Role of the spleen in the exaggerated polycythemic response to hypoxia in chronic mountain sickness in rats

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Kam, H. Y., L. C. Ou, C. D. Thron, R. P. Smith, and J. C. Leiter. Role of the spleen in the exaggerated polycythemic response to hypoxia in chronic mountain sickness in rats. J. Appl. Physiol. 87(5): 1901–1908, 1999.—In a rat model of chronic mountain sickness, the excessive polycythemic response to hypoxic exposure is associated with profound splenic erythropoiesis. We studied the uptake and distribution of radioactive iron and red blood cell (RBC) morphology in intact and splenectomized rats over a 30-day hypoxic exposure. Retention of $^{59}$Fe in the plasma was correlated with peak splenic erythropoiesis. We studied the uptake and distribution of radioactive iron and red blood cell (RBC) morphology in intact and splenectomized rats over a 30-day hypoxic exposure. Peak splenic $^{59}$Fe uptake and splenomegaly occurred at the most intense phase of erythropoiesis and coincided with the rapid appearance of $^{59}$Fe-labeled RBCs in the blood. The bone marrow remains the most important erythropoietic organ under both resting and stimulated states, but inordinate splenic erythropoiesis in this rat strain accounts in large measure for the excessive polycythemia during the development of chronic mountain sickness in chronic hypoxia.

interaction of splenic and medullary erythropoiesis; high altitude; splenomegaly; splenectomy; leukocytosis; thrombocytosis; thromboctopenia

WE HAVE CHARACTERIZED a rat model of human chronic mountain sickness (CMS). As in the human disease, the rats manifest excessive polycythemia and hypervolemia (16). In our investigations, we studied two strains of Sprague-Dawley rats: the Hilltop strain is hypoxia sensitive and develops CMS, whereas the Madison strain is relatively resistant to hypoxia and does not develop CMS. Splenic erythropoiesis contributes significantly to the overall polycythemic response to hypoxia in the Hilltop strain (17, 18). In contrast, the polycythemic response to the identical hypoxic conditions is only moderate in the Madison strain, and splenic erythropoiesis plays a minimal role (17).

Splenic erythropoiesis has been well documented in rodents, particularly in mice (5). The prominence of splenic erythropoiesis in adult rodents during erythropoietic stimulation was attributed to the limited marrow space, which might restrict the production of red blood cells (RBCs) (3). Thus splenectomy reduced the polycythemic response after bleeding or led to death when medullary erythropoiesis was chemically ablated (23). On the other hand, a normal and complete polycythemic response to chronic hypoxia was found in splenectomized mice (13) and rats (24), suggesting that splenic erythropoiesis serves only as an accessory function. Hence, there is an intricate relationship between splenic and medullary erythropoiesis that remains to be investigated. The goal of the present study was to determine the relative contributions of splenic and medullary erythropoiesis in the development of the excessive polycythemic response to chronic hypoxia in the CMS-susceptible, Hilltop rat strain. The temporal patterns of splenic and medullary erythropoiesis were followed in intact and splenectomized animals over a period of 30 days of hypoxic exposure by means of radioactive-iron uptake and morphological analyses of the marrow and spleen. The effect of splenectomy and hypoxia on other formed elements of the blood during hypoxic exposure was also examined.

MATERIALS AND METHODS

Radioactive-iron uptake studies. Adult male Sprague-Dawley rats weighing 270–290 g were purchased from the Hilltop Breeding Laboratories (Scottsdale, PA). Six separate groups of rats were exposed to a simulated altitude of 18,000 ft (5,500 m or an inspired PO$_2$ = 73 Torr) for 0, 1, 3, 5, 10, or 30 days in a large environmental chamber (24). Each group of 10 animals consisted of 2 subgroups with equal numbers of intact and splenectomized rats. Splenectomy was performed while the rats were under ether anesthesia. After surgery, the rats were treated with penicillin (100,000 U daily im) for 5 days, although exposure to high altitude began after a 2- to 3-day postsurgical recovery period. In the intact group, one or two animals received sham operations for each time subgroup. No significant effect of the sham operation was observed in any of the measured variables, and the data from animals with and without sham operations were pooled to form the intact subgroup.

