The Effect of Erythropoietic Stimulation on the Hemopoietic Colony-forming Cells of Mice

By W. R. Bruce and E. A. McCulloch

It is generally assumed (see for example, ref. 1) that the continuing supply of mammalian blood cells depends on the perpetuation of one or more primitive progenitor cell types from which differentiated cells are derived. These primitive progenitor cells may be assumed to have the capacity to give rise to at least one type of differentiated descendant, and, in addition, to perpetuate their own kind by producing new undifferentiated cells. However, study of primitive progenitor cells has proved difficult, for their unequivocal morphologic recognition has not been achieved. Thus, functional tests based on the recognition of the descendants of the primitive progenitor cells must be employed, and, as these tests require that progenitor cells divide, the cells under test are necessarily lost in the process of their detection. For example, Jacobson, and Gurney and their collaborators3,4 have developed a test for erythropoietic progenitor cells based on the response of these cells to the hormone erythropoietin. Following injection of erythropoietin into plethoric mice, an increase is measured in the number of cells capable of incorporating iron into hemoglobin, or in the number of reticulocytes in the peripheral blood. These iron-incorporating cells and reticulocytes are relatively differentiated, and their number may be used to infer the activity of ancestor cells triggered into the production of differentiated descendants by the action of erythropoietin.

Another approach to the problem of studying progenitor cells has been provided by the observation that normal mouse hemopoietic tissue contains a class of cells capable of undergoing a sufficient number of divisions to give rise to macroscopic colonies in the spleens of irradiated mice.5,6 These colonies, which have been shown by direct cytologic means7 to be derived from single cells, are usually found to contain recognizable differentiating cells, and often erythrocytic, granulocytic and megakaryocytic cells are found in the same colony.8 In addition, at least some of the colonies have been shown to contain cells which are themselves capable of giving rise to spleen colonies,9 that is, they are capable of self-perpetuation. Thus, colony-forming cells possess two fundamental properties of progenitor cells: the ability to give rise to differentiated descendants and the capacity for self-perpetuation.

If capacity to form colonies is to be considered as a criterion for the recognition of progenitor cells, then cells must lose this capacity upon undergoing differentiation. This hypothesis might be tested by applying a differentiating pressure to a population of cells containing colony-forming cells. Since many
of the cells present in colonies may be recognized as erythroblasts, an erythropoietic stimulant is an appropriate tool to use in such a test of the hypothesis. Jacobson et al., Alpen et al., and Erslev have suggested that erythropoietin acts on the stem cells of the erythropoietic series, causing their differentiation. If colony-forming cells respond to the hormone in this way, the response might be detected as a loss of colony-forming capacity. Such a result would imply the existence of a close relationship between colony-forming cells and the stem cells of the erythroid series.

In the experiments to be described, hypoxia was used as an erythropoietic stimulant. Reduced oxygen tension has been shown to stimulate erythropoiesis by causing increased production of erythropoietin. By using endogenous erythropoietin produced in this way, an intense and prolonged stimulation could be achieved under physiologic conditions. Therefore, the number of colony-forming cells in the spleen and marrow of control mice and mice exposed to hypoxia for varying lengths of time was measured. The results were compared with the effect of hypoxia on the number of circulating reticulocytes and red cells, and the uptake of radioiron by suspensions of cells from marrow and spleen. The results of these experiments indicated that erythropoietic stimulation does indeed result in a marked decrease in colony-forming cells, although this decrease was observed only in spleen and not in femoral marrow. However, the kinetics of the changes observed are not consistent with the view that colony-forming cells are identical with erythropoietin-sensitive cells.

**Materials and Methods**

(a) **Mice:** Eight to 12 week old C57BL/Ha and (C3Hf/Ha X C57BL/Ha)Fl mice bred at this laboratory and C57BL/J bred commercially (Jackson Memorial Laboratory, Bar Harbour, Me.) were used in these experiments. Experimental groups used for the hematologic studies and as donors in the preparation of bone marrow and spleen suspensions consisted of 5 to 10 animals of one sex. The groups used as recipients for the examination of colonies were 30 animals of either sex and the same strain as the donor. Unirradiated animals were housed 5 animals to the cage; irradiated animals were housed 2 to 3 per cage. All were allowed food (Rockland Mills mouse diet) and water as desired. As the results were found not to depend on the source or strain of mice used, these will not be designated separately.

