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Effects of Hypoxia on Thrombocytopoiesis and Thrombopoietin Production of Mice[1] (40445)

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Hypoxia of prolonged duration causes marked thrombocytopenia in laboratory animals (1–5). The thrombocytopenia has been associated with decreased megakaryocyte concentration (4–5) and reduced isotope incorporation into platelets (2, 3, 6), supporting the conclusion that reduced platelet counts result largely from decreased platelet production. Hypoxia could cause thrombocytopenia by either stem-cell competition between the erythrocytic and megakaryocytic cell lines (2) or by an alteration in the production of a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) (6). If either of these hypotheses is correct, hypoxia should reduce the proliferation of a primitive megakaryocyte precursor cell, and consequently the rate of platelet production. In these experiments, platelet production after short-term hypoxia was measured as an indirect indication of precursor status. The results show that 24 hr of hypoxia followed by 2–3 days at the ambient O_2 level decreased ^{35}S-sodium sulfate incorporation into platelets and platelet counts of mice without altering the ability of mice to produce TSF. These findings are consistent with the hypothesis that thrombocytopenia is caused by stem-cell competition.

Materials and methods. Male C3H mice, 10–12 weeks of age and weighing approximately 25 g were used. The mice were exposed to hypoxia by enclosure in cages covered with dimethyl-silicone rubber membranes (7–9) for 24- to 96-hr periods. After equilibration of about 8 hrs, the oxygen levels in the cages were between 6 and 7%.

Platelet production was measured by determining the amount of radiosulfate incorporation into newly-formed platelets (10). Thirty μCi of Na_2 $^{35}SO_4$ diluted into 0.5 ml of saline were injected intraperitoneally (ip) into each mouse and the % ^{35}S incorporation into platelets was measured 24 hr later. Percent ^{35}S incorporation by platelets was calculated as previously described (10) using the appropriate blood volume for the corresponding time after hypoxia or rabbit anti-mouse platelet serum (RAMPS) treatment (6, 11).

RAMPS was prepared and absorbed with mouse RBC as previously described (10, 12) and injected ip (0.1 ml diluted with saline to 0.5 ml per mouse).

Peripheral platelet counts were determined from a single drop of blood obtained from the retroorbital sinus of mice. The blood was diluted with ammonium oxalate solution in red cell pipettes; the platelets were counted by use of phase microscopy.

Plasma from normal mice, mice exposed to 24 hr of hypoxia, and mice exposed to 24 hr of hypoxia followed by RAMPS-treatment was tested for TSF content. Exhypoxic mice were injected with RAMPS and were bled 4 hr later (13). For each experiment, platelet counts, WBC counts, and packed cell volumes were determined on five mice selected at random from each of the treatment groups (13) consisting of 20 mice each. Blood was collected into cold-50 ml conical centrifuge tubes containing sodium citrate; the plasma was separated from the blood cells by centrifugation at 4° and stored at 4° overnight. The plasma samples were assayed for TSF content by use of the immunothrombocythemic mouse assay (10). On days 5 and 6 after RAMPS administration, TSF assay mice were injected subcutaneously twice daily with 0.5 ml of test substances (0.8 ml of plasma, corrected for sodium citrate dilution, diluted to 2 ml with saline). Thirty μCi of Na_2 $^{35}SO_4$ in 0.5 ml of saline were injected intravenously on day 7 and the % ^{35}S incorporation of injected dose into circulating platelets was measured 24 hrs later (day 8) from blood

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samples obtained by cardiac puncture. The % ^{35}S incorporation was calculated as previously described (10).

The data were compared by use of Student's *t* test.

Results. Figure 1 shows the effects of 24-hr hypoxia on sulfate incorporation into platelets and platelet counts of mice. Hypoxia alone for this short duration had no effect on % ^{35}S incorporation into platelets of mice, regardless of whether the ^{35}S was given 48 or 24 hr after return to normal atmospheres, at removal to normal atmospheres or at time of initiation of hypoxia. The platelet counts of these mice were also unaltered except for those in Group 3, which were treated with hypoxia between days −1 and 0 and returned to normal atmospheres for 3 days before counting ($P < 0.05$). After mice had been injected with RAMPS to make them thrombocytopenic and to initiate increased production of platelets either after 24 hr of hypoxia (Group 4) or before 24 hr of hypoxia (Group 6), a decrease in ^{35}S incorporation into platelets ($P < 0.05$) and in platelet counts ($P < 0.0005$) was found when compared with RAMPS-treatment alone (Group 2). However, exposure to 24 hr of hypoxia beginning 1 or 2 days after RAMPS-treatment had no effect on the ^{35}S incorporation into platelets or platelet counts of RAMPS-treated hypoxic mice when compared with RAMPS-treated control mice, indicating that the effect of hypoxia must be on a primitive megakaryocyte or on its precursor cell.