After altitude exposure, the animals were given 15 mCi $^{59}$Fe (29 µCi/mg) via the jugular vein under ether anesthesia and returned to room air [sea-level (SL) controls] or to the hypoxic environment (experimental groups). Sixty minutes after isotope injection, the animals were killed while under ether anesthesia by exsanguination via the vena cava. The half-life of $^{59}$Fe incorporation is 40 min in rats (22). A sampling time of 60 min allowed adequate $^{59}$Fe incorporation and was consistent with previous studies. Radioactivity was assayed in tissue samples (−1 g) from spleen, liver, femur, whole blood, and plasma (Beckman Gamma Counter, Irvine, CA). Radioactivity was expressed as counts per minute (cpm) per unit volume in the case of whole blood, plasma, and RBCs. Radioactive counts were consistently >10,000 cpm/sample, and background activity was low. For the organ-uptake data, radioactivity was expressed per whole spleen or femur per 100 g body weight. The narrow distribution of $^{59}$Fe radioactivity has been studied in rats (9), and the ratio of femur $^{59}$Fe
The temporal patterns of the changes in $^{59}$Fe activity of whole blood, plasma and packed RBCs 1 h after $^{59}$Fe injection are shown in Fig. 1. After a latency of 24 h, the radioactivity of the whole blood increased slightly, but significantly, at 72 and 120 h of hypoxia and then returned to normal control values (Fig. 1A). Retention of injected $^{59}$Fe in the plasma dropped precipitously after 72 h of hypoxia and remained low throughout the entire period of exposure (Fig. 1B). The $^{59}$Fe retained in the plasma in intact animals was consistently lower than in splenectomized animals, indicating that removal of the injected $^{59}$Fe was more rapid in the intact group. The $^{59}$Fe uptake of the packed RBCs measured 1 h after injection increased greatly after 72 h of hypoxia, then declined somewhat, but remained significantly elevated above the SL control values (Fig. 1C). More rapid removal of injected $^{59}$Fe from the plasma was associated with a more rapid increase in the RBC $^{59}$Fe in intact animals. In contrast, $^{59}$Fe radioactivity persisted longer in the plasma of the splenectomized rats, and RBC $^{59}$Fe rose more slowly and to a lesser extent.

The net total tissue $^{59}$Fe uptake for the marrow, spleen, and liver are summarized in Fig. 2. The total marrow $^{59}$Fe uptake 1 h after injection in splenectomized animals (Fig. 2A) exceeded total marrow $^{59}$Fe uptake in intact, control animals at SL. Subsequently, there was a dip in $^{59}$Fe uptake at 24 h, after which marrow activity increased ~40% over the SL control values. Furthermore, $^{59}$Fe uptake in splenectomized animals remained significantly higher than in the SL controls even after 30 days of hypoxia. In the intact animals (Fig. 2A), marrow $^{59}$Fe uptake, measured 1 h after injection, increased immediately, reached a maximum uptake (50% increase over the SL control level) by the third day, and then gradually decreased toward the control values. Except for the first 24 h of hypoxia, the
mean marrow $^{59}\text{Fe}$ uptake values were consistently higher in the splenectomized than in the intact animals. Most strikingly, hypoxic exposure greatly stimulated splenic $^{59}\text{Fe}$ uptake in the intact animals (Fig. 2B). The $^{59}\text{Fe}$ uptake increased ~2-fold after 24 h of hypoxia, reached a value 10-fold greater than the SL control level at the end of 72 h, and declined slowly to ~5-fold the control value after 30 days. Under SL conditions, splenic $^{59}\text{Fe}$ uptake was ~10% ($49 \times 10^3$ vs. $545 \times 10^3$ cpm/100 g body wt) of the total marrow uptake, but it increased to ~60% ($500 \times 10^3$ vs. $750 \times 10^3$ cpm/100 g body wt) of the total marrow uptake after hypoxic exposure. There was a hypoxia-related decrease in $^{59}\text{Fe}$ uptake, measured 1 h after injection, in the liver that was greater in the intact rats compared with the splenectomized animals (Fig. 2C).

