Hypoxia Increases Erythropoiesis and Decreases Thrombocytopoiesis in Mice: A Comparison of Two Mouse Strains (43253)

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Abstract. **Several previous studies have shown that hypoxia increases erythropoiesis and decreases thrombocytopoiesis in mice. It has been postulated that the thrombocytopenia is caused by stem cell competition between the erythrocytic and megakaryocytic cell lines. In the present work, we compared the effects of severe hypoxia (5.5-6.0% O_2) in both male and female C3H and BALB/c mice by measuring their abilities to produce red blood cells and platelets. All mice had significant increases in packed cell volumes and marked decreases in platelet production after hypoxia; however, there were significant differences in the degree of stimulation in the two mouse strains. After 14 days of hypoxia, the percentage of ^{35}S incorporation into platelets, total circulating platelet counts and total circulating platelet masses were lower in C3H mice than in BALB/c mice, but platelet sizes were larger. Also, hypoxia caused greater changes in male mice than in female mice, with male C3H mice showing the greatest increase in packed cell volumes and the lowest platelet counts of all mice tested. The least responses were observed in female BALB/c mice. BALB/c mice had higher P50 (right-shifted O_2 dissociation curves) and lower erythrocyte 2,3-diphosphoglycerate values than C3H mice, indicating a lower hemoglobin O_2 affinity for BALB/c mice. The results indicate that the effects of hypoxia are not direct upon platelet production, but that the thrombocytopenia is a result of stimulation of erythropoiesis. These data support the stem cell competition hypothesis and illustrate that the degree of the inverse relationship between red blood cells and platelet production of hypoxic mice is dependent, to a large degree, upon the sex and strain of mice that are used.** [P.S.E.B.M. 1991, Vol 197]

Hypoxia has been shown to cause marked thrombocytopenia in laboratory animals (1-8). Although normal platelet survival values have been found (4), the thrombocytopenia has been associated with decreased platelet production (1, 5), reduced megakaryocyte concentration (4, 6-7), a reduction in the number of small acetylcholinesterase-positive cells (3), and a decreased number of colony-forming units megakaryocyte (9). It has been hypothesized that the thrombocytopenia is caused by stem cell competition between the erythrocytic and megakaryocytic cell lines (1, 3, 6, 10), since the degree of thrombocytopenia was inversely related to the level of erythropoiesis (1, 10). In support of this view, previous studies showed that after 14 days of hypoxia, C3H mice have severe thrombocytopenia and significantly elevated packed cell volumes (PCV), whereas BALB/c mice given the same degree of hypoxia and duration did not have increased red blood cell counts or reduced platelet numbers (1). BALB/c mice were thought to have a defect in erythropoietin (Epo) production that prevented them from responding to hypoxia with increased erythropoiesis (11-12). However, in these previous studies, rather mild oxygen levels were utilized (6-8%) and only a few mice were investigated. The possibility exists that more severe hypoxic conditions than those used in the previous study (1) might increase erythropoiesis in these mice. A recent study by Jackson *et al.* (13) showed that male and female C3H mice had different platelet counts and megakaryocyte ploidies. Therefore, the purpose of the present study was to compare platelet production in

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both male and female C3H and BALB/c mice after utilizing lower levels of oxygen (5.5–6%) than were used in the previous study. The results showed significant differences between C3H and BALB/c mice in their abilities to produce platelets and red blood cells. In agreement with previous studies (1), hypoxia had greater effects in C3H mice than in BALB/c mice. Likewise, greater differences were noted in male mice than in female mice. The mice with the greatest degree of thrombocytopenia also had the greatest change in PCV, a finding that supports the stem cell competition hypothesis.

Materials and Methods

Animals. Both male and female C3H/HENHSD (C3H) and BALB/CANNHSD (BALB/c) mice (from Harlan Sprague-Dawley, Indianapolis, IN; 4–5 weeks of age, weighing approximately 15 g each) were used in these experiments. The mice were exposed to hypoxia for 14 days by enclosure in cages covered with dimethylsilicone rubber membranes as described previously (14). After equilibration for approximately 8 hr, the oxygen levels inside the cages were between 5.5 and 6.0%. Since female mice had smaller body sizes than male mice, one additional female mouse was placed in each membrane cage to lower the oxygen levels to the same degree as for male mice. In addition, C3H and BALB/c female mice were exposed to identical O_2 levels by enclosing them in the same cage for the 14-day period. Likewise, both strains of male mice were also kept in the same hypoxic atmosphere. Mice were removed from the hypoxic chamber at 14 days and blood was collected from the retroorbital sinus for platelet counts and PCV determinations.

