Erythropoietin Concentration During the Development and Recovery From Iron Deficiency in the Rat

By Lennart T. Jansson, Mikko V. Perkkiö, Gisela Clemons, Canio J. Refino, and Peter R. Dallman

The concentration of plasma erythropoietin was determined by radioimmunoassay during the progression of and subsequent recovery from iron-deficiency anemia in the rats. During the development of anemia, the plasma erythropoietin level rose as the hemoglobin (Hgb) concentration declined, reaching maximal levels when the Hgb was lowest. During the recovery from iron-deficiency anemia after institution of the control diet, the plasma erythropoietin concentration rapidly declined to baseline or below baseline levels even before the Hgb had completely returned to control values. This early fall in the erythropoietin level was associated with a sustained decrease in blood oxygen affinity (increase in \( P_50 \)). The rise in \( P_50 \) was associated with an increase in the number of circulating reticulocytes in addition to and independently of an increase in the concentration of 2,3-diphosphoglycerate (DPG) in red cells. Therefore, reticulocytosis may play a part in the recovery from anemia, not only by replenishing the red cell pool but also by temporarily facilitating oxygen delivery to the tissues.

**Erythropoietin** is the major stimulus to red cell production in response to hypoxia. Under conditions of anemia, the elevation in the concentration of plasma erythropoietin not only reflects the decrease in oxygen-carrying capacity of the blood, but is also a function of the position of the oxygen dissociation curve of the blood. A decrease in the oxygen affinity of hemoglobin (Hgb) is one of the adaptations that helps to compensate for anemia by facilitating the release of oxygen to tissues. The oxygen affinity of Hgb is regulated by the concentration of 2,3-diphosphoglycerate (DPG) within the red cell, but it may also be related to the rate of erythropoiesis, since young red cells have been found to have a decreased oxygen affinity. The changes in DPG and the proportion of young red cells during the progression and repair of anemia may therefore influence oxygen delivery, plasma erythropoietin secretion, and adaptation to anemia.

During the development and reversal of dietary iron deficiency in the rat, there are rapid changes in the oxygen-carrying capacity and the oxygen affinity of the blood. The purpose of the present study was to determine the relationships of anemia and some adaptations to anemia (reticulocyte count, DPG, and blood oxygen affinity) to the plasma erythropoietin concentration during progression and reversal of anemia.

**MATERIALS AND METHODS**

**Experimental Animals**

All experiments were performed with male Sprague-Dawley rats purchased from Simonsen Laboratories (Gilroy, Calif). They were housed in rust-free cages under a 12-hour light–dark cycle. Food and distilled water were given ad libitum.

**Experimental Diet**

A semipurified diet (Teklad, Madison, Wis) was used in all experiments. It was identical in composition to that proposed by the American Institute of Nutrition for growing rats, except that cellulose was omitted because of its variable iron content. The rats were given either of two diets containing 6 mg iron/kg diet (iron-deficient diet) and 50 mg iron/kg diet (control diet), respectively.

**Experimental Design**

**Experiment 1: Progression and reversal of iron-deficiency anemia.** Rats were obtained at 21 days of age and randomly assigned to either the iron-deficient diet or the control diet. Eight rats in each group were exsanguinated by heart puncture at 28 and 35 days of age under pentobarbital anesthesia (5 mg/100 g body weight). Blood was collected in heparinized syringes for determination of Hgb, reticulocyte count, and erythropoietin. At 42 days of age, the iron-deficient diet was changed to the control diet. Blood samples were collected from tail vein under light ether anesthesia (eight rats in each group) on days 42, 43, 44, 45, 48, 50, and 52 (0, 1, 2, 3, 6, 8, and 10 days after initiation of the control diet regimen in the iron-deficient group).