We assume that the marrow and the spleen represent the primary erythropoietic organs, and $^{59}\text{Fe}$ uptake by these organs relates directly to erythropoietic activity. Hence, the total marrow $^{59}\text{Fe}$ uptake represents the total erythropoietic activity in the splenectomized animals, and the combined $^{59}\text{Fe}$ uptake of the spleen and marrow represents the total erythropoietic activity in the intact animals. The changes in the total erythropoietic activity with time of hypoxic exposure in intact and splenectomized animals are shown in Fig. 3. The temporal patterns of total erythropoietic activity were similar to those of the packed-RBC $^{59}\text{Fe}$ activity on the one hand (Fig. 1C), but mirror images of the $^{59}\text{Fe}$ retained in the plasma on the other hand (Fig. 1B). The uptake and disposition of exogenous $^{59}\text{Fe}$ in plasma are best revealed in Fig. 4. The concentration of injected $^{59}\text{Fe}$ retained in the plasma was inversely related to the total erythropoieticorgan $^{59}\text{Fe}$ uptake (Fig. 4A), but the appearance of the $^{59}\text{Fe}$-labeled RBCs in the blood was linearly related to the total erythropoietic organ $^{59}\text{Fe}$ uptake (Fig. 4B).

In the intact animals (Fig. 5), splenic erythropoietic activity was linearly correlated with the packed-RBC $^{59}\text{Fe}$ activity. The comparable relationship for the medullary component, however, was not linear; a second-order polynomial fit the points significantly better than did a straight line. The initial component of the marrow relationship paralleled the splenic relationship; marrow activity increased as the packed-RBC $^{59}\text{Fe}$ activity increased, but, at the most intense phase of erythropoiesis, the increased $^{59}\text{Fe}$ content of packed RBCs was not reflected by increased $^{59}\text{Fe}$ uptake by the marrow. The splenic erythropoietic activity correlated more closely with the release of young RBCs into the circulation at this time. The two-component marrow
response occurred, however, only in intact animals. Medullary erythropoietic activity in splenectomized animals was linearly correlated with the packed-RBC $^{59}$Fe activity. This observation suggests that spleen was the major erythropoietic organ providing new RBCs during the early phase of intense hypoxia-stimulated erythropoiesis.

Morphological changes in the spleen and marrow during hypoxia. Figure 6 depicts the morphological changes in the spleen as a function of the time of hypoxic exposure. The spleen became significantly hypertrophied after 72 h of hypoxia and remained so for the rest of the exposure period except at the 10th day of hypoxia, when the splenic weight did not differ significantly from the SL control value. Increased numbers of NRBCs in splenic sections demonstrated that splenic erythrocytic activity was also significantly increased after 72 h of hypoxia. This increase was sustained throughout the subsequent course of the hypoxic exposure. The histological evaluation was based on the NRBC population density, and the twofold increase in splenic volume further augmented the total NRBC population.

Figure 7 summarizes the morphological changes in the marrow. On exposure to hypoxia, the NRBC population of both the intact and splenectomized rats increased rapidly and reached maximal and constant levels after 72 h of hypoxia (Fig. 7A). The increase in NRBC populations in both intact and splenectomized rats was more than twice the SL control values. Although the mean increase in the NRBCs was consistently higher in the splenectomized than in the intact animals, the differences were not statistically significant. Total bone marrow cellularity in intact and splenectomized animals followed strikingly different patterns (Fig. 7B). In the intact rats, the total cell counts decreased after 24 h of hypoxia but then increased linearly up to twice the control value. In contrast, the total cell counts in splenectomized animals increased immediately and reached a peak after 5 days of hypoxia. Thereafter, they declined slightly. Thus the total cell counts in the splenectomized animals were higher in the early phase and lower in the later phase of hypoxic exposure compared with intact animals. The mature RBC count in the marrow of intact animals dipped initially (Fig. 7C) but increased steadily to a level significantly above both the SL controls and marrow RBC counts in splenectomized animals at 30 days of exposure. There were no significant changes in the mature RBC counts in splenectomized animals over the period of hypoxic exposure.