Figure 2 shows the results of continuous hypoxia on % ^{35}S incorporation into platelets and platelet counts of mice. As shown, hy-

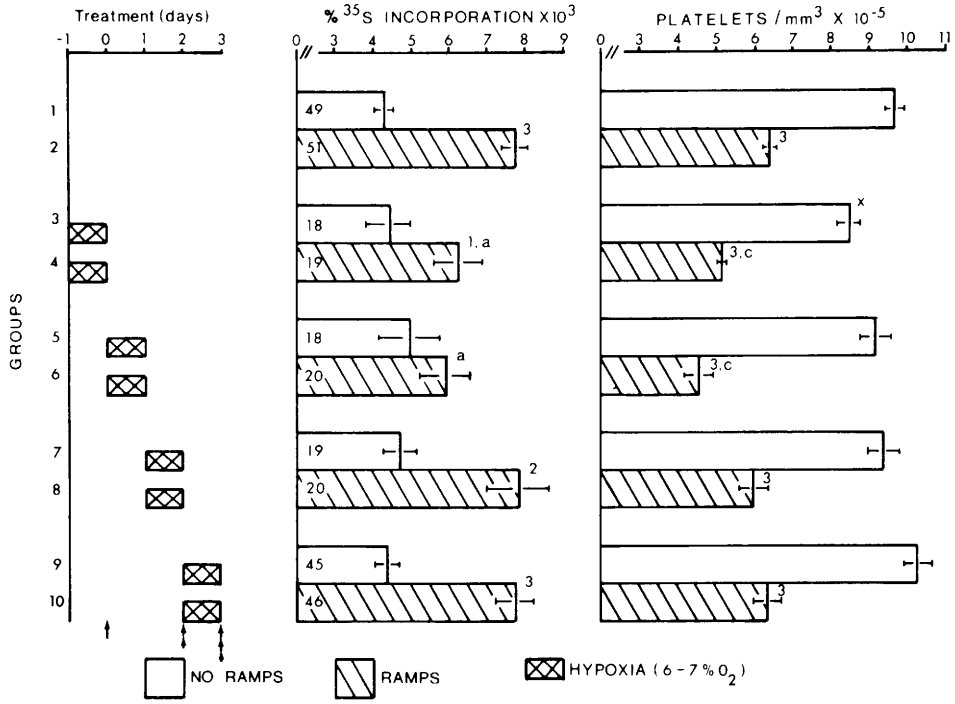

FIG. 1. The effect of 24 hrs of hypoxia on % ^{35}S incorporation into platelets and platelet counts of mice. The times of treatment are shown at the left for each treatment group. ↑ time of RAMPS injection (day 0), ↟ time of ^{35}S-sodium sulfate injection (day 2), and ↡ time of measurement of % ^{35}S incorporation into platelets and platelet counts (day 3). The numbers on the bars represent the number of mice per treatment, and the horizontal lines represent the standard errors. For measurements of platelet count or ^{35}S incorporation, values were significantly different from control: Normal control mice (Gp 1) versus hypoxia (Gps 3, 5, and 9) $^{x}P < 0.05$, $^{y}P < 0.005$, $^{z}P < 0.0005$; RAMPS-treated control mice (Gp 2) versus RAMPS plus hypoxia (Gps 4, 6, 8, and 10) $^{a}P < 0.05$, $^{c}P < 0.0005$; hypoxia control versus hypoxia and RAMPS (Gp 1 vs Gp 2, Gp 3 vs Gp 4; Gp 5 vs Gp 6; Gp 7 vs Gp 8; Gp 9 vs Gp 10) $^{1}P < 0.05$, $^{2}P < 0.005$, $^{3}P < 0.0005$.