Additional female C3H and BALB/c mice were purchased from Cumberland View Farms (Clinton, TN) for determination of hemoglobin (Hb), hemoglobin-oxygen affinity (P50), and erythrocyte 2,3-diphosphoglycerate (2,3-DPG) values.

PCV and Platelet Counts. PCV were determined using heparinized microhematocrit capillary tubes and a centrifuge; platelet counts of mice were obtained using direct phase microscopy.

Percentage of ^{35}S (%^{35}S) Incorporation into Platelets and Platelet Sizes. For determination of the 24-hr %^{35}S incorporation into platelets, blood was taken by cardiac puncture after injection of a heparin-sodium pentobarbital solution (15–16). Approximately 0.5 ml of blood was collected into syringes containing 1.0 ml of 3.8% sodium citrate solution, and the diluted blood was expressed into plastic tubes. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 160*g* for 4 min at 22°C. The PRP was capped to minimize pH changes, which have been shown in previous studies to alter platelet sizes.

Platelet size measurements were made using a Particle Data Instrument (Particle Data, Inc., Elmhurst, IL) with a logarithmic scale, as described previously (16). The settings for the instrument were log 10 and current 6. At frequent intervals, calibration was monitored by standardizing the instrument with latex particles of known size (2.02-μm diameter). The PRP was diluted into isotonic-buffered saline to give concentrations in the range of $1–1.2 \times 10^4$ platelets/100 μl of sample in order to reduce the coincidence error to less than 1%.

After platelet sizing, platelets from PRP preparations were washed and the %^{35}S incorporation into platelets measured as described previously (15).

Total Circulating Platelet Counts (TCPC) and Total Circulating Platelet Masses (TCPM). Blood volumes were determined as described previously utilizing the ^{59}Fe-labeled erythrocyte dilution technique (17). Both normal and hypoxic C3H donor mice were injected with ^{59}Fe, and red blood cells (RBC) were collected. After injection of ^{59}Fe-labeled erythrocytes, blood volume was estimated using an isotope dilution technique (17). Values were expressed as milliliter of blood per 100 grams of body weight. A highly significant correlation ($r^2 = 0.99$) was found between PCV and blood volumes of hypoxic mice. Therefore, blood volume was calculated for each hypoxic mouse based upon its PCV. The TCPC and the TCPM were calculated utilizing formulas published previously (17). The TCPC was calculated by multiplying the peripheral platelet count per milliliter of blood by the total blood volume (in milliliters). The TCPM was calculated by multiplying TCPC by the average platelet volume (in cubic micrometers).

Hb, P50, and 2,3-DPG. Hemoglobin values (g/dl) were determined by a standard hemoglobin procedure (DATA Medical Associates Inc., Arlington, TX) using heparinized blood obtained via cardiac puncture. The same blood specimen was utilized for determination of P50 (mm Hg) and 2,3-DPG values. P50 was determined using a HEM-O-SCAN oxygen dissociation analyzer (American Instrument Co., Silver Spring, MD). Values for 2,3-DPG (μM/g Hb) were determined by use of a kit (No. 35-UV) purchased from the Sigma Chemical Co. (St. Louis, MO).

Statistical Analysis. Student's *t* test was used to determine statistical differences between means of these data.

Results

Figure 1 records the results of measuring PCV, platelet counts, %^{35}S incorporation into platelets, and platelet sizes of both male and female C3H and BALB/c mice. As shown, hypoxia caused significant ($P < 0.0005$) increases in the PCV of both male and female C3H and BALB/c mice (see Table I). An unexpected finding was that both untreated and hypoxic C3H