Changes in circulating RBCs during hypoxia. The whole blood hemoglobin concentration (Fig. 8A) and the hematocrit (Fig. 8B) increased over the time of hypoxic exposure in both intact and splenectomized rats as expected. In most cases, the mean values for these variables were significantly higher in the intact than in the splenectomized animals. The circulating reticulocyte numbers (Fig. 8C), after a 24-h latency, increased rapidly and reached peak values in both...
groups of animals after 120 h of hypoxia. The reticulocyte count rapidly declined in the intact animals, but the decline was delayed in the splenectomized animals, and reticulocyte counts were significantly higher in splenectomized compared with intact rats after 10 days of hypoxia.

The mean corpuscular volume increased by ~15% after 72 h of hypoxia and remained elevated throughout the entire period of exposure in intact and splenectomized animals. The mean corpuscular hemoglobin content increased along with the increase in cell size so that the mean corpuscular hemoglobin concentration remained unchanged during the hypoxic exposure. The temporal pattern of changes in mean corpuscular volume and mean corpuscular hemoglobin content were similar in the intact and splenectomized rats (data not shown). Electrophoretic analysis of the hemoglobin species in circulating RBC showed no differences between the intact and splenectomized animals.

Changes in other blood cells during hypoxia. As shown in Fig. 9A, hypoxic exposure resulted in differing early responses in marrow white blood cell (WBC) counts between the intact and splenectomized animals. WBC counts decreased in intact rats, but increased in the splenectomized animals. The transient decrease in marrow WBC counts in the intact animals was primarily due to a fall in lymphocytes (data not shown). The increase in marrow WBC in the splenectomized animals, however, was due to increased lymphocytes and polymorphonuclears (data not shown). Both circulating WBC (Fig. 9B) and platelet counts (Fig. 9C) increased during the first few days of hypoxic exposure. Thereafter, WBC counts declined toward, and platelet counts fell below, the SL control values. Splenectomy significantly increased both the circulating WBC and platelet counts under SL normoxic conditions and augmented the hypoxia-induced transient leukocytosis and thrombocytosis.

**DISCUSSION**

Flow of exogenous $^{59}$Fe in rats during hypoxic exposure. The present study demonstrates that $^{59}$Fe was rapidly used by the erythropoietic organs for the synthesis of hemoglobin and returned to the circulation in the form of newly released young RBCs. The evidence for this is that, first, the rapid disappearance of plasma $^{59}$Fe was closely associated with a rapid increase in $^{59}$Fe uptake by the erythropoietic organs (marrow and spleen; Figs. 1, 2, and 4). In fact, the retention of plasma $^{59}$Fe was linearly and inversely related to $^{59}$Fe uptake by the erythropoietic organs (Fig. 4). Second, the appearance of newly formed RBCs in the circulation correlated well with either the total $^{59}$Fe uptake of the erythropoietic organs (bone marrow and spleen) in the intact animals or with the marrow $^{59}$Fe uptake in the splenectomized animals (Fig. 5).

Relative roles of splenic and medullary erythropoiesis in the polycythemia response to hypoxia. Under normoxic conditions the total splenic $^{59}$Fe uptake represents only 10% of the total marrow uptake ($49 \times 10^3$ vs. $550 \times 10^3$ cpm/100 g body wt). Hypoxic exposure increased the splenic uptake by ~10-fold, but increased the medullary uptake by ~50%. The maximum increase in $^{59}$Fe uptake occurred after 3 days of hypoxia in both the spleen and marrow. Because the basal uptake in the marrow was much higher than in the spleen, the absolute $^{59}$Fe uptake remained significantly higher in the marrow than in the spleen during the entire period of hypoxic exposure. Consistent with these ferrokinetic findings, the studies of splenic and marrow morphology showed that nucleated RBC numbers increase mark-
edly at the onset of hypoxic exposure, reached a peak after 3 days of hypoxia, and remained elevated in both the spleen and the bone marrow throughout the remainder of the 30-day hypoxic exposure. Thus the present study demonstrated that hypoxic exposure rapidly and simultaneously stimulated both splenic and medullary erythropoiesis. As expected, the polycythemic response to hypoxia was consistently greater in the intact compared with the splenectomized animals (Fig. 8). As mentioned earlier, Hilltop rats develop CMS, which is characterized by excessive polycythemia and profound splenic erythropoiesis. In contrast, Madison rats are resistant to CMS and develop only moderate polycythemia and minor splenic erythropoiesis (17). These previous observations suggest that the inordinate splenic erythropoiesis seen in the Hilltop rats during chronic hypoxia may account for the excessive polycythemia seen in the Hilltop rat strain; the spleen is necessary as an additional erythropoietic site that facilitates full expression of the polycythemic response to hypoxia. This conclusion is consistent with the notion of a limited marrow space in rodents (3).