(b) **Hypoxia:** Mice to be exposed to an hypoxic environment were placed in lucite and plywood boxes 30 x 85 x 85 cm. An air and nitrogen mixture was introduced into each box at four openings at one side and removed by a symmetrical collecting system at the opposite side of the box. The flow rate was such as to replace the volume of the container every 3 hours. Flow from the compressed air source and water-pumped nitrogen tank were controlled by flow regulators (Model 1350, Brook Instrument of Canada, Scarborough) to yield an atmosphere of 10.5 ± 0.5 per cent oxygen. Oxygen levels were checked closely with a commercial pyrogallol absorption oxygen analyzer. Control animals were placed in a similar box loaded with an equivalent number of mice and with an equivalent controlled flow rate of air (21 per cent oxygen). The cages of all animals were changed every 2nd day to ensure that degradation products of the excreta would not accumulate in the atmosphere.

(c) **Preparation of bone marrow and spleen suspensions:** Marrow cells were obtained from the femora of the mice, suspended in CMRL 1066 and the nucleated cells counted.
in a hemocytometer as previously described. Spleen cell suspensions were prepared as follows: Spleens were minced finely in CMRL 1066 and then gently aspirated up and down 10 to 20 times in a 5 ml. plastic syringe. Coarse clumps were allowed to settle, and the cell suspension pipetted off. Microscopic examination of the cell suspensions obtained showed predominantly single cells with very few visible clumps of cells. The suspensions were diluted with CMRL 1066 to achieve the concentration required for injections or incubations. All suspensions were kept at ice water temperature prior to use. Each suspension was obtained from the femora and spleens of at least 10 mice.

(d) Iron incorporation in vivo: The experimental animals were injected intravenously with 0.5 µc. of Fe59 of high specific activity (25 mc./mg.) present as ferric chloride. At intervals following the injection, the animals were sacrificed by cervical fracture and the femora, spleens and blood samples were obtained. Each blood sample was diluted in a 0.15 sI solution of phosphate-buffered saline (PBS) and was then centrifuged at 1000 rpm for 10 minutes to yield two fractions—the supernatant serum sample and the sedimented cellular fraction. The cellular fraction and the intact spleens and femora were counted in an automatic scintillation counter (model C-120 Nuclear Chicago, Chicago, Ill.) and the activities reported as per cent of the injected activity.

(e) Iron incorporation in vitro: A protein bound solution of radioactive iron was prepared by adding Fe59 as ferric chloride to normal horse serum at a concentration of 1 µg./ml. This did not saturate the iron binding capacity of the horse serum. One and five-tenths ml. of this solution was added to 15 ml. of a bone marrow or spleen cell suspension containing 10⁶ to 10⁷ cells/ml. The resulting suspension was pipetted into a number of 5 ml. plastic test tubes and incubated in a water bath at 37 C. The cells were fixed by adding 20 per cent (V/V) of 10 per cent (V/V) formalin solution, centrifuged and washed twice with PBS. The radioactivity of the cells was then measured using a scintillation counter. Samples were incubated in triplicate, three samples being fixed immediately after the addition of the isotope and three after 1 hour of incubation. The results were expressed as the difference between the activity of the incubated sample and the activity of the unincubated sample.