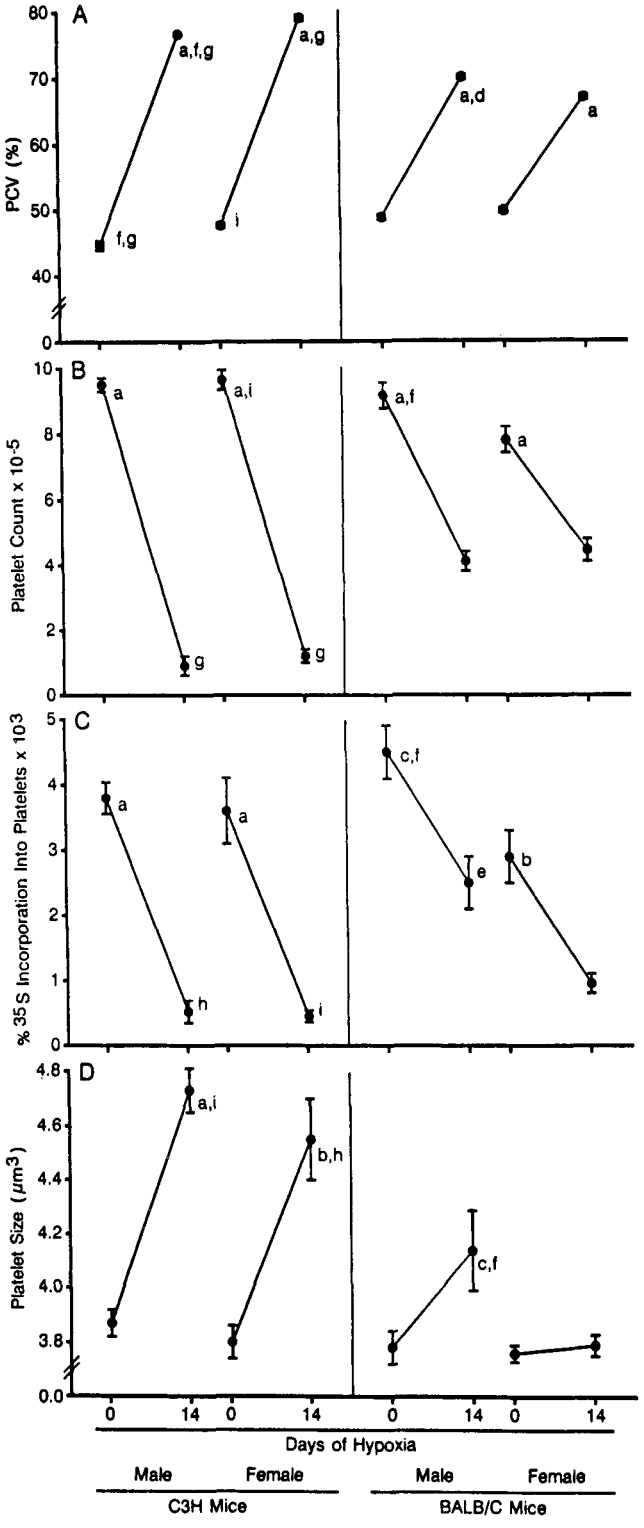

Figure 1. Effects of 14 days of hypoxia on PCV (A), platelet counts (B), $\%^{35}S$ incorporation into platelets (C), and platelet sizes (D) of male and female C3H and BALB/c mice. Each point is the average of five to six mice and the vertical lines represent the SE. Within sexes and strains of mice, values for mice kept at ambient air (21% O_2) were significantly different from mice enclosed in cages covered with silicone-rubber membranes (5.5–6% O_2): a = $P < 0.0005$, b = $P < 0.005$, c = $P < 0.05$; values for male mice were significantly different from female mice: d = $P < 0.0005$, e = $P < 0.005$, f = $P < 0.05$; values for C3H mice were significantly different from values for BALB/c mice: g = $P < 0.0005$, h = $P < 0.005$, i = $P < 0.05$.

female mice had higher PCV than did C3H male mice ($P < 0.05$). However, after 14 days of hypoxia, BALB/c male mice had significantly higher PCV than did BALB/c female mice ($P < 0.0005$). However, PCV of untreated BALB/c male mice and BALB/c female mice were not significantly different. In addition to the differences noted due to the hypoxia and sex of these mice, significant differences were found in PCV due to the strain of mice, i.e., untreated C3H male and female mice had significantly lower PCV ($P < 0.0005$ and $P < 0.05$) than did untreated BALB/c male and female mice. However, C3H male and female mice that had been exposed to hypoxia for 14 days had significantly higher ($P < 0.0005$) PCV than did hypoxic BALB/c male and female mice.

