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Effects of Hypoxia on the Small Acetylcholinesterase-Positive Megakaryocyte Precursor in Bone Marrow of Mice[1] (42394)

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Abstract. The number of small acetylcholinesterase-positive (SAChE+) cells in the marrow of hypoxic mice was measured. Mice were exposed to 6–7% O_2 levels by enclosure in cages covered with dimethyl-silicone rubber membranes for 1–14 days. The mice showed a linear increase in packed cell volumes with time in the hypoxic atmosphere, but platelet counts showed a characteristic biphasic response, i.e., increased platelet counts were observed after 1–3 days of hypoxia, and significantly ($P < 0.05$–$P < 0.0005$) decreased platelet counts were observed thereafter (6–14 days). The total number of megakaryocytes in the marrow of hypoxic mice decreased significantly ($P < 0.005$) with time. In agreement with the data on platelet counts, hypoxia caused the total number of SAChE+ cells in the marrow of mice to be biphasic. At Day 2 there was a significant increase ($P < 0.05$) in the total number of SAChE+ cells/mm^3 of bone marrow; however, by days 10–14 the numbers had decreased markedly ($P < 0.005$). These data indicate that hypoxia decreases platelet production by action on a precursor cell to the SAChE+ cell. The hypoxia-induced thrombocytopenia is probably caused by stem-cell competition between the erythrocytic and megakaryocytic cell lines.

Hypoxia has been shown to cause marked thrombocytopenia in laboratory animals (1–5). The thrombocytopenia has been associated with reduced platelet production (2, 3, 6), while normal platelet survival values have been found (4). It has been hypothesized that the thrombocytopenia is caused by stem-cell competition between the erythrocytic and megakaryocytic cell lines (2, 5, 6). Moreover, the thrombocytopenia has been associated with decreased megakaryocyte concentration (4, 5), but the number of small acetylcholinesterase-positive (SAChE+) cells has not been measured. Several previous studies have shown that SAChE+ cells are early cells in the megakaryocytic series, and measurement of these cells appears to be a sensitive indicator of early changes in megakaryocytopoiesis and thrombocytopoiesis (7–10). Therefore, if stem-cell competition is the mechanism by which hypoxia causes thrombocytopenia, the number of SAChE+ cells should be decreased following hypoxia.

Materials and Methods. Male C3H mice, 8–10 weeks of age and weighing approximately 23 g, were used. The mice were exposed to hypoxia by enclosure in cages covered with dimethyl-silicone rubber membranes (11) for up to 14 days. After equilibration of about 8 hr, the oxygen levels inside the cages were between 6 and 7%. At various times, mice were removed from the hypoxic chamber and blood was collected from the retroorbital sinus for platelet counts and hematocrit determinations.

After bleeding, the mice were killed and marrow was taken from one of the femurs and dispersed into a single cell suspension by mixing with a plasma expander (polyvinyl-pyrrolidone, 3.5% in saline). Marrow smears were made and stained for the presence of acetylcholinesterase (AChE) activity using the method of Karnovsky and Roots (12). For staining, marrow smears were rinsed in 0.1 *M* sodium phosphate, pH 6.0, for 1 min and then incubated in the acetylcholine substrate mixture at room temperature for 3 hr (9). After staining, the smears were postfixed in absolute methanol for 10 min and 50% methanol for 30 sec. All AChE+ cells on the slide were counted. The SAChE+ cells are cells that stain positively for AChE, are usually round, stain evenly without granulation, and are less than 13 μm in diameter (9). For each mouse, ~500 AChE-positive cells were examined (except at Days 10–14 of hypoxia, where ~100 cells were counted); the number of SAChE+ cells and

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the total number of cells that stained positively for AChE were recorded.

The other femur was removed, fixed in 10% phosphate-buffered Formalin, decalcified, and embedded in methacrylate (JB-4, Polysciences). One, 2 μm thick, longitudinally oriented bone marrow section was cut from each femur and stained with hematoxylin and eosin. Sections were examined with a light microscope at a magnification of 1200× and the diameters of megakaryocyte profiles and number of profiles/mm^2 of section area were determined via video overlay onto the X-Y digitizing tablet of a Zeiss Videoplan Image Analysis System. The number of megakaryocytes/mm^3 of bone marrow, corrected for errors due to section thickness and optically lost cell profiles, was then calculated as previously described (13). The number of SAChE+ cells/mm^3 of bone marrow was calculated using

$$\text{Total number of SAChE+ cells/mm}^3\text{ of marrow} = \frac{(\text{number of SAChE+ cells } (<13\ \mu\text{m diam}) \text{ on smears}) \times (\text{number of recognizable megakaryocytes in sections/mm}^3)}{\text{number of } >13\ \mu\text{m diam AChE+ cells on smears}}.$$

The data were evaluated by use of Student's t test and one-way analysis of variance.

