The Mechanism by Which Plethora Suppresses Erythropoiesis

By T. M. Kilbridge, W. Fried and P. Heller

ERYTHROPOIETIN is believed to be the prime physiologic regulator of erythropoiesis. It is generally assumed that erythropoietin production is chiefly controlled by the demands of tissues for oxygen relative to their oxygen supply. According to this hypothesis transfusion-induced polycythemia results in suppression of erythropoietin production (and consequently of erythropoiesis) by virtue of its ability to increase the oxygen supply to tissue. However, transfusion-induced polycythemia of a marked degree is associated with an increase in viscosity which might actually diminish oxygen supply to the tissues. A compensatory increase in cardiac output or fall in peripheral resistance could overcome this rheological disadvantage of higher blood viscosity following hypertransfusion. Recently, however, it has been suggested that marked post-transfusion polycythemia results in an inhibition of erythropoietin production by a mechanism which is independent of the increased oxygen content of blood. The present study was designed to demonstrate in mice the effect of an increase in hematocrit on erythropoietin production without a concomitant augmentation of the oxygen content of blood. This purpose was accomplished either by dehydrating mice or by transfusing them with methemoglobinized cells from a patient with hereditary methemoglobinemia due to deficiency of NADH-methemoglobin reductase (diaphorase). Such cells were chosen because methemoglobin of normal nitrite-treated human or mouse red cells was shown to be completely reduced within one to two hours after transfusion. The same was experienced with cyanmethemoglobin-containing cells.

The results of the studies to be reported indicate that factors other than the ratio of oxygen supply and requirement play an important role in the regulation of erythropoiesis in severely plethoric mice.

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MATERIALS AND METHODS

Blood was collected in ACD solution from a patient with methemoglobinemia due to hereditary deficiency of NADH-methemoglobin reductase and stored at 4°C until use. Blood from a normal individual was obtained on the same day and similarly stored.

The mice used in these experiments were 10 to 12 weeks old CF1 females weighing approximately 22 Gm.

Red cells were separated from enzyme deficient blood by centrifugation, washed with saline, and exposed for 20 minutes to an equal volume of a 1 per cent sodium nitrite solution at 5°C. Afterwards the red cells were washed ten times with cold isotonic saline to remove all excess nitrite. These cells contained 100 per cent methemoglobin and had no oxygen-carrying capacity. Normal human red cells stored for the same time were treated similarly except that they were not exposed to nitrite. Erythropoietin in plasma of heparinized blood was assayed according to the method of DeGewin et al. by measuring 59Fe uptake into the hemoglobin of newly formed red cells of plethoric mice.

Hypoxia was produced by placing mice into a 3' X 2' x 2' steel chamber evacuated by a vacuum pump and maintained at a pressure equivalent to 0.4 atmospheres. The mice were maintained in this hypoxic atmosphere for four and one-half hours.

The red cell mass was measured 15 minutes after the intravenous injection of isologous red cells labeled in vivo with 59Fe. Red cell mass (RCM) was calculated according to the formula:

RCM = \frac{X}{Y} \times \text{hematocrit} - Z

where X = counts per minute (CPM) injected, Y = CPM/ml. of blood removed and Z = volume of red cells injected.

Methemoglobin-containing erythrocytes on blood films were visualized by the slide elution test of Kleihauer and Betke.

Hemoglobin concentration was determined colorimetrically by the cyanmethemoglobin technic. The oxygen carrying capacity of hemoglobin was measured by the method of Van Slyke and Neill. The amount of hemoglobin incapable of oxygen transport was calculated by subtracting the functional hemoglobin concentration as determined by its oxygen carrying capacity from the total hemoglobin concentration measured by the colorimetric technic.

A. Comparison of the Effects of Transfused Mouse and Human Red Cells on Erythropoietin Production.

Various amounts of either washed normal human or mouse erythrocytes were given intravenously to groups of 20 to 30 mice. Immediately thereafter the animals and a control group of uninjected mice were placed into the hypobaric chamber which was then evacuated to 0.4 atmosphere. After four and one-half hours the animals were sacrificed and their plasma collected for erythropoietin assay.