Figure 1B and Table I record the results of platelet counts of the same mice presented in Figure 1A. As shown, hypoxia caused significant decreases in platelet counts of both C3H and BALB/c male and female mice ($P < 0.0005$). However, when comparing sexes of mice, i.e., C3H male versus C3H female mice and BALB/c male versus BALB/c female mice at both 0 and 14 days of hypoxia, there were no significant differences in platelet counts except for a small difference ($P < 0.05$) that was noted between untreated BALB/c male mice and untreated BALB/c female mice. When comparing differences between strains of mice, it was shown that both male and female hypoxic C3H mice had significantly lower ($P < 0.0005$) platelet counts than were found for male and female BALB/c mice also exposed to 14 days of hypoxia. In addition, untreated C3H female mice had significantly higher platelet counts than did untreated BALB/c female mice ($P < 0.05$). However, we found that untreated C3H male mice had platelet counts that were not significantly different from those of untreated BALB/c male mice.

Figure 1C and Table I show the effects of hypoxia on $\%^{35}S$ incorporation into platelets. Hypoxia caused significant decreases in $\%^{35}S$ incorporation into platelets of both male and female C3H mice ($P < 0.0005$). Also hypoxic BALB/c male and female mice had significantly ($P < 0.05$–$P < 0.005$) lower $\%^{35}S$ incorporation values than did untreated BALB/c male and female mice after 14 days of hypoxia. Hypoxic BALB/c male mice had higher $\%^{35}S$ incorporation values ($P < 0.005$) than did BALB/c female mice given the same level of hypoxia. In addition, untreated BALB/c male mice showed greater $\%^{35}S$ incorporation values ($P < 0.05$) than did untreated BALB/c female mice. There were no significant differences in $\%^{35}S$ incorporation values of untreated and hypoxic C3H male mice and untreated and hypoxic C3H female mice. In addition to differences in $\%^{35}S$ incorporation into platelets of mice that were due to the hypoxia and sex of the mouse, it was shown that additional differences existed due to the strain of the mouse. For example, hypoxic C3H male

Table I. Statistical Analysis of Data Presented in Figure 1

	PCV	Platelet counts	Percentage of ^{35}S incorporation into platelets	Platelet size
Hypoxia				
U C3H male versus H C3H male[a]	a[b]	a	a	a
U C3H female versus H C3H female	a	a	a	b[c]
U BALB/c male versus H BALB/c male	a	a	c[d]	c
U BALB/c female versus H BALB/c female	a	a	b	NS[e]
Sex				
U C3H male versus U C3H female	f	NS	NS	NS
H C3H male versus H C3H female	f	NS	NS	NS
U BALB/c male versus U BALB/c female	NS	f	f	NS
H BALB/c male versus H BALB/c female	d	NS	e	f
Strain				
U C3H male versus U BALB/c male	g	NS	NS	NS
U C3H female versus U BALB/c female	i	i	NS	NS
H C3H male versus H BALB/c male	g	g	h	i
H C3H female versus H BALB/c female	g	g	i	h

[a] U, untreated mice; H, hypoxic mice.
[b] a, d, g = $P < 0.0005$.
[c] b, e, h = $P < 0.005$.
[d] c, f, i = $P < 0.05$.
[e] NS, not significantly different.

and female mice had significantly lower $\%^{35}S$ incorporation values than did BALB/c male ($P < 0.005$) and female ($P < 0.05$) mice exposed to the same level of hypoxia. However, both untreated male and female C3H mice did not have significantly different $\%^{35}S$ incorporation values when compared to untreated male and female BALB/c mice.

When comparing platelet sizes of these mice (Fig. 1D), it was shown that hypoxic C3H male and female mice had significantly ($P < 0.005$–$P < 0.0005$) larger platelets than did untreated C3H male and female mice (Table I). Likewise, hypoxic BALB/c male mice had significantly larger platelets than did untreated BALB/c male mice ($P < 0.05$), but platelet sizes of untreated BALB/c female mice were not different from platelet sizes of BALB/c female hypoxic mice. It should also be noted that when comparing platelet sizes of mice, the sex of the mouse only made a difference when comparing hypoxic BALB/c male mice to hypoxic BALB/c female mice ($P < 0.05$). Moreover, hypoxic C3H male and female mice had larger platelets than did hypoxic BALB/c male and female mice ($P < 0.05$–$P < 0.005$). There were no differences in platelet sizes of both normal male and female C3H mice when compared to untreated BALB/c male and female mice.