Results. Figure 1 shows the hematocrits and platelet counts of mice exposed to hypoxia. As shown in Fig. 1A, there was a linear increase in hematocrits of mice with time in hypoxic atmospheres. After 14 days of hypoxia the hematocrits had increased to 76%. Also shown in Fig. 1B are the platelet counts of these same mice. The platelet count showed a characteristic biphasic response, i.e., increased platelet counts were seen after 1–3 days of hypoxia and significantly ($P < 0.05$–$P < 0.0005$) decreased platelet counts were observed thereafter (Days 6–14). By Day 14 the platelet counts had decreased to only 17% of the pretreatment levels.

The total number of recognizable megakaryocytes and the calculated values for SAChE+ cells found in the bone marrow of hypoxic mice are recorded in Fig. 2. The total number of recognizable megakaryocytes in the marrow of untreated mice was ~4600/mm^3 (Fig. 2A). However, after exposure to hypoxia there was a marked drop in the numbers of megakaryocytes in the marrow of mice. The numbers of megakaryocytes seem to level off at about 44% of the pretreatment level after 2–8 days of hypoxia. However, with continued time in hypoxic atmospheres, the numbers decreased significantly ($P < 0.005$) to about 18% of the pretreatment values on Days 10–14. Figure 2B shows the numbers of SAChE+

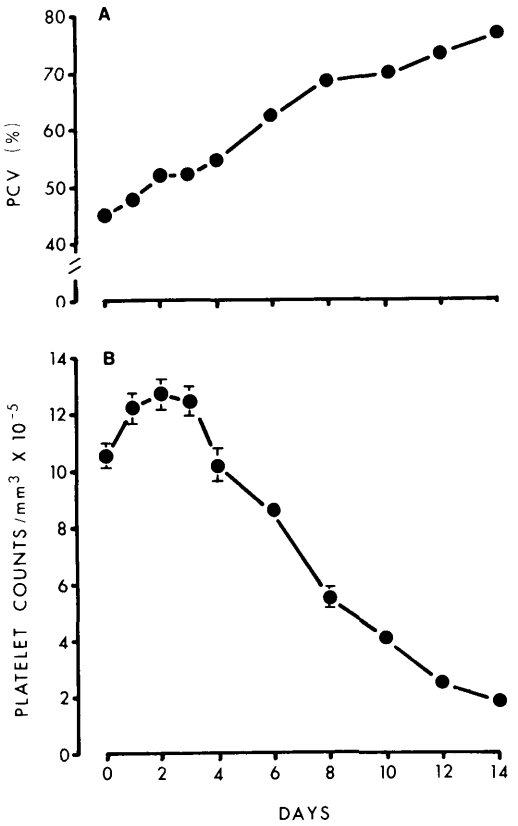

FIG. 1. Hematocrits (A) and platelet counts (B) of mice exposed to hypoxia of 6–7% O_2 for 1–14 days. Each point is the average of six mice and the vertical bars represent the standard errors. In those cases where bars are not shown the standard errors were smaller than the size of the point.

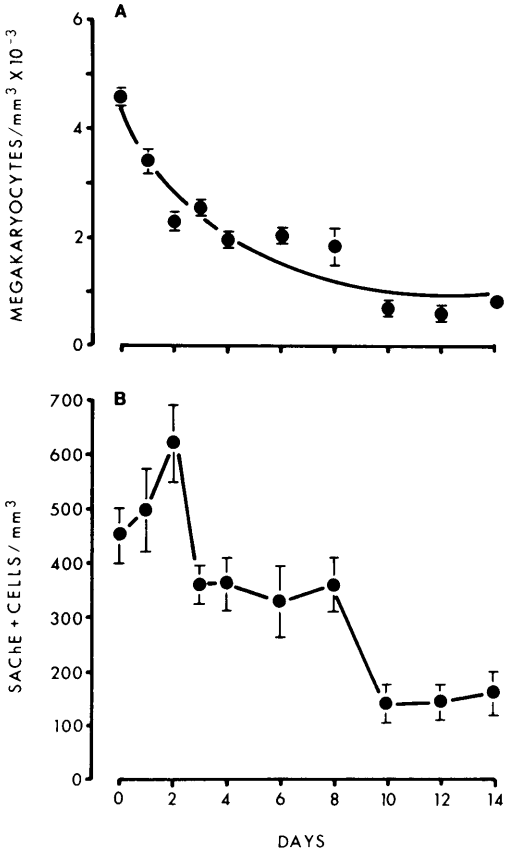

FIG. 2. The number of megakaryocytes/mm^3 (A) and the total number of small acetylcholinesterase-positive (SAChE+) cells/mm^3 (B) of bone marrow of mice after exposure to 1–14 days of hypoxia. Each point is the average of six mice and the vertical bars represent the standard errors. In the case where a bar is not shown, the standard error was smaller than the size of the point.