(f) Irradiation procedure: Mice were irradiated in groups of 30 in a 30 cm. diameter circular Lucite container, which was rotated in the x-ray beam at a distance of 60 cm. from the target. Either 280 kvp x-rays having an HVL of 1.12 mm. of Cu, at an exposure dose rate of 100 r/min., or 200 kvp x-rays having a HVL of 1 mm. Cu. at an exposure dose-rate of 50 r/min., were used. The radiation dosages used were sufficiently high to reduce the average number of endogenous colonies in the spleen of the recipient mice to below 0.5 colonies per spleen. Total body irradiation of the mice with gamma rays was carried out in a rotating Lucite holder with a 5 mm. Lucite top at a distance of 40 cm. from a Co60 gamma ray source. A Victoreen condenser ionization chamber calibrated by the U. S. National Bureau of Standards was used to determine the exposure dose. The absorbed dose rate was approximately 50 rads per minute.

(g) Assay for colony-forming cells: The assay for colony-forming cells depends on the enumeration of macroscopic colonies in the spleens of irradiated mice. Colonies may be obtained either by transplanting cell suspensions containing colony-forming cells into heavily irradiated isologous mice (transplantation method) or by permitting endogenous colony-forming cells to form colonies in the spleens of sublethally irradiated mice (endogenous colony method). These methods may be described briefly as follows: for the transplantation method an appropriate number of nucleated cells is injected intravenously into heavily irradiated (900-1000 r) recipient mice. After 10 days, survivors are killed, and their spleens fixed in Bouin’s solution. This fixation makes the colonies stand out as round or oval yellow nodules, which are easy to count. The results are expressed as average number of colonies produced per 10⁵ nucleated cells injected (CFU ratio). The endogenous colony method depends on the observation that the spleens of mice surviving 800-800 rads of gamma rays contain colonies, and a negative exponential relationship is obtained between the average number of colonies per spleen and the dose of radiation.
given. The slope of this negative exponential is very similar to the slope of the exponential portion of the survival curve obtained for colony-forming cells irradiated in vivo and assayed by the transplantation method. The endogenous colony method may be used to study changes in the number of colony-forming cells in the spleen by comparing the number of spleen colonies that are observed following the same dose of radiation administered to control and experimental groups of mice. In the present experiments, mice were exposed to hypoxia for varying lengths of time, and then irradiated with either 650 or 700 rads of gamma rays. Eleven days later their spleens were examined, and the number of colonies determined.

The transplantation technic and the endogenous colony technic complement each other. Using the transplantation method, precise data over a wide range of numbers of colony-forming cells may be obtained. However, the method has the disadvantage that cell suspensions must be prepared, and artefacts may be introduced in the preparation of these suspensions. The endogenous colony method yields less precise data, and countable colonies may only be observed over a narrow range of radiation dose. However, no manipulation of the cells is required, and artefacts resulted from handling are avoided.

(h) Hematology: Blood samples of animals exposed to hypoxia were examined for erythrocyte concentration and for percentage of reticulocytes. A sample from each animal was diluted in PBS and counted by means of both the hemocytometer and the electronic cell counter (Coulter cell counter). The counts obtained by the two methods seldom differed significantly and the values given were obtained by the latter method. Blood samples were also stained with brilliant cresyl violet and examined for reticulocytes. The results are expressed as percentage of red blood cells, and are based on the examination of 1000 cells.

Tissue for histologic examination was fixed in Bouin’s solution, sections prepared and stained with hematoxylin and eosin.

RESULTS

As our experimental plan depended on the observation that exposure of animals to hypoxia results in an increased production of erythroid elements by the hematopoietic system, it was necessary to measure erythropoiesis in order to be sure that the expected increase in fact occurred. Observations were made on the histologic appearance of marrow and spleen following hypoxia, the numbers of red cells and reticulocytes in the peripheral blood and the radioiron incorporation by hematopoietic cells in vitro and in vivo. The results of these experiments will be described first.

Histologic Examination

Examination of the sections, prepared from spleens and femora of mice exposed to hypoxia for varying lengths of time, showed that very little recognizable erythropoiesis was present in the spleens of normal mice, but following 4–6 days of hypoxia, numerous collections of erythroblasts could be seen. Later, the erythroid activity receded. Normal femoral marrow contains numerous erythroblasts, and no qualitative change was observed in the marrow of animals exposed to hypoxia. The histologic observations suggest that mice exposed to hypoxia rapidly increase the degree of erythropoiesis occurring in their spleens.

