On the Mechanism of Erythropoietic Action of Hemoglobin and Its Derivatives[1] (35367)

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Injections of hemolyzed red blood cells have been shown to stimulate erythropoiesis in dogs and rats, and the effect has been suggested to play a role in sustaining high rates of erythropoiesis in patients with compensated hemolytic anemias (1–3). Brown and associates (4) have found hemoglobin of several species to be equally effective in rats and identified the heme moiety as the active principle. The same investigators also showed iron-free heme-related compounds like protoporphyrin IX, bilirubin, or chlorophyllin to have erythropoiesis-stimulating effects, thus confirming reports in the earlier literature. Labardini and associates (5) demonstrated more than twofold normoblast increases in marrow and spleen of hemolysate-treated rats. This finding indicates that the increases in reticulocytes and ^{59}Fe incorporation observed after hemoglobin administration were due to a true increase in the rate of erythropoiesis and not caused by an accelerated release of reticulocytes. The mechanism of action underlying the erythropoietic effect of heme compounds has remained obscure. The present study investigated whether the effect involves increases in erythropoietin levels.

Methods. Female Sprague-Dawley rats (250 ± 20 g), gerbils (50 ± 5 g), or MF_1 mice (25 ± 3 g) were made polycythemic by 3 weeks exposure at barometric pressures between 380 and 320 mm Hg. Five days after their return to ambient pressure they received the first of 3 daily injections of hemoglobin or of hemin. Eight animals were used in each experimental group. One day after the last injection of the test material, mice received 0.5 μCi of ^{59}Fe iv; gerbils, 1.0 μCi ip; and rats, 2.0 μCi iv. Forty-eight hr later, heart blood was obtained for measurements of ^{59}Fe incorporation. Blood volume was calculated as 7% of body weight. Reticulocytes were counted on tail blood. Crude hemoglobin solutions were prepared from rat or mouse blood by freezing and thawing washed red cells. After dilution with water, cell debris were removed by centrifugation. Hemoglobin concentrations were measured by the cyanmethemoglobin method. Recrystallized hemin (Nutritional Biochem. Corp.) was suspended in saline. All injections were given subcutaneously. Antihuman erythropoietin serum (obtained through the generosity of Dr. John C. Schooley, University of California) was injected ip in two doses of 0.5 ml each on the 2 days preceding ^{59}Fe injection. *In vitro*, 1 ml of the antiserum inactivated 20 units of human erythropoietin, but its neutralizing action against rat erythropoietin was estimated to be one twentieth of that.[6] Sheep erythropoietin (Connaught Laboratories, Toronto) was injected subcutaneously, and its dosage is expressed in International Reference Units. Plasma for erythropoietin bioassay was obtained by exsanguination of rats 6 hr after the last of 4 daily injections of 750 mg/kg hemoglobin. Heparin was used as anticoagulant. Plasma from nontreated rats served as control. Each plasma was assayed in a group of 6 posthypoxic polycythemic mice. Each mouse received 1 ml of plasma sc on 2 consecutive days. ^{59}Fe was injected on day 3, and heart blood was collected 72 hr later for determination of ^{59}Fe incorporation.

Results. Three injections of 500 mg/kg of rat hemoglobin caused significant increases in reticulocytes and iron incorporation of polycythemic rats but had no effect in polycythemic mice (Table I). Injections of mouse hemoglobin or of hemin, as well as increases

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TABLE I. Effect of Injection of Hemoglobin or Hemin on ^{59}Fe Incorporation and Reticulocytes of Posthypoxic Polycythemic Mice and Rats.

Treatment	Mice; (%)[a]		Rats; (%)[a]	
	^{59}Fe	Retic.	^{59}Fe	Retic.
Saline control	0.3 ± 0.1	<0.05	6.9 ± 2.1	0.3 ± 0.2
Hemoglobin, 3 × 500 mg/kg	0.3 ± 0.2	<0.05	16.7 ± 3.1	1.4 ± 0.4
+ 2 × 0.15 units of E[b]	11.2 ± 2.1	0.9 ± .2		
E,[b] 2 × 0.15 units	12.9 ± 2.4	1.1 ± .3		
Hemin, 3 × 50 mg/kg	0.4 ± 0.1	<0.05	13.4 ± 3.4	1.2 ± 0.3

[a] Mean and SEM, 8 animals/group.
[b] Units of sheep erythropoietin.

in dose and duration of treatment were likewise ineffective in mice. Concomitant injections of erythropoietin and hemoglobin into polycythemic mice were not more effective than injections of erythropoietin alone. Injections of hemoglobin or hemin in nonpolycythemic mice also failed to induce significant increases in iron incorporation or reticulocytes. A third species, gerbils, was therefore included in the study. Posthypoxic polycythemia suppressed their erythropoiesis to a greater degree than that of rats, but the suppression was still incomplete as indicated by reticulocyte counts around 0.2%. Injections of rat hemoglobin caused significant rises in their reticulocytes and iron incorporation (Table II). Concomitant injection of hemoglobin and erythropoietin induced increases in erythropoiesis approximately equal to the sum of the increments induced by separate injection of the two agents. Antierythropoietin serum was injected to determine whether the hemoglobin-induced erythropoiesis stimulation was mediated by erythropoietin. Four injections of 0.5 ml of antiserum reduced the iron incorporation in hemoglobin-treated polycythemic gerbils from 11.1 to 3.4%. A similar drop, from 13.1 to 3.1%, occurred when the same dose of antiserum was injected into polycythemic gerbils which had received two injections of 0.6 units of sheep erythropoietin.

Table III shows the results of measurements of the erythropoietin in the plasma of hemoglobin-treated rats. The difference in the iron incorporations of assay mice injected with plasma from normal and from hemoglobin-treated mice is not significant.

