reticulocyte and PVC levels and concomitantly depressed splenic CFU by about 80% of nonhypoxic controls. This can also be interpreted as another example of severe hypoxia acting at the level of the stem cell.

In addition to validating the above-stated hypothesis, our studies on chronic hypoxia offer support to the thesis put forward by Morley et al., "...that negative feedback loops control erythropoiesis, granulopoiesis, and thrombopoiesis," and extend this to the control of the size and turnover rate of the stem cell compartment itself.

Normal homeostasis shows evidence of oscillation in the numbers of neutrophils and platelets in normal men and in erythropoiesis in normal male dogs. This oscillatory phenomenon is also evident when perturbation of the steady state is introduced. Cyclic neutropenia was induced by feeding dogs cyclophosphamide, and the injection of an incompatible red cell isoantibody in rabbits experimentally produced not only hemolytic anemia but also oscillation of erythropoiesis, as does sublethal irradiation. Furthermore, clinical dyscrasias, such as chronic granulocytic leukemia and polycythemia vera, also appear to be cyclic in nature.

Information on the controlling mechanisms of these cellular responses is far from adequate. Nevertheless, the kinetics of erythropoietin production in the stressed state may afford some insight. Cyclical oscillation in plasma levels of erythropoietin have been reported by a number of investigators. Camiscoli's work is seminal to our findings of oscillation in CFU numbers, as well as in their population turnover rate. The increased rate in the turnover of CFU after 3 days of hypoxia is preceded by a peak in the plasma titer of erythropoietin. The next peak of erythropoietin occurs at 132 hr of hypoxia. Plotting the midpoints of each slope, one finds a lag of about 20 hr before splenic CFU are triggered into increased turnover, and this is followed about 26 hr later by an increase in the mean of the total splenic CFU. The interval between mean elevation of plasma erythropoietin and mean splenic CFU increase is, therefore, approximately 48 hr. The same analysis carried out on femoral CFU kinetics is not as simple, because the total CFU seem to plateau between 144 and 168 hr of hypoxia. Nevertheless, the mean increase in erythropoietin precedes by about 72 hr the mean increase in femoral CFU. It is tempting to ascribe the 24-hr plateau to cell migration, shunting of erythropoiesis to spleen, or some other factor(s), for subtracting that period results in the precise time parameters as evidenced by the spleen.

One should not place too much emphasis on these comparisons simply
because they are comparative and not necessarily causal. At the same time one perhaps ought not to regard them as irrelevant coincidence.

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