Effects of Hypoxia on Numbers of Reticulocytes and Red Cells in the Blood

Twelve groups, each consisting of five C57BL mice, were exposed to hypoxia (10.5 per cent oxygen) for varying lengths of time. At the end of
exposure, the animals were killed, and the concentration of reticulocytes and red cells in their blood determined. Controls consisted of animals not exposed to hypoxia, and animals kept for 28 days in a box similar to the one used to produce hypoxia but flushed with room air. The results of the experiment are shown in figure 1. In this figure, per cent reticulocytes and the red cell counts are plotted as a function of time of exposure to hypoxia. From the figure it is clear that a reticulocyte peak was observed at 4 days, and that by 8 days the percentage of reticulocytes had returned to normal. During the first 4–6 days of exposure the red cell count rose very sharply from $8.2 \times 10^8$ cells/ml. to $10.2 \times 10^8$ cells/ml., and these increased levels of red cells were maintained throughout the rest of the observation period. No increase in reticulocytes or red cells was observed in animals exposed only to air. These observations are similar to those of others, and indicate that the maximum change in erythrocyte production in animals exposed to hypoxia occurs in the first 4–6 days of exposure.

**Radionor Incorporation In Vivo**

The changes observed in the peripheral blood of animals exposed to hypoxia should be preceded by changes in the rate of red cell formation in the spleen and bone marrow. A quantitative technic for measuring such changes was therefore desirable. Many investigators have used the incorporation of parenterally injected radioiron for this purpose. The suitability of this method for the study of hypoxic animals was tested in the following experiment.

Eighty C57BL mice were exposed to hypoxia for 4 days, and an equivalent number of mice were kept as controls. At the end of the exposure period, all animals were injected intravenously with Fe$^{59}$. Thereafter, they were kept in a normal atmosphere, and at intervals over the next 48 hours, groups of animals were killed, and the radioactivity present in spleen, femoral marrow and washed peripheral red cells was determined. The results are shown in figure 2, in which the percentage incorporation into the various organs is shown as a function of time after administration of the isotope. Figure 2 (left) represents the control results, and figure 2 (right) the results obtained from animals that had been exposed to hypoxia for 4 days.

The results (fig. 2) reveal a great difference in the iron incorporation between normal animals and animals exposed to 4 days of hypoxia. The difference was most apparent in the spleen. While the iron incorporation of the normal spleen was similar to that of the femur, iron incorporation in the spleen of the hypoxic mouse was more than 6 times that of femur measured 1 hour after the injection. The radioactivity of the spleen then decreased over the following 2 days as iron migrated out of the organ. The activity of the peripheral red cells of untreated animals increased over a period of 12 hours following the injection of iron, but in hypoxic animals it increased more rapidly over a period of only a few hours. Though similar results might have been expected for femoral marrow, this was not the case. As may be seen from the figure, very little Fe$^{59}$ was incorporated into femoral marrow, and no increase in incorporation was observed in the animals exposed to hypoxia.
Fig. 1.—The effect of hypoxia (10.5 per cent oxygen) on the per cent of reticulocytes and erythrocyte concentration of mice. Ninety-five per cent confidence intervals are indicated.

Two conclusions may be drawn from this experiment. First, exposure to hypoxia results in a pronounced increase in the incorporation of radioiron into mouse spleen, but not into mouse marrow. Second, exposure to hypoxia results in a marked change in the kinetics of iron incorporation into spleen and into peripheral erythrocytes. In the control animals, iron incorporation into the spleen reached a peak at 4 hours, and then very gradually fell off. In contrast, in the hypoxic animals, the peak incorporation in spleen was found when the first measurement was made at 1 hour, and subsequent measurements showed a rather rapid decrease in radioactivity. Changes also occurred in the iron incorporated in peripheral red cells. These observations indicated that the kinetics of iron uptake in control and hypoxic animals was different, and that a single measurement of the incorporation of Fe⁵⁹ in vivo did not provide a reliable index of erythropoiesis when only a single time following the injection of radioiron was used. This difficulty could be avoided by measuring Fe⁵⁹ incorporation by cell suspensions incubated in vitro.

