Quantitative and Qualitative Aspects of Steady State Erythropoiesis Induced in Protein-Starved Rats by Long-Term Erythropoietin Injection

By Kenjiro Ito and Kurt R. Reissmann

The absence of protein from the food of rats results within a few days in a depression of red cell formation, and the latter proceeds during the first 4 dietary weeks at approximately one-tenth of its normal rate.1 If protein starvation is continued, a gradual recovery of erythropoiesis is noted, and the red cell mass tends to stabilize around one-half of its normal value. Evidence has been presented2,3 in support of a diminished erythropoietin formation as the cause of the erythropoietic depression, and experiments employing short-term injection of erythropoietin have failed to detect any rate-limiting effect of the protein deficiency on red cell synthesis per se. The present study attempts to determine whether rabbit erythropoietin, injected daily over a period of 1 month, can induce in protein-starved rats a steady state erythropoiesis which is quantitatively and qualitatively equal to that in normal rats. Such demonstration would lend further support to the dependency of normal erythropoiesis on erythropoietin. It would also prove that erythropoietin, extracted from plasma of anemic rabbits, provides complete substitution for lacking endogenous erythropoietin in the rat, and would permit an estimate of erythropoietin requirements during normal steady states. Accordingly, a daily dose of erythropoietin was selected which sustained reticulocytes in the protein-starved rats at levels equal to those in normal controls. The quantity of red cells formed in these animals was assessed by comparing their red cell mass at the end of a period of 32 days with that in normal controls or in untreated protein-starved rats. As it will be shown, nearly 40 per cent of the cells present were formed in response to the injected rabbit erythropoietin. The quality of these cells could thus be assessed with a high degree of confidence, because abnormalities in regard to physical properties or longevity of such a large fraction of the total cell population should be readily detectable.

Methods

Female Sprague-Dawley rats (180–200 Gm.) were given ad libitum Purina Rat Chow or a Protein Depletion Diet (Nutritional Biochemical Corp.) containing 84 per cent dextrin, 9 per cent corn oil, 2 per cent agar, 1 per cent cod liver oil and 4 per cent vitamin-salt mixture. Erythropoietin was obtained according to Borsook’s method4 from plasma of rabbits made

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rapidly anemic by bleeding plus injections of phenylhydrazine. The erythropoietin in the plasma extract was calibrated against Standard B (N.I.R. London), and the dosage is expressed as units of this standard. The experimental groups received daily subcutaneous injections of erythropoietin from the first to thirtieth day of protein starvation. The nitrogen content of the extract was 0.8 mg. N per unit. Protein-starved controls received saline injections containing an equivalent amount of nitrogen in the form of rat albumin.

Reticulocyte counts were made on 2000 red cells on wet preparations stained with brilliant cresyl blue. Hematocrits were determined by micromethod and hemoglobin concentrations by cyan-methemoglobin method. Price-Jones curves were obtained by measuring the diameter of 500 cells by means of a Leitz micrometer. Osmotic fragility was determined according to the method of Parpart. Red cell mass was measured by means of Cr\textsuperscript{51} labeled cells as previously described.

On the thirty-second experimental day, blood from various groups was labeled in vitro by adding 5 &micro;g. Cr\textsuperscript{51} (Rachromate) per ml. of blood. The washed cell suspensions were injected in aliquots of 1 ml. per recipient. and 4 normal rats were used for survival