cells in the marrow of these mice with time in hypoxic atmospheres. As shown, there was a significant increase ($P < 0.05$) in the numbers of SAChE+ cells at Day 2, but by Days 10–14 there was a highly significant decrease ($P < 0.005$) in the numbers of SAChE+ cells/mm^3 of bone marrow; at the later time period the values were decreased to 36% of the pretreatment levels.

Discussion. The present study has shown that hypoxia causes a significant increase in hematocrits and significant decreases in the numbers of platelets, the numbers of recognizable marrow megakaryocytes, and the numbers of SAChE+ cells. Moreover, the biphasic platelet response (4, 14) that is caused by hypoxia was confirmed. Previously, it was shown that hypoxia causes decreased platelet counts (1, 2, 14) and lowered platelet production, as measured by incorporation of radioisotopes into platelets (2, 3). The hypoxic-induced thrombocytopenia was not caused by excess sequestration of platelets by an enlarged spleen (1, 2) or expanding blood volumes (14), and the effect of hypoxia does not appear to be direct upon thrombocytopoiesis, since stimulated RBC production was required for reduced platelet production (2, 6). Also, exposure of Balb/c mice (which have a defective erythropoietin production mechanism) to hypoxia resulted in unaltered platelet counts and normal RBC counts (2).

Other work (5), showed that platelet production in exhypoxic-thrombocytopenic mice was reduced when compared with the results of mice recovering from thrombocytopenia induced with RAMPS, probably because of diminished megakaryocyte precursor cells in the marrow of exhypoxic mice. Moreover, it seems clear 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 (15). Therefore, all of these previous studies, along with the findings of the present work, support the hypothesis of stem-cell competition between precursors of megakaryocytic and erythrocytic cell lines as the cause of the thrombocytopenia in hypoxic mice. This hypothesis states that hypoxia (or erythropoietin released in response to hypoxia) causes progenitor cells (either multi or bipotential progenitor cells) to differentiate into erythroid cells at the expense of cells of the megakaryocytic series. Although our hypothesis appears to differ from the theory that cellular commitment to a specific cell line of differentiation is a stochastic process, the data of the present work support the conclusion that commitment of a large number of cells from a limited stem-cell pool into the erythroid series reduces the number of cells that can differentiate into the megakaryocytic series.

In the present study, the numbers of SAChE+ cells in bone marrow were elevated at Day 2, but thereafter the numbers of these precursor cells were significantly reduced. The reason for the increase in SAChE+ cells at this

early time period is not known, but this finding may be related to the biphasic platelet response. We showed earlier (14) that the total circulating platelet counts and masses were increased at this early time period, lending support to the claim that it is a true increase in platelet numbers in the circulation. It seems possible that megakaryocytes may "shed" platelets into the circulation in response to the stress of hypoxia, very much like platelets are released into the circulation by postsurgical thrombocytosis. These mature platelet-producing megakaryocytes may, in the process of "shedding" platelets, release substances that cause an increase in the number of SAChE+ cells.

In further support of this feed-back mechanism, the present work showed that the number of megakaryocytes in the marrow of mice was reduced ($P < 0.025$) at one day after exposure to hypoxia. This reduction in megakaryocyte number may partially be the result of destruction of megakaryocytes by platelet "shedding," since, Jackson and Edwards (4) found increased numbers of naked megakaryocyte nuclei in the marrow of rats exposed to discontinuous hypobaric hypoxia. It seems possible that a feed-back mechanism by these "spent-megakaryocytes" that "shed" platelets on a precursor cell caused a temporary influx of SAChE+ cells in the marrow at day 2. However, the most obvious finding in the present study was a highly significant decrease in the number of SAChE+ cells of mice after exposure to hypoxia. The reasons for the sudden precipitous drops in the number of SAChE+ cells after day 2 and again after day 8 are unknown, but may be related to competition, to a similar feedback mechanism discussed above, or other factors. However, the findings of the present work support the hypothesis that hypoxia causes thrombocytopenia by stem-cell competition between the erythrocytic and megakaryocytic cell lines.

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