Iron Incorporation in Vitro

Spleen cell suspensions exposed to Fe⁵⁹ for from 0 to 4 hours incorporate activity as shown in figure 3. Iron becomes bound to the cells immediately; thereafter the incorporation rate is constant for a period of several hours. This result suggested that a meaningful incorporation rate might be obtained by taking the difference between the activity of a sample after 1 hour of incubation and the activity of a similar sample immediately following the
addition of iron. This measure of incorporation rate was further examined by measuring the incorporation rate of spleen cell suspensions with from 0.5 x 10^7 to 3.5 x 10^7 cells/ml. The result for four such experiments (fig. 4) shows a linear relation between iron incorporation and cell number. Iron incorporation in vitro may therefore be used as a measure of erythropoietic activity following exposure to hypoxia.

The effect of hypoxia on erythropoietic activity was assayed as follows: Groups of mice were exposed to hypoxia for from 0 to 24 days. They were then sacrificed and pools of bone marrow and spleen cells were prepared from animals of each group. The in vitro iron incorporation for each sample was determined and is expressed in units of counts per minute (cpm) incorporated per hour per spleen or per two femora.

The results are given in figure 5, in which cpm/two femora or cpm/spleen are plotted against time of exposure to hypoxia. It is clear from the figure that a seven- to eight-fold increase in iron incorporation occurred in the spleen cells after 3 days of hypoxia. Thereafter the incorporation decreased gradually, until normal values were approached after 10 days. This peak of iron incorporation is very similar to the reticulocyte peak shown in figure 1, except that the maximum value for iron incorporation was reached 1 day earlier than the reticulocyte peak. Iron incorporation by marrow cells also showed an increase, although the increase was much less than that observed for suspensions of spleen cells. The increased iron uptake by marrow cells was observed by the 2nd day, and had not fallen to normal by the 10th day. The definite increase in Fe^59 uptake of marrow cells observed by the in vitro method was not detected by the in vivo method described earlier, presumably because the latter method is less sensitive.

**Measurement of Colony-forming Units in Hypoxic Mice**

The experiments described above confirmed the expectation that exposure of mice to an atmosphere of 10.5 volumes per cent oxygen would produce a
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marked increase in erythropoiesis. Measurements were made, therefore, of the content of colony-forming units in the spleens and marrows of these animals, after varying exposures to hypoxia. Both the transplantation method and the endogenous colony method were used.

Results of Transplantation Experiments

Groups of 10 animals were exposed to hypoxia for varying lengths of time. After exposure they were killed, and cell suspensions were prepared from their spleens and femoral marrow. The number of colony-forming units in these suspensions was then measured as previously described. Results obtained in five separate experiments are given in table 1 and presented graphically in figure 6. The table and figure show that a marked decrease was observed in the number of colony-forming units in the spleens of hypoxic mice. This decrease occurred over a period of 10 to 15 days of hypoxia, and appeared to occur most rapidly after the 4th day. After the 15th day, little subsequent decrease was noted, and the content of colony-forming units in the spleens of hypoxic animals remained at or about 10 per cent of the control value until the end of the experiments at day 28. The number of colony-forming units did not change in the spleens of mice exposed to normal atmosphere.

Fig. 3.—Radioiron incorporation of $5 \times 10^7$ spleen cells incubated at 37 C. in CMRL 1066, 10 per cent normal horse serum and Fe$^{3+}$ as ferric chloride.
Fig. 4.—Radioiron incorporation of spleen cells incubated for 1 hour with Fe$^{59}$ in vitro as a function of cell number. The four symbols are for the results of separate experiments in which different cell suspensions were used.

under the same conditions as animals exposed to hypoxia. In contrast with the marked change observed in the spleen, no change was found in the number of colony-forming units in the marrow of mice exposed to hypoxia for 10 days (fig. 6).