B. Comparison of the Effect of Methemoglobinized Diaphorase Deficient Red Cells on Erythropoietin Production.

Twenty-five mice were injected with 0.25 ml. of methemoglobin-containing cells (Met-RBC). In addition, ten mice were given an equal amount of normal human red cells (Oxy-RBC) and 15 mice were not transfused. Following four and one-half hours of hypoxia ten of the mice given Met-RBC and the ten mice which received Oxy-RBC were exsanguinated. The blood from each of these two groups was pooled and the oxygen carrying capacity and hemoglobin concentration determined. The red cell mass was measured in the remaining 15 mice transfused with Met-RBC and in the 15 uninjected mice. The difference between these two groups was taken to represent the volume of transfused erythrocytes present in the circulation after four and one-half hours of hypoxia. Any loss in volume was considered to be due to hemolysis; this was maximally 25 per cent (vide infra). Peripheral blood films were obtained from several mice at the conclusion of the hypoxic period and the slide elution test for methemoglobin performed.

To study the effect of the degree of plethora on the production of erythropoietin in response to hypoxia groups of mice were transfused with varying amounts (0.15, 0.3, 0.45
Fig. 1.—Plasma erythropoietin levels of hypoxic mice transfused with various amounts of normal mouse or human erythrocytes. Values are expressed as percent of erythropoietin levels in nontransfused mice. Brackets indicate one standard error. Control values (100 per cent) represent a 72 hour radioiron utilization of 11.1 per cent obtained in plethoric assay animals injected with 0.5 ml. of plasma from control mice (see text).

ml.) of either Met-RBC or Oxy-RBC. These groups and a control (untransfused) group of 25 animals were exposed to hypoxia for four and one-half hours. Immediately after exposure blood samples for hematocrit determinations were obtained and subsequently the animals were exsanguinated and plasma collected for erythropoietin assay.

C. The Effect of Hemolysates on Erythropoietin Production.

Hemolysates of methemoglobin-containing cells were prepared by freezing and thawing suspensions of cells five times. The stroma was not removed and the suspension was agitated prior to injection. The effect of these disrupted Met-RBC on the ability of hypoxic mice to produce erythropoietin was compared to the effect of intact Met-RBC. Control mice received no transfusions. One group of 30 mice was given 0.50 ml. of intact Met-RBC. A second group received an amount of disrupted Met-RBC equal to the amount of expected in vivo hemolysis (25 per cent or 0.14 ml.). Two additional groups of mice received larger amounts of disrupted Met-RBC (0.19 ml. and 0.22 ml.). All mice were then exposed to hypoxia for four and one-half hours following which they were exsanguinated and the plasma frozen for erythropoietin assay. In addition, a small aliquot of plasma from each group was collected for determination of the plasma hemoglobin concentration. To determine the actual degree of hemolysis of injected Met-RBC, red cell volumes were obtained in this group following hypoxia as well as in another group of mice 10 minutes after receiving 0.5 ml. of intact cells. The difference between the two red cell volumes was taken to represent the volume of red cells hemolyzed during the four and one-half hour period of hypoxia.
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Table 1.—Comparison of Oxygen-Carrying Capacity and Total Hemoglobin Concentration After Transfusion with OXY-RBC or MET-RBC

<table>
<thead>
<tr>
<th>Hemoglobin Concentration (Gm.%) at 4½ Hours</th>
<th>% Methemoglobin Colorimetric Method</th>
<th>Oxygen-Capacity Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 ml. Normal Human RBC</td>
<td>18.2</td>
<td>18.2</td>
</tr>
<tr>
<td>0.25 ml. Methemoglobin Containing RBC</td>
<td>17.2</td>
<td>13.9</td>
</tr>
</tbody>
</table>

D. The Effect of Dehydration on Erythropoietin Production

A decrease in plasma volume due to dehydration was accomplished by withholding water, but not food, from 15 mice for three days. These animals and a control group of mice were then subjected to four and one-half hours of hypoxia. A second experiment was performed in which animals dehydrated for three days were acutely rehydrated by intraperitoneal injection of 3 ml. of normal saline four hours before hypoxia and 1 ml. of normal mouse plasma 30 minutes prior to hypoxia. These animals were subjected to hypoxia simultaneously with a group of normal mice. Microhematocrits were taken after the period of hypoxia and plasma was collected and frozen for erythropoietin assay.

RESULTS

A. Transfusion of mice with either normal human or mouse erythrocytes suppressed their ability to produce erythropoietin when made hypoxic (Fig. 1). Increasing amounts of either produced a progressive reduction of erythropoietin levels.