Figure 2 and Table II show the results of determining TCPC and TCPM (expressed as cubic micrometers) of the same mice presented in Figure 1. The data show that both male and female C3H and BALB/c mice had significantly ($P < 0.005$–$P < 0.0005$) decreased TCPC and TCPM values after exposure to hypoxia. When considering the effects of sex of mice on TCPC and TCPM, it was shown (Fig. 2 and Table II) that untreated C3H male and untreated BALB/c male mice had significantly greater TCPC and TCPM than did C3H and BALB/c female mice ($P < 0.05$–$P < 0.005$). When comparing hypoxic male BALB/c mice to hypoxic BALB/c female mice, no differences were found in TCPC, but TCPM differed significantly ($P < 0.05$). However, hypoxic C3H male mice had TCPC and TCPM that were not significantly different from values of C3H female mice exposed to hypoxia. The effects of strain of mouse showed that untreated C3H male mice had TCPC and TCPM values similar to untreated BALB/c male mice (Fig. 2 and Table II). Moreover, TCPC of untreated C3H female mice did not differ from values of untreated BALB/c female mice; however, TCPM was significantly different ($P < 0.05$). Hypoxic C3H male and female mice had significantly lower TCPC ($P < 0.005$) and TCPM ($P < 0.0005$) than did hypoxic BALB/c male and female mice.

The results of measuring Hb, P50, and 2,3-DPG of female mice of both strains are shown in Table III. Significantly lower ($P < 0.025$) P50 values and higher ($P < 0.05$) 2,3-DPG values were found in C3H mice when compared to values of BALB/c mice. However, Hb values were not different.

Discussion

The present work showed that normally C3H and BALB/c mice have similar platelet production indices. However, in untreated mice, PCV were slightly lower in C3H mice than in BALB/c mice, and untreated female C3H mice had higher platelet counts and TCPM than did untreated female BALB/c mice. Also, untreated female BALB/c mice had higher P50 and lower

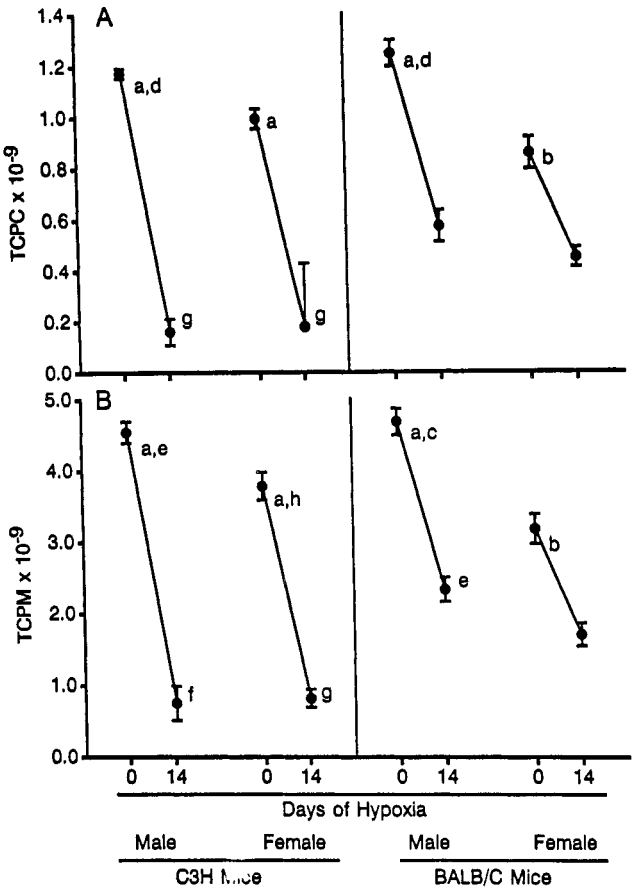

Figure 2. TCPC (A) and TCPM (B), given in cubic micrometers, of mice presented in Figure 1. Vertical lines represent SE. For formulas for calculation of TCPC and TCPM, see Materials and Methods. Within sexes and strains of mice, values for hypoxic mice were significantly lower than untreated control mice: a = $P < 0.0005$, b = $P < 0.005$; values for male mice were significantly greater than for female mice: c = $P < 0.0005$, d = $P < 0.005$, e = $P < 0.05$; and values for C3H mice were significantly different from values of BALB/c mice: f = $P < 0.0005$, g = $P < 0.005$, h = $P < 0.05$.