Results of Assay for Endogenous Colonies

We have shown that a drop in the number of CFU observed per spleen occurs by 10 days after animals are exposed to an hypoxic environment. This is presumably due to a decrease in the number of colony-forming cells in this organ. It may, however, be only a reflection of differences by which spleen-cell suspensions may be prepared at that time. A procedure in which the spleen cells are not subjected to any manipulation would meet this objection. Such a procedure is afforded by the endogenous colony technic.

Before using this method it was necessary to show that the number of colonies observed by this method was not a function of changes in the environmental conditions in the irradiated animals. This was done as follows: Three groups of 20 animals not otherwise treated were irradiated to a whole body absorbed dose of 700 rads. They were then placed in atmospheres of 80 per cent oxygen, air (21 per cent oxygen) and 10.5 per cent oxygen. The results are given in table 2. The mean numbers of colonies in the spleens of
Fig. 5.—Radioiron incorporation of spleen and femur cells incubated for 1 hour with Fe\(^{59}\) in vitro as a function of the duration of exposure of the donor animals to hypoxia. The ordinates give the incorporation for the number of cells recovered from a donor spleen or from two donor femora. Ninety-five per cent confidence limits are indicated.

Each group, when sacrificed 11 days later, were not greatly different and there was no significant effect observed when the animals were exposed to a higher oxygen atmosphere following the irradiation than they had been exposed to before. The use of the spontaneous colony technic to examine changes in the number of cells capable of giving rise to colonies in the spleens of animals exposed to hypoxia was therefore justified.

In the next experiments, animals exposed to hypoxia for from 0 to 28 days were irradiated (700 rads). After 11 days at normal atmosphere, the animals were killed and their spleens examined. The mean number of colonies observed for two such experiments is shown in figure 7. The results also showed a decrease in spleen colony-forming cells following exposure to hypoxia.

**Discussion**

The cells which give rise to macroscopic colonies in the spleens of irradiated mice may be considered to be one (but not necessarily the only one) of the progenitor cells of hematopoietic tissue, for these colony-forming cells have the capacity to produce differentiated descendants\(^8\) and to perpetuate their own cell type.\(^9\) The experiments presented in this paper were designed to test the hypothesis that an increased demand for differentiated cells would reduce the supply of progenitor cells and thus result in a loss of cells with colony-forming ability. The method of increasing production of differentiated cells employed was exposure of mice to hypoxia, for lowered oxygen tension is known to increase production of erythrocytes. The use of hypoxia has the additional advantage that it is known that its effect is mediated through the humoral substance erythropoietin.\(^{12}\) and evidence has been presented from several laboratories to suggest that erythropoietin acts by inducing the differentiation of stem cells.\(^{8,4,10,11}\)
Table 1.—Results of Assay for CFU in the Spleens and Femoral Marrow of Mice Exposed to Hypoxia (10.5% Oxygen)

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* Ninety-five per cent confidence interval.

The prediction, based on the hypothesis that increased demand for differentiated cells would decrease the number of colony-forming cells, was fulfilled in part. After 10-day exposure to hypoxia, approximately 90 per cent of the colony-forming cells were lost from the spleen. The loss of colony-forming cells from the spleens of hypoxic mice is consistent with the view that during differentiation, the capacity to form macroscopic colonies is lost. However, no change in the number of colony-forming cells in femoral marrow was observed, and the possible significance of this result will be discussed later.

A further objective of our experiments was to examine the relationship between colony-forming cells and the erythropoietin-sensitive stem cells of the erythroid series. The latter have been studied extensively by Jacobson and his colleagues. These workers have established an assay for stem cells...
Fig. 6.—The number of colony-forming units (CFU) per spleen and femur (assayed by the injection technic) as a function of the duration of exposure of the donor mice to hypoxia. The ordinate is given as a percentage of the number of colony-forming cells in the spleen and femur of unexposed animals. Ninety-five per cent confidence intervals are indicated.