B. As shown in Table 1 essentially 100 per cent of the hemoglobin in erythrocytes of mice injected with normal human blood was capable of carrying oxygen after four and one-half hours whereas about 19 per cent of the total hemoglobin of mice injected with methemoglobin-containing red cells was incapable of carrying oxygen. This value compares with a predicted concentration, calculated from red cell volume studies immediately after transfusion and following four and one-half hours of hypoxia, of 13 per cent ± 1.8 (mean ± S.E.). Staining of peripheral blood films for methemoglobin after hypoxia (Fig. 2) showed that the cells of human origin remained filled with methemoglobin. There was therefore no apparent reduction of methemoglobin to oxyhemoglobin during the four and one-half hour hypoxic period.

C. The influence of intact Met-RBC's and of disrupted Met-RBC on erythropoietin production is compared in Table 2. Following injection of 0.5 ml. of
Fig. 2.—Peripheral blood film from mouse after hypoxia. Methemoglobin has been eluted from the cells. Cells taking up stain contain oxyhemoglobin. The larger cells are of human origin.

Met-RBC (sufficient to increase the hematocrit to 62 per cent at four and one-half hours) blood volume determinations showed 0.12 (1 S.E. ± 0.02) ml. of the cells to have been lysed. Injection into mice of the hemolysate of 0.14 ml. and 0.19 ml. of Met-RBC resulted in no significant suppression of erythropoietin production, although the latter resulted in a higher plasma hemoglobin concentration than did injection of intact cells. Injection of 0.22 ml. of hemolysate did, however, suppress erythropoietin production.

D. As shown in Figure 4, the hematocrits of dehydrated mice were increased (57.3 ± 0.7) and the production of erythropoietin in response to hypoxia was decreased. Rehydration of such mice normalized the hematocrit (48.0 ± 0.1) and completely restored the ability to produce erythropoietin in response to hypoxia.

Discussion

There is a large body of information indicating that the regulation of erythropoiesis and of erythropoietin production is dependent on the relation
Fig. 3.—Plasma erythropoietin levels of hypoxic mice transfused with various amounts of either oxyhemoglobin-containing or methemoglobin-containing cells. Brackets indicate one standard error. Plasma of control mice (0.5 ml.) produced a 12.2 per cent radioiron utilization in plethoric assay animals.

The increased oxygen-carrying capacity per volume of whole blood which is produced by transfusion of small amounts of blood may explain the subsequent suppression of erythropoietin production and erythropoiesis and, indeed, it has been shown that polycythemic states decrease the plasma erythropoietin titre and the activity of extractable renal erythropoietic factor (REF) in rodents exposed to hypoxia. However, the situation following transfusion of larger amounts of blood is made more complex by changes in blood viscosity. The viscosity of blood as measured in a Brookfield microviscometer at a hematocrit of 60 per cent is more than three times that of blood with a hematocrit of 40 per cent and one and one-half times that of blood with a hematocrit of 49 per cent. It has been assumed that these differences in viscosity at varying hematocrits would be even more striking in the microcirculation. Such an increased viscosity, without compensatory cardiovascular response, would result in a decreased delivery of oxygen to tissue. If this premise is valid, polycythemic states should be self-perpetuating.
The data presented here demonstrate that in two experimental situations in which the hematocrit is increased without apparent increase of oxygen transport to tissues erythropoietin production is suppressed. Elevated hematocrits were produced either by the transfusion of nonfunctional red cells or by dehydration without alteration of red cell mass. Whereas transfusion of small amounts of oxygenated human erythrocytes significantly suppressed erythropoietin production, transfusion of identical amounts of methemoglobin-containing erythrocytes did not. It, therefore, is likely that at these degrees of plethora, increased oxygen carrying capacity provided by transfused blood was critical to suppression of erythropoietin production. When, however, larger numbers of erythrocytes were transfused, both Oxy-RBC and Met-RBC decreased erythropoietin production in response to hypoxia to less than 15 per cent of normal. Seventy-five per cent of transfused human red cells containing only methemoglobin remained in the circulation at four and one-half hours. It was shown that the amount of hemolysis taking place during the hypoxic period could not, by itself, be responsible for suppression of erythropoietin production, since injection of disrupted methemoglobin containing cells, in excess of maximal amounts destroyed in vivo during transfusion studies, did not alter the erythropoietin response to hypoxia.