Table II. Statistical Analysis of Data Presented in Figure 2

	Probability	
	TCPC[a]	TCPM
Hypoxia		
U C3H male versus H C3H male[b]	a[c]	a
U C3H female versus H C3H female	a	a
U BALB/c male versus H BALB/c male	a	a
U BALB/c female versus H BALB/c female	b[d]	b
Sex		
U C3H male versus U C3H female	d	e[e]
H C3H male versus H C3H female	NS[f]	NS
U BALB/c male versus U BALB/c female	d	c
H BALB/c male versus H BALB/c female	NS	e
Strain		
U C3H male versus U BALB/c male	NS	NS
U C3H female versus U BALB/c female	NS	h
H C3H male versus H BALB/c male	g	f
H C3H female versus H BALB/c female	g	g

[a] TCPC, total circulating platelet counts; TCPM, total circulating platelet mass in cubic micrometers.
[b] U, untreated mice; H, hypoxic mice.
[c] a, c, f = $P < 0.0005$.
[d] b, d, g = $P < 0.005$.
[e] e, h = $P < 0.05$.
[f] NS, not significantly different.

Table III. Hemoglobin, Hemoglobin-Oxygen Affinity, and Erythrocyte 2,3-Diphosphoglycerate Values of Female Mice[a]

Strain of mice	Hb (g/dl)	P50 (mm Hg)	2,3-DPG (μM/g Hb)
C3H	11.9 ± 0.3	46.0 ± 0.7[b]	31.06 ± 0.72[c]
BALB/c	12.5 ± 0.3	48.0 ± 0.4	28.95 ± 0.67

[a] Data are expressed as mean ± SE. Ten mice were used in each treatment group.
[b] Values for C3H mice were significantly different from those for BALB/c mice: $P < 0.025$.
[c] $P < 0.05$.

2,3-DPG values than did female C3H mice. In all mice, hypoxia caused significant changes in RBC and platelet production indices, with greater increases occurring in C3H mice rather than in BALB/c mice. However, hypoxia resulted in platelet sizes being larger in all mice except BALB/c female mice, presumably because of the lack of thrombocytopenia found in these mice. Based upon our hypothesis of stem cell competition, this lack of thrombocytopenia was probably due to the poor RBC production rates.

In agreement with previous work (1–8), the present study showed that hypoxia causes significant increases in PCV and decreases in platelet production of mice. We believe that these results provide additional evidence for support of the stem cell competition hypothesis as the cause of decreased platelet production in hypoxic mice. As shown herein and previously, hypoxia causes decreased platelet counts (1–8) and lowered platelet production, as measured by incorporation of radioisotopes into platelets (1, 5). Moreover, we showed earlier that the hypoxia-induced thrombocytopenia was not caused by expanding blood volumes or excess sequestration of platelets by an enlarged spleen (17), and normal platelet survival values have been found (4). The effect of hypoxia does not appear to be direct upon thrombocytopoiesis, since stimulated red blood cell production was required for reduced platelet production (1, 10). Previous work (1) showed that exposure of BALB/c mice to hypoxia at mild oxygen levels (between 6 and 8%) resulted in unaltered platelet counts and normal red blood cell counts at Day 14. However, as shown in the present work, if BALB/c mice were exposed to more severe hypoxic conditions, they would respond with increased PCV. BALB/c mice produced increased RBC levels as did C3H mice, but the changes were significantly less than those found in C3H mice.