Discussion. Erythropoietin exerts its main regulating action by increasing the number of proerythroblasts transformed per unit of time from undifferentiated precursors. Each proerythroblast then undergoes a number of successive divisions, and the number of erythrocytes produced is thus a multiple of the number of proerythroblasts. From a cell kinetic viewpoint, therefore, an erythropoietic agent could act through one or more of the following processes: (i) increase in erythropoietin level; (ii) induction of proerythro-

TABLE II. Effect of Injections of Hemoglobin and Erythropoietin or Antierythropoietin Serum on Erythropoiesis of Posthypoxic Polycythemic Gerbils.

Treatment	^{59}Fe incorp. (%)[a]	Retic. (%)[a]
Saline control	2.4 ± 1.1	0.2 ± 0.1
Hemoglobin, 3 × 750 mg/kg	11.1 ± 2.1	0.9 ± 0.3
+ 4 × 0.5 ml of antiserum	3.4 ± 1.4	0.4 ± 0.2
+ 2 × 0.4 units of erythropoietin	15.1 ± 2.0	1.3 ± 0.4
Erythropoietin, 2 × 0.4 U	7.3 ± 1.4	0.6 ± 0.2

[a] Mean and SEM.

TABLE III. Erythropoietin Content of Plasma from Hemoglobin-Treated Rats as Measured in Polycythemic Mice.

Material assayed	^{59}Fe incorp. (%)[a]
Saline	0.3 ± 0.1
Plasma, normal rats	1.1 ± 0.5
hemoglobin-treated rats	1.6 ± 0.6
Saline + 0.3 units of erythropoietin	12.2 ± 2.4

[a] Mean and SEM.

blast formation through an agent other than erythropoietin; (iii) increase in the response to erythropoietin either at the level of proerythroblast formation or during subsequent erythroid cell divisions. The most direct proof of an increased rate of erythropoiesis being due to erythropoietin can be obtained by demonstrating elevated plasma erythropoietin by bioassay, and by *in vitro* inactivation of the active plasma by antierythropoietin antibody. However, normal and moderately elevated plasma levels induce a response in the bioassay mice, which lies on the nearly flat part of the dose–response curve. Small plasma elevations of erythropoietin may thus escape detection by the bioassay, although they may have caused significant increases in the erythropoiesis of the plasma donor. The absence of demonstrable elevated plasma erythropoietin levels in the hemoglobin-treated rats is thus inconclusive. As an alternative approach, therefore, antierythropoietin serum was injected in hemoglobin-treated gerbils. This markedly lowered their reticulocytes and iron incorporation in comparison with the group which received hemoglobin only. However, the interpretation of this finding is complicated by the fact that posthypoxic polycythemia did not completely suppress erythropoiesis and thus presumably erythropoietin formation in the gerbils. An increase in their erythropoiesis would result if the hemoglobin injections would merely increase the response to erythropoietin without actually increasing the latter. Such an increased response could conceivably be effected either by an increase in the number of proerythroblasts formed at a given erythropoietin level, or by an increase in the number of their successive divisions. The presence of erythropoietin would be prerequisite to either of these processes and its inactivation by antierythropoietin serum would thus abolish the hemoglobin-stimulated erythropoiesis. The results of the antiserum experiments are thus compatible with both a hemoglobin-induced increase in erythropoietin and an increase in the response to it. However, in the latter case, the percentile increase over control levels should be roughly the same at two different erythropoietin levels. This was tested after raising the erythropoietin level of polycythemic gerbils by injections of small doses of erythropoietin. Injection of hemoglobin increased their iron incorporation and reticulocytes by about 100%, whereas gerbils whose erythropoietin level had not been raised responded with a 400% increase to hemoglobin injections. Moreover, the observed increase in the former could be accounted for as a simple addition of the effects of separately injected erythropoietin and hemoglobin. There was thus no evidence of an altered responsiveness, and consequently, the suppressing effect of antiserum in hemoglobin-treated animals is interpreted as resulting from inactivation of elevated erythropoietin levels which had been induced by the administration of hemoglobin. It remains to be seen whether the latter stimulated erythropoietin formation or whether its elevation was due to a diminished rate of its disappearance from plasma or tissues.

The reason for the complete absence of erythropoietic action of hemoglobin and its derivatives in mice is unknown. The finding itself may provide some explanation of the well-known differences in erythropoietic suppression of polycythemic rats and mice. In the latter, posthypoxic or hypertransfusion polycythemia results in a practically complete suppression of erythropoietin formation as indicated by the absence of erythroblasts from their marrow; whereas the same degree of polycythemia leads to only incomplete suppression of erythropoiesis in rats whose iron incorporations remained between 2 and 6%. The breakdown of red cells in a polycythemic rat liberates approximately 100 mg of hemoglobin/24 hr. This amount, when in-

jected, significantly stimulates erythropoiesis. It is conceivable, therefore, that the breakdown of red cells provides, in rats and gerbils, sufficient stimulus to override the hyperoxemic suppression of erythropoietin formation. Hemoglobin injections are ineffective in mice and the suppression of erythropoietin formation by polycythemia is therefore much more severe.

Summary. Injections of hemoglobin or hemin stimulated erythropoiesis in rats and gerbils but were without effect in normal or polycythemic mice. Attempts to demonstrate increased erythropoietin levels in plasma of hemoglobin-treated rats were unsuccessful. Injection of antierythropoietin serum abolished in gerbils the hemoglobin-induced increase in erythropoiesis. No evidence was found that hemoglobin increased the erythropoietic response to erythropoietin. It is concluded that hemoglobin exerts its erythropoiesis-stimulating effect by inducing small increases in erythropoietin.

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