During moderate hypervolemic plethora (hematocrit < 60 per cent) the mechanical disadvantage of increased blood viscosity may be overcome by a series of cardiovascular adjustments to transfusion. A decrease in peripheral vascular resistance and an inconstant increase in cardiac output during hypervolemia was documented in dogs by Murray et al. The overall effects of these conflicting influences on oxygen delivery at various degrees of plethora are uncertain. Campbell determined the oxygen tension of air in subcutaneous pockets of rabbits and reported increased oxygen tension at all hematocrits. Thorling and Erslev showed that the oxygen tensions in pneumoperitoneums of mice increased as the hematocrits were raised from 20 per cent to 50 per cent. Elevations in hematocrit beyond this point were not associated with statistically significant changes in oxygen tension. Although tissue oxygen ten-

### Table 2.—The Effect of Transfusion of Disrupted MET-RBC on Erythropoietin Production

<table>
<thead>
<tr>
<th>% $^{55}$Fe uptake in (±1S.D.) assay mice</th>
<th>% of control uptake</th>
<th>Plasma Hgb 4½ hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>No transfusion (CONTROL)</td>
<td>9.3±4.0</td>
<td>.09 Gm.%</td>
</tr>
<tr>
<td>0.5 ml RBC * (Intact)</td>
<td>2.3±0.9</td>
<td>25% .48</td>
</tr>
<tr>
<td>0.14 ml RBC (Lysed)</td>
<td>8.8±2.3</td>
<td>95% .44 Gm.%</td>
</tr>
<tr>
<td>0.19 ml RBC (Lysed)</td>
<td>9.5±2.8</td>
<td>102% .71</td>
</tr>
<tr>
<td>0.22 ml RBC</td>
<td>3.6±1.1</td>
<td>39% .97</td>
</tr>
</tbody>
</table>

* 0.12 ml±.03 of these cells had been lysed at the end of the experiment.
Fig. 4.—The effect of dehydration and rehydration on erythropoietin production by hypoxic mice. Brackets indicate one standard error. Plasma from control mice (0.5 ml.) produced a 12.2 per cent utilization of radioiron in plethoric assay mice.

sions could not be measured under the conditions of the present study, our observations on mice transfused with nonfunctioning erythrocytes indicate that increased tissue oxygen supply is instrumental in depressing erythropoiesis at mild degrees of polycythemia, but at higher hematocrits factors other than oxygen supply influence the control of erythropoiesis. Such factors might be increased blood volume, increased red cell mass, or increased blood viscosity.

The studies in dehydrated mice indicate that the suppression of erythropoietin production in these animals was related to increased hematocrit or viscosity, and not to an increase in red cell mass or blood volume. Animals denied water, but fed, for 72 hours demonstrated reduced erythropoietin production (49 per cent of normal) during hypoxia. That this was not a non-specific toxic or metabolic effect of dehydration is suggested by the fact that rehydration, and restoration of hematocrit to normal, just before exposure to hypoxia, completely restored the ability of dehydrated mice to produce erythropoietin under stress. The usefulness of dehydrated rodents for bioassay of erythropoietin probably depends on the decrease of endogenous erythropoietin in such animals and the subsequent increased sensitivity to small amounts of exogenous erythropoietin.

CONCLUSION

Erythrocytes incapable of oxygen-transport were obtained by exposing human red cells deficient in NADH-methemoglobin reductase to nitrite. The
effect of transfusions of such cells (Met-RBC) and of normal human erythrocytes (Oxy-RBC) on erythropoietin production was compared in mice kept at 0.4 atmospheres pressure for four and one-half hours. At a mild degree of plethora (hematocrit 52 per cent) the ability of transfused cells to carry oxygen appeared critical to suppression of erythropoietin production. With increasing degrees of plethora, however, the difference between the response of animals transfused with Oxy-RBC and Met-RBC disappeared so that at a hematocrit of 60 per cent both types of cells reduced erythropoietin production to less than 15 per cent of that in non-plethoric hypoxic mice.

Dehydrated mice also had decreased production of erythropoietin during hypoxia, indicating that factors related to increased hematocrit rather than an expanded blood volume or red cell mass assume an important role in the suppression of erythropoietin and erythropoiesis.

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REFERENCES

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