**Experiment 2: Correction of iron deficiency with \( P_50 \) added as a variable.** Rats were obtained at 21 days of age and randomized to the iron-deficient (6 mg iron/kg diet) and control diets (50 mg iron/kg diet). At 42 days of age, the iron-deficient animals were changed to the control diet. Blood for determination of Hgb, reticulocyte count, red cell DPG, erythropoietin, and blood gases was obtained from the abdominal aorta under pentobarbital anesthesia (5 mg/100 g body weight) on days 42, 43, 46, 47, 48, 49, 51, and 54 (0, 1, 4, 5, 7, 9, and 12 days after initiation of the control regimen). There were eight iron-deficient and six control rats in each group sacrificed daily, except on day 42 (14 deficient, 12 control).

**Experiment 3: Red cell age and oxygen affinity.** Rats with a body weight of approximately 250 g were bled 10% of the estimated blood volume on three successive days from the orbital plexus under...
pentobarbital anesthesia (dose as above). Two days after the bleeding, blood from the abdominal aorta was collected into heparinized syringes under pentobarbital anesthesia. The erythrocytes were fractionated according to buoyant density by ultracentrifugation on a Stractan gradient. The following concentrations of Stractan were used: 28%, 26%, 24%, 23%, 22%, and 21%. After separation, the red cells were found in the 24%, 23%, 22%, and 21% layers (the 21% and 22% layers were sampled as the top layer, and the other two red cell layers were combined as the bottom layer). Both fractions were washed twice in buffered saline with potassium and glucose, pH 7.4 as described by Corash et al., resuspended in plasma, and analysed for reticulocyte count, Hgb, DPG and P0. The P0 and the DPG assays were done in triplicate, and the P0 values were corrected to a base excess of zero and to pH 7.4 (in vitro P0).

**Analyses**

Arterial blood gases and pH were measured within two minutes after the withdrawal of blood on a Corning 158 pH, Blood Gas Analyzer (Corning, Medford, Mass). The position of the oxygen dissociation curve was expressed in terms of the P50, the oxygen tension at which Hgb is 50% saturated with oxygen. The in vivo P50 and the in vitro P0 (corrected in pH 7.40; PCO2 40 torr) were determined on an automatic oxygen dissociation analyzer (Hem-O-Scan: American Instrument Company, Silver Spring, Md). DPG was determined by the method described in Sigma Technical Bulletin No. 35-UV (Sigma Chemical Co, St Louis). Erythropoietin levels were measured by radioimmunoassay as described by Garcia et al., utilizing 100 μL plasma for the analysis. Means of erythropoietin values were calculated after logarithmic transformation. Hgb concentration was determined spectrophotometrically after conversion to cyanmethemoglobin. Reticulocyte counts were done on slides stained with new methylene blue. The reticulocyte counts in the iron-deficient groups were calculated with correction for anemia, except when the association of the percentage of reticulocytes or DPG with P0 was estimated.

**Statistics**

Differences between means were calculated by Student's t test. The correlations between P50 levels and the reticulocyte count and DPG concentration were calculated by linear regression analysis and analyses of variance (ANOVA).

**RESULTS**

**Experiment 1**

After the initiation of the diets at 21 days of age, the mean concentration of Hgb in the control group increased from 8.5 to 12.5 g/dL at 42 days of age (Fig 1). This is in accord with previously observed age-related changes. The mean reticulocyte count during this period decreased from 20% to 10%. In the iron-deficient animals, the mean concentration of Hgb gradually decreased to 6.5 g/dL on day 42. After day 42, when the animals were given the control diet, the Hgb concentration rose following a sigmoid curve with a steep rise after between two and five days and a slower subsequent increase. On the tenth posttreatment day, there was no significant difference in mean Hgb levels between the groups.