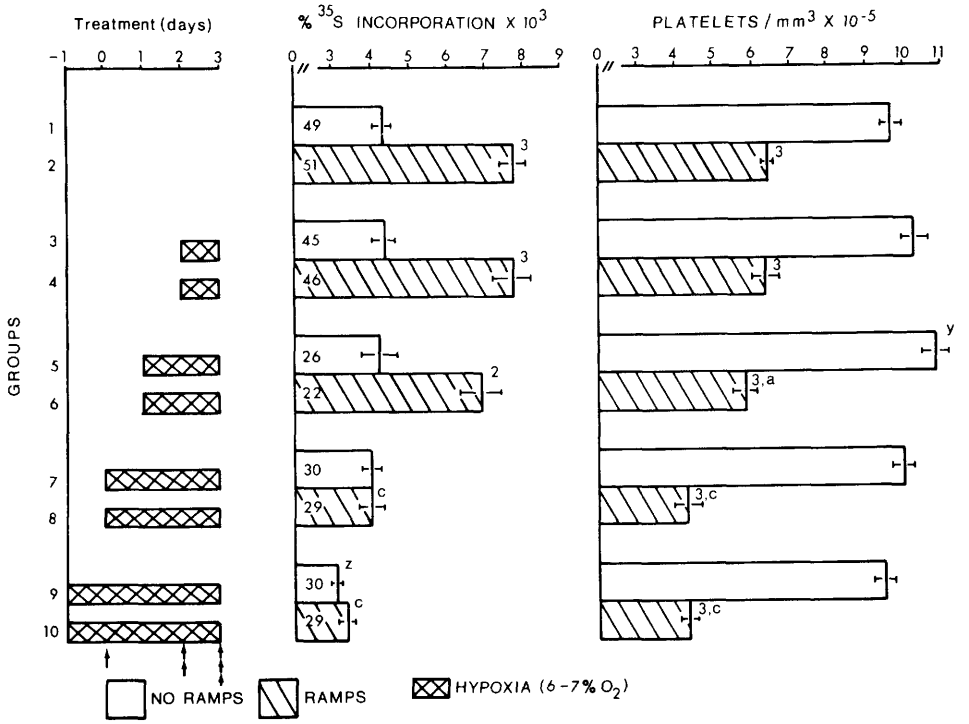

FIG. 2. The effect of continuous hypoxia (24 hrs to 96 hrs) on % ^{35}S incorporation into platelets and platelet counts of mice. Experimental conditions, statistical significance, and abbreviations are as indicated in the legend of Fig. 1.

poxia alone caused reduced ^{35}S incorporation into platelets only after 4 days (Group 9); when compared with normal control mice, increased platelet counts after 2 days of hypoxia (Group 5) were also seen. However, in mice given RAMPS, a reduction in sulfate incorporation was observed after 3 days (Group 8) and 4 days (Group 10) of hypoxia, but ^{35}S incorporation into platelets was unaltered after 24 or 48 hr of hypoxia. Likewise, hypoxia prevented the platelet counts of RAMPS-injected mice from returning to normal in Groups 8 and 10 ($P < 0.0005$) and in Group 6 ($P < 0.05$) when compared with values of RAMPS-treated control mice. The effects of hypoxia, therefore, require 3 days before measurable decreases can be seen in platelet counts or platelet production.

The results found after treating mice with hypoxia and RAMPS and assaying their plasma for TSF content are shown in Table I. RAMPS caused thrombocytopenia in hypoxic mice without altering PCV; whereas, in comparison to normal mice, hypoxia alone slightly elevated PCV but decreased WBC counts of donor mice. Plasma obtained from exhypoxic–thrombocytopenic mice caused increased ($P < 0.025$) ^{35}S incorporation into platelets of TSF-assay mice when compared with values of other mice injected with plasma from normal or exhypoxic mice. Hypoxia, therefore, did not prevent the production of thrombopoietin.

Discussion. The data of the present study show that 24 hr of hypoxia prior to or immediately after stimulation of platelet production by platelet specific antisera reduced platelet production rates 3 days later, indicating that the effect of hypoxia is on a precursor cell or on an early megakaryocyte. However, when thrombocytopoiesis was stimulated by antiserum injection 24–48 hr before exposure to hypoxia, there was little or no effect on platelet production rates; this finding demonstrates that hypoxia does not affect the differentiated megakaryocyte or its production of platelets. Four days of continuous hypoxia were required before platelet production rates were decreased in normal mice, but mice made hypoxic and injected with

TABLE I. Alterations in Platelet Count, Packed Cell Volume and WBC Count and in Plasma TSF-Activity of Mice Treated with Hypoxia or Hypoxia and RAMPS[a].