Interaction between splenic and medullary erythropoiesis in chronic hypoxia. In the murine model of CMS, both splenic and medullary erythropoiesis were stimulated concomitantly during hypoxic exposure in these rats (as shown by the radioactive-iron uptake and the increased population of NRBCs). There is less evidence...
Augmented $^{59}$Fe uptake by the marrow in splenectomized animals (note the surge in $^{59}$Fe uptake in the marrow; Fig. 2). Uptake was established in the splenectomized animals during hypoxic exposure in the intact animals, whereas counts of mature RBCs in the splenectomized animals remained constant (Fig. 7). Because hypoxic exposure increased the NRBC population comparably in both the intact and splenectomized animals, the gradual accumulation of mature RBCs with time of exposure in the intact animals probably reflects decreased release of mature RBCs with time of exposure in the intact animals. The cause of the accelerated maturation of the precursor cells and the increased release of mature RBCs from the marrow in the absence of the spleen is not known. However, EPO is known to accelerate the maturation and release of the RBCs from the marrow, and one of the characteristic responses of Hilltop rats to hypoxia is inordinate expression of the EPO gene, leading to exaggerated production and sustained high titers of EPO (19). It is conceivable that elevated EPO might influence both the marrow and spleen to stimulate erythropoietic activity. In addition, it has long been known that the spleen can influence various aspects of hematopoiesis, including the erythropoiesis in the marrow (5, 11, 21), and, therefore, some other unknown mechanisms cannot be excluded.

Another characteristic of the polycythemic responses to hypoxia of both the intact and splenectomized animals was a sustained macrocytosis associated with a higher mean corpuscular hemoglobin content after 24 h of hypoxic exposure. Because there was also a sustained reticulocytosis under these conditions in both groups of animals (Fig. 8), and reticulocytes have a greater average diameter than the average circulating RBCs (8, 22), the persistent reticulocytosis may account for the sustained macrocytosis. In addition, reticulocytes released under stress (such as high EPO titers or severe anemia) are considerably larger than ordinary reticulocytes, and these larger reticulocytes may also have greater hemoglobin content (22). Thus the abnormal and persistently elevated level of EPO in the Hilltop strain of rats during hypoxic exposure (17, 19) may contribute to the morphological features of the hematologic responses to hypoxia in this particular rat strain.