During the progression of iron deficiency, the mean reticulocyte count, corrected for anemia, was initially below that of the control group at 28 and 35 days of age (Fig 1). At 42 days of age, it had risen to a mean of 8.4%, and it was not significantly different from the control value. During the recovery from iron deficiency, the reticulocyte count rose to a mean value of 24.1% on the fourth posttreatment day, and it remained significantly higher compared with control levels until the eighth posttreatment day. During the progression of anemia, the plasma erythropoietin level rose sharply as the Hgb concentration declined. The peak geometric mean erythropoietin level of 1,130 μU/mL was reached on day 42, the day with the lowest Hgb values. After the introduction of the control diet, the erythropoietin concentration rapidly decreased to a mean level of 16.1 μU/mL, compared with 36 μU/mL in the control group on day 6 posttreatment. This difference was statistically significant (P < .02). Thereafter, the erythropoietin levels increased in the iron-deficient group to become similar to that of the control group on the tenth posttreatment day.

**Experiment 2**

Figure 2 shows the mean ± SEM values for Hgb, erythropoietin, and P50 during the recovery from iron-deficiency.
ER Ty ropl oetin in Iron Deficiency

Fig 2. Hgb, \( P_{50} \) and erythropoietin in rats during recovery from iron-deficiency anemia (days 42 to 54). Values for iron-deficient (○) and control (■) groups are shown as mean ± SEM. The last point during recovery from iron deficiency when mean values for the groups still differed significantly (\( P < .05 \)) is indicated (+).

deficiency anemia and in the control group. The Hgb levels were similar to those found in experiment 1. From day 7 after the institution of the control diet, there were no significant differences in mean Hgb between the groups. The erythropoietin concentration was highest on day 42 and thereafter rapidly decreased to become equal to the control values from day 5 posttreatment.

Figure 3 shows the changes in reticulocyte count, \( P_{50} \), and DPG concentration during the recovery from iron deficiency. The reticulocyte count in the iron-deficient group increased to reach maximum values on days 4 to 7 after initiation of the control diet (a broader peak than in experiment 1). The \( P_{50} \) was significantly increased in the iron-deficient animals compared with the control group, and remained increased during the seven days after initiation of the control regimen. This was also the case when \( P_{50} \) values were not corrected to a base excess of zero and to pH 7.4 (in vivo \( P_{50} \)); there was no significant difference between the mean in vivo and in vitro \( P_{50} \) values. Neither was there a significant difference between groups at any of the time points in either base excess or pH.

The DPG level decreased during the reversal of anemia from a mean of 21.3 \( \mu \)mol/g Hgb on day 42 to a mean of 16 \( \mu \)mol/g Hgb nine days later. The iron-deficient animals had a significantly increased mean DPG concentration compared with controls on days 0 and 1 posttreatment (\( P < .05 \)).

The relationships between \( P_{50} \) and DPG and between \( P_{50} \) and reticulocyte count are shown in Fig 4. In the iron-deficient rats and during recovery, there was a significant positive correlation between DPG and in vitro \( P_{50} \) (\( r = .55; P < .001 \)) and between reticulocyte count and \( P_{50} \) (\( r = .58; P < .001 \) (Fig 4). There was no significant relationship between reticulocyte count and DPG (\( r = .22; P < .1 \).

The independent effects of the reticulocyte count and DPG on \( P_{50} \) were statistically significant (\( P < .001 \). The relative contributions of the independent variables to the \( P_{50} \) could be weighted as 35% from reticulocyte count, 18% from DPG, and 23% from an interaction between the two (sum of squares of the variable/sum of squares explained).

Experiment 3

The \( P_{50} \), reticulocyte count, and DPG were measured in red cell fractions separated on the basis of buoyant density after centrifugation on a Stractan gradient (Table 1). In the top, low-density layer, the reticulocyte count was 35% and the \( P_{50} \) was 38.5 torr. In the bottom, high-density layer, the reticulocyte count (2%) and the \( P_{50} \) (33.4 torr) were both lower than in the top layer. The DPG values were similar,
Fig 4. The relationship between DPG and P₅₀ (upper panel) and reticulocyte count and P₅₀ (lower panel) during recovery from iron deficiency. Linear regression and correlation coefficients are shown.

with the slightly lower value in the high P₅₀, reticulocyte-rich, top layer.