Based on the response of plethoric mice to the administration of erythropoietin. Since this assay depends on response to erythropoietin, an essential characteristic of the cell it measures is sensitivity to erythropoietin. A second characteristic of erythropoietin-sensitive cells may be deduced from the Fe$^{59}$ radioautographic studies of Alpen et al. They showed that the pool of cells capable of incorporating iron was diluted by non-labeled cells more rapidly in dogs stimulated by hemorrhage than in control animals. If the

Table 2.—Comparison of Environmental Conditions on the Development of Endogenous Colonies in the Spleen after 700 Rads Whole Body Irradiation

<table>
<thead>
<tr>
<th>Environment</th>
<th>Colonies per Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>21% oxygen (air)</td>
<td>3.6 ± 2.1*</td>
</tr>
<tr>
<td>10.5% oxygen</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>80% oxygen</td>
<td>3.6 ± 1.4</td>
</tr>
</tbody>
</table>

*Ninety-five per cent confidence interval.
Fig. 7.—The average number of colonies observed per spleen following a dose of 650 or 700 rads (endogenous colony technique) in mice exposed to hypoxia for varying lengths of time. Ninety-five per cent confidence intervals are indicated.

Non-labeled cells appearing in these experiments are derived from erythropoietin-sensitive cells, it follows that erythropoietin-sensitive cells lack the capacity to incorporate iron, although they give rise to descendants that do incorporate iron.

In our studies, changes in the number of cells in spleen and femoral marrow with the capacity to incorporate iron, and changes in the number of reticulocytes in the peripheral blood were measured. These measurements are very similar to those employed to detect the activity of erythropoietin-sensitive cells. The reticulocyte peak, observed at 4 days, with rapid return of the reticulocytes to normal levels is very similar to the reticulocytosis observed by Gurney et al.16 following erythropoietin injection. The increase in Fe-incorporating cells in the spleen, occurring before the reticulocyte peak, may be assumed to represent the appearance in the spleen of progeny of erythropoietin-sensitive cells with iron-incorporating capacity. An increase in iron-incorporating capacity was also observed in cells from femoral mar-
row, but it is interesting to note that this increase was much less than that observed in the spleen, indicating either that the marrow contains fewer erythropoietin-sensitive cells than the spleen, or that these cells are in some way protected from the action of the hormone.

As expected, the changes that mirror the responsiveness of cells to erythropoietin occurred very rapidly after the exposure of the animals to hypoxia. Indeed the sequence of events observed, the initial increase in iron-incorporating cells in spleen and marrow followed by a reticulocyte peak in the peripheral blood, and then the return of all these values to normal levels may be interpreted as a wave of cellular activity occurring following the triggering of a population of sensitive cells. If this is true, then the first event in the series should be a sharp decrease in the number of cells susceptible to erythropoietin. Thus, if the erythropoietin-sensitive stem cell of the erythron is identical with the colony-forming cell, then the first event to be observed after stimulation should be a decrease in the number of colony-forming cells present. However, the experimental results showed that the number of colony-forming cells continued to decrease after the peak activity of descendants of erythropoietin-sensitive cells was found. The minimum value for spleen colony-forming cells was observed after 10–15 days of hypoxia, several days past the peak of reticulocytes and iron-incorporating cells. And in the femoral marrow, where some increase in iron-incorporating cells was observed, no change in the content of colony-forming cells was found. These results appear to us to be incompatible with the view that the colony-forming cell is highly sensitive to erythropoietin, and suggests that colony-forming cells are not identical with the erythropoietin-sensitive stem cells measured by Gurney et al.

As mentioned previously, erythroblasts are found frequently within spleen colonies. The presence of these cells indicates that their progenitor, the erythropoietin-sensitive stem cell of the erythroid series, though unrecognizable by morphologic methods, may also be present within colonies. These considerations suggest that the relationship between colony-forming cells and erythropoietin-sensitive cells may be described as follows: Colony-forming cells are not themselves sensitive to erythropoietin, but erythropoietin-sensitive cells are included among the progeny of colony-forming cells. From this point of view the slow decline in colony-forming cells in the spleens of hypoxic mice may be a consequence of a depletion of the pool of erythropoietin-sensitive cells, with colony-forming cells differentiating in order to restore the pool.