In other work (10), transfusion of RBC to mice

prior to making them thrombocytopenic by injection of rabbit anti-mouse platelet serum did not impair their rebound-thrombocytotic patterns. However, when the erythrocythemic mice were returned to the hypoxic environments after being made thrombocytopenic, marked inhibition of platelet production occurred, indicating that it is the hypoxia (and not the presence of elevated RBC) that decreases platelet production in mice (10). Moreover, it was shown that platelet production in ex-hypoxic thrombocytopenic mice was reduced when compared with that of mice recovering from thrombocytopenia induced with rabbit anti-mouse platelet serum (6), probably because of diminished megakaryocyte precursor cells in the marrow of ex-hypoxic mice (3, 4, 18).

The present work showed a marked reduction in the number of platelets being produced after 14 days of hypoxia, with the greatest effect occurring in the C3H mice. This finding, coupled with greater differences in PCV of the C3H mice, leads to the conclusion that the greater the increase in red blood cell production, the more severe will be the thrombocytopenia. Our results support this hypothesis and reveal that C3H male mice showed the greatest effect, the C3H female mice showed the second greatest effect, BALB/c male mice the third, and BALB/c female mice the least. It is interesting to note that in a recent study, Jackson *et al.* (13) showed that C3H male mice had the highest megakaryocyte DNA content of all mouse strains examined and the BALB/c female mouse had the lowest. It is tempting to speculate that a relationship exists between the DNA content of the mouse's megakaryocytes and its ability to produce RBC.

The fact that our previous work (18) showed that large doses of Epo (15 units of Epo per mouse over a 2-day period) caused increased platelet production in mice seems to argue against the stem cell competition hypothesis. However, previous work (19) found much smaller Epo levels in plasma of hypoxic mice than were used in the above study. We found that exposure of mice to hypoxia by enclosure in silicone rubber membranes resulted in Epo levels of about 2.5 units of Epo/ml of plasma at 24 hr (19). The circulating Epo levels then decreased to < 1 unit/ml as PCV values increased. These findings of greatly different Epo levels might explain the apparent discrepancy between the previous study (18) and the present work. These data might also explain the cause of the biphasic response in platelet counts of mice exposed to hypoxia (3–8).

It should not be surprising that male mice responded to hypoxia with greater changes in PCV and platelet counts than did female mice. Fried and Gurney (20) showed a striking sex difference in the production of Epo in response to androgen secretion some years ago, and Gurney *et al.* (21) reported that testosterone administered to female DBA/2J mice enhanced red cell production while in hypoxia. The results of the present work are in agreement with these previous findings and show that the degree of red cell production is inversely related to the level of thrombocytopenia of both male and female mice, a finding that supports the stem cell competition hypothesis.

As recorded in Table III, BALB/c female mice have higher P50 values (right-shifted curves) than do C3H female mice, but lower 2,3-DPG values were found. We interpret this right-shifted hemoglobin dissociation curve of BALB/c mice (decreased affinity for oxygen) as facilitating oxygen unloading without compromising uptake from the environment. In agreement with this hypothesis, we show herein that BALB/c mice have lower RBC production rates while in hypoxia than do C3H mice (Fig. 1). We believe this is because of decreased hemoglobin affinity for oxygen, which leads to higher O_2 levels in tissues of BALB/c mice than were found in C3H mice, resulting in lower Epo production (11,12) and smaller increases in PCV (Fig. 1). It may be suggested, therefore, that the lower RBC production resulted in a less severe thrombocytopenia of BALB/c mice than was found in the C3H mouse (Fig. 1).

The defect in BALB/c mice could be in the inability to produce Epo, as proposed previously (11,12), or a combination of these two factors. However, the fact that severe hypoxia leads to increased erythropoiesis in BALB/c mice seems to argue against a defective Epo production mechanism. In disagreement with our findings, Hebbel *et al.* (22) showed that individuals with left-shifted oxygen dissociation curves did not produce as much Epo or develop such severe thrombocytopenia upon exposure to high altitude as did normal subjects. The present work showed that BALB/c mice, whose P50 values were higher than those of C3H mice (Table III), did not show thrombocytopenia as severely as did C3H mice. The differences in hypoxic environments (altitudes of 3,100 m versus enclosure in silicone rubber membrane cages with 5.5–6.0% O_2 levels) may account for part of this discrepancy. However, based on our stem cell competition hypothesis, it seems reasonable that mice with the lower O_2 affinity hemoglobin should have lower Epo production rates with reduced RBC production, leading to a less severe thrombocytopenia.

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