The present observations of normal circulating WBC levels and the development of thrombocytopenia after prolonged hypoxia in intact animals corroborated our early findings (17). Splenectomy appeared not to affect this chronic hypoxic effect. The transient leukocytosis and thrombocytosis during the acute phase of the hypoxic exposure are also consistent with observations of splenic erythropoiesis in adult humans with CMS. Nonetheless, the relative contribution of the spleen and marrow to the increased circulating RBC count remains an issue, particularly the mechanism(s) of coordination between these two sites of erythropoiesis. During early hypoxia, the rapid release of young RBCs into the circulation correlated more with the increase in $^{59}$Fe uptake in the spleen than with $^{59}$Fe uptake in the marrow (Figs. 1, 2, and 5). The spleen may be the primary immediate source of new RBCs on exposure to hypoxia. A similar correlation between the rapid appearance of new RBCs in the circulation and medullary $^{59}$Fe uptake was established in the splenectomized animals (note the surge in $^{59}$Fe uptake in the marrow; Fig. 2). Augmented $^{59}$Fe uptake by the marrow in splenectomized animals could result simply from the absence of competition for the available $^{59}$Fe or from an inhibitory influence of the spleen over medullary erythropoiesis. Furthermore, medullary $^{59}$Fe uptake increased steadily during hypoxic exposure in the intact animals, but fell below the control level on the first day of hypoxia in the splenectomized animals (Figs. 2 and 3). The conspicuous fall in medullary $^{59}$Fe uptake in splenectomized animals was associated with a parallel fall in the number of the labeled RBCs in the circulation (Fig. 1). This observation seems to indicate a depletion of some precursor cells that normally take up iron, become mature RBCs, and are released into the circulation in response to erythropoietic stimulation, as was seen in the intact animals. The apparent depletion of this particular stage of the precursor cells in splenectomized animals may be the consequence of accelerated maturation of these precursors in the marrow in the absence of the spleen. Finally, the number of mature RBCs in the marrow, after a slight fall, accumulated during the time of hypoxic exposure in the intact animals, whereas counts of mature RBCs in the splenectomized animals remained constant (Fig. 7). Because hypoxic exposure increased the NRBC population comparably in both the intact and splenectomized animals, the gradual accumulation of mature RBCs with time of exposure in the intact animals probably reflects decreased release of mature RBCs in the presence of the spleen. The cause of the accelerated maturation of the precursor cells and the increased release of mature RBCs from the marrow in the absence of the spleen is not known. However, EPO is known to accelerate both the maturation and release of the RBCs from the marrow, and one of the characteristic responses of Hilltop rats to hypoxia is inordinate expression of the EPO gene, leading to exaggerated production and sustained high titers of EPO (19). It is conceivable that elevated EPO might influence both the marrow and spleen to stimulate erythropoietic activity. In addition, it has long been known that the spleen can influence various aspects of hematopoiesis, including the erythropoiesis in the marrow (5, 11, 21), and, therefore, some other unknown mechanisms cannot be excluded.
in humans and animals (1, 25). The causes of these changes remain unclear. Blood volume contracts during acute hypoxia (25) and expands during chronic hypoxia (16). These blood volume changes could conceivably affect the blood cell counts. The life span of WBC and platelets remains unchanged under hypoxic conditions (1, 14). The transient circulating leukocytosis coexisted with a decrease in WBC population in the marrow, suggesting that increased release of marrow leukocytes contributed to the circulating leukocytosis in intact animals. The rate of thrombocyte production increases in acute hypoxia and decreases during chronic hypoxia (2). Thus it seems likely that the transient early thrombocytosis during hypoxia resulted from increased production, and the thrombocytopenia during chronic hypoxia resulted from depressed platelet production.

Splenectomy augmented both the hypoxia-induced transient leukocytosis and thrombocytosis. Because there was a conspicuous transient rise in the marrow WBC population after splenectomy (Fig. 9), increased production and release are likely involved, at least for the WBC. Splenectomy induces prolonged leukocytosis and thrombocytosis under SL conditions (7, 11, 15), whereas leukocytosis and thrombocytosis were short lived under hypoxic conditions. All blood cells are derived from multipotent stem cells (12). Competition among different cell lines occurs under conditions when a particular cell line is specifically stimulated (2, 10). Thus, when erythropoiesis is stimulated under hypoxic conditions, the common stem cells are directed toward production of RBCs, and fewer stem cells are available to support production of other cell types. This may explain the short-lived leukocytosis and the development of thrombocytopenia during chronic hypoxia after splenectomy.

In summary, radioactive-iron and morphological analyses in both intact and splenectomized rats during 30 days of exposure to hypoxia revealed an important role of splenic erythropoiesis in the excessive polycythemic response in the Hilltop strain of Sprague-Dawley rats. Although exaggerated gene expression and production of EPO is a characteristic response to hypoxia in this rat strain, the capacity for splenic erythropoiesis also appears to be essential, either as an extramedullary site or an extra source of progenitor cells.

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