Treatment	Donor mice			TSF-Assay mice
	Platelets/mm^3 × 10^{-5} ± SE	PCV % ± SE	WBC/mm^3 × 10^{-3} ± SE	^{35}S Incorporation × 10^4 ± SE
Normal, no treatment	11.1 ± 0.3	47.0 ± 0.3	9.8 ± 0.4	44.5 ± 2.7
Hypoxia	11.0 ± 0.6	49.8 ± 0.4	5.4 ± 0.3	38.0 ± 3.4
Hypoxia and RAMPS Injection	1.3 ± 0.4	49.8 ± 0.3	7.6 ± 0.4	56.9 ± 3.7[b]

[a] Groups of 20 mice were exposed to 24 hr of hypoxia (6–7% O_2); RAMPS (rabbit anti-mouse platelet serum) was injected at time of removal from hypoxia. Donor mice were killed 4 hr after treatment and their plasma harvested. The results are the average of 2 separate experiments. In both experiments, 0.80 ml of plasma from donor mice was injected in 4 equal doses into groups of 5 TSF-assay mice.

[b] Significantly higher than values for normal mouse plasma ($P < 0.025$).

RAMPS showed evidence of decreased platelet production after 3 days of hypoxia. In addition, exhypoxic mice still retained the ability to generate thrombopoietin when rendered thrombocytopenic by platelet specific antisera. These findings support the hypothesis of stem-cell competition between the precursor cells of the megakaryocytic and erthrocytic cell lines.

Other previous data support the hypothesis that stem-cell competition causes diminished platelet production in hypoxic mice. Hypoxia causes decreased platelet counts of mice (1, 2, 14) and lowered incorporation of radioisotopes into platelets (2, 3). The hypoxia-induced thrombocytopenia was not caused by expanding blood volumes (11) or excess sequestration of platelets by an enlarged spleen (1, 2). Moreover, hypoxia does not appear to have direct effects on platelet production since stimulated RBC production was required for reduced platelet production (2, 6). It has been shown (2) that exposure of Balb/c mice (which have a defective erythropoietin production mechanism) to hypoxia resulted in unaltered platelet counts and normal RBC counts. In other work (6), RBC transfusion of mice prior to making them thrombocytopenic by injection of RAMPS did not impair their rebound-thrombocytotic patterns. However, when the mice were returned to an hypoxic environment 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. Other studies (5) have shown that platelet production in exhypoxic– thrombocytopenic mice was reduced when compared with that of mice recovering from thrombocytopenia induced with RAMPS, because of diminished megakaryocyte precursor cells in the marrow of exhypoxic mice.

Stem-cell competition between erythrocytic and megakaryocytic cell lines could account for hypoxia-induced thrombocytopenia. However, since platelet depletion of hypoxic mice did not show increased thrombocytopoiesis to the same degree as did RAMPS-treatment alone, and since TSF production is apparently normal (Table I), it is tempting to speculate that the erythropoietin released in response to hypoxia may override the influence of thrombopoietin and its control over platelet production (competition for stem cells between the action of "poietins"). It is also possible that hypoxia may directly alter the status of stem cells by decreasing their ability to differentiate into megakaryocytes or by altering their microenvironment or their "space". However, the work with Balb/c mice (2) which showed that the effects of hypoxia in reducing platelet counts required accelerated erythropoiesis seems to argue against the possibility of direct effects. Any one of these hypotheses, nevertheless, could account for the results of the present study, i.e., inhibition of platelet production at a precursor cell stage. However, the most likely mechanism of hypoxia-induced thrombocytopenia appears to be competition among a limited pool of stem-cells.

If the above hypothesis of stem cell competition between the erythrocytic and megakaryocytic cell lines caused by increased erythropoietin production in hypoxic mice is correct, then one could predict that injection of physiological doses of erythropoietin

should depress platelet production in mice as occurred with hypoxia. Indeed, in previous work (15) small amounts of erythropoietin significantly depressed thrombocytopoiesis in rebound-thrombocytotic mice.

Summary. Mice were placed in hypoxia chambers for various lengths of time prior to and after injection of platelet specific antiserum to determine the cause of thrombocytopenia in hypoxic mice. The results indicate that hypoxia decreased platelet production by action on a precursor cell or a primitive population of megakaryocytes without altering the ability of mice to produce thrombopoietin. Hypoxia may cause thrombocytopenia by stem-cell competition between the erythrocytic and megakaryocytic cell lines.

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