Our results may contribute to the solution of another problem in the control of erythropoiesis. The problem is: how is the stem cell pool preserved under conditions of continuing demand for differentiated cells? A solution to this problem has been proposed by Lajtha, who has suggested that stem cells exist in two physiologic states: in the first state they are inactive and responsive to a differentiating stimulus; in the second state they are triggered into the cell cycle as a result of a feedback mechanism, and while in the cycle are unresponsive to differentiating stimuli. Direct evidence in support
of this model is lacking, and our results suggest two different mechanisms by which the stem cell pool could be protected against excessive depletion. First, the observation that colony-forming cells in the marrow do not decrease during hypoxia suggests that colony-forming cells in certain locations may be less responsive, or receive a smaller stimulation than cells in other sites. Cells in such a protected location would constitute a reservoir from which the pool of progenitor cells could regenerate. Second, we interpret our results as indicating that erythropoietin does not act directly upon colony-forming cells, and that the decrease of their number in the spleens of hypoxic mice is a secondary phenomenon, occurring only when the pool of erythropoietin-sensitive cells is depleted. If this interpretation is correct, erythropoietin-sensitive cells would represent a buffer between the progenitor cells and the effect of stimulation. As long as cell proliferation maintains this buffer, the pool of progenitor cells might be protected from falling to dangerously low levels.

As methods for measuring in functional terms the activity of cells with the characteristics of primitive progenitor cells of the hemopoietic system are devised and improved, the proposed models for the organization of the blood-forming system may be tested. In this way an experimentally verified model may be expected to emerge.

**Summary**

The effect of prolonged hypoxia on the mouse hemopoietic system has been examined in order to ascertain the effect of this intense stimulus on quantitative measures of differentiation and proliferation on the erythropoietic system.

The first change that was observed (2–3 days after hypoxia) was a marked increase in the in vitro iron incorporation of the spleen cells and a less marked increase of that of the marrow cells. Later (at 4 days), there was an increase in peripheral reticulocytes. Following both of these events there was a marked decrease in the number of colony-forming cells in the spleen of the hypoxic mice but no change in the number in the marrow.

We conclude that the primitive erythroid precursor that is sensitive to erythropoietic stimulation is not the colony-forming cell. We suggest, instead, that the late decrease in colony-forming cells in the spleen is a response to a depleted pool of cells sensitive to erythropoietin and that colony-forming cells are differentiated to produce more of these cells.

**Summario in Interlingua**

Le effecto de un prolongate hypoxia super le systema hematopoietic murin esseva examinate pro determinar le effecto de iste intense stimulo del mesuras quantitative de differentiation e proliferation in le systema erythropoietic.

Le prime alteration notate (2 a 3 dies post le hypoxia) esseva un marcate augmento in vitro del incorporation de ferro in le cellulas del splen e un minus marcate tal augmento in le cellulas medullari. Plus tarde (al fin de 4 dies), il occurreva un augmento in le reticulocytos peripheric Post iste occurrentias,
THE EFFECT OF ERYTHROPOIETIC STIMULATION

un marcate augmento esseva notate in le numero del cellulas a formation de colonias in le splen del muses in hypoxia, sed nulle tal augmento esseva notate in le medulla.

Nos conclude que le primitive precursor erythroide que es sensibile pro le stimulation erythropoietic non es le cellula a formation de colonias. Como these alternative nos propone le explication que le tardive declino del numero de cellulas a formation de colonias in le splen es un responsa al depletion in le reservior de cellulas sensible pro erythropoietina e que le cellulas a formation de colonias es differentiate pro producer numeros additional de tal cellulas.

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The Effect of Erythropoietic Stimulation on the Hemopoietic Colony-forming Cells of Mice

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