DISCUSSION

In this study we monitored the plasma erythropoietin response during the progression and reversal of iron deficiency in the rat. In addition we examined some of the factors that are involved in the adaptation to anemia and the possible influence of these factors on the erythropoietin concentration. During the progression of anemia, we found successively increasing erythropoietin levels (Fig 1), reaching a maximum on the day when the Hgb concentration was lowest. This high concentration of erythropoietin was not associated with an increase in reticulocyte count, presumably because the lack of iron for Hgb production restricts the rate of erythropoiesis. On introduction of the iron-sufficient diet, the reticulocyte count increased to reach a maximum after four to five days (Figs 1, 2). During the first four days posttreatment, the erythropoietin concentration decreased and was below (experiment 1) or equal to control values (experiment 2) after five days. This fall in erythropoietin levels during the treatment of iron-deficiency anemia is in accord with the observation in iron-deficient humans by de Klerk et al. As in our study, those authors found that erythropoietin levels fell during the period of reticulocytosis. Indeed, the decline in erythropoietin was evident even before there was a substantial Hgb response. It was suggested that this early fall in erythropoietin might indicate increased removal of erythropoietin from the circulation by erythropoietin receptors in the bone marrow. Our results suggest an additional or alternative explanation. The circulating level of erythropoietin depends not only on the Hgb concentration but more directly on the adequacy of oxygen delivery to the tissue or tissues that control the synthesis and release of erythropoietin. An important adaptation to anemia is a rightward shift of the oxygen dissociation curve that facilitates oxygen delivery to tissues. This change in the oxygen affinity of blood can occur within minutes due to a decrease in pH, within hours due to an increase in red cell DPG, and within days due to an increased proportion of young blood cells. The latter relationship has not been studied in relation to anemia or recovery from anemia.

In the present study, the P₅₀ was increased (a rightward shift in the oxygen dissociation curve) not only prior to iron treatment, but also through the first seven days of treatment. Initially, a high red cell DPG contributed to the elevated P₅₀, but later the elevation in P₅₀ was associated primarily with an increased number of circulating young red cells. This conclusion was strengthened by observations on red cells separated according to buoyant density and age by centrifugation on a Stractan gradient. Our finding of an elevated P₅₀ in the young red cells was in accord with the observation in human erythrocytes by Edwards and Rigas. The cause of this difference in P₅₀ is not clear, but it cannot be attributed entirely to corresponding differences in DPG. In this study, the reticulocytosis that occurred between four and seven days of treatment of iron-deficiency anemia was associated with a persistent elevation in P₅₀ at a time when the DPG concentration had returned to control values (Fig 3). Since the reticulocyte count was highest when the anemia was still profound, the reticulocyte- or young red cell-associated elevation in P₅₀ represented a temporary adaptation that enhanced oxygen delivery until oxygen-carrying capacity returned to normal. However, this adaptation, which is related to a cohort of reticulocytes (or young red blood cells), can apparently be reversed only gradually, as red cells mature. The

Table 1. P₅₀, Reticulocyte Count, and DPG in Low- and High-Density Fractions of Red Blood Cells Separated by Ultracentrifugation on a Stractan Gradient

<table>
<thead>
<tr>
<th>Layer</th>
<th>P₅₀ (torr)</th>
<th>Reticulocyte Count (%)</th>
<th>DPG (μM/g Hgb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top, low density layer</td>
<td>38.5</td>
<td>35</td>
<td>25.5</td>
</tr>
<tr>
<td>Bottom, high density layer</td>
<td>33.4</td>
<td>2</td>
<td>27.8</td>
</tr>
</tbody>
</table>
fall of erythropoietin levels before Hgb concentration is normalized might be explained by the persistence of an elevated P50 when the anemia has almost completely resolved. Our results are in accord with a highly sensitive feedback mechanism for the regulation of erythropoietin production that is responsive to both anemia and oxygen affinity. Furthermore, the persistent elevation in P50 appears to result in a more rapid correction of impaired oxygen delivery than would be possible through the slower reversal of anemia.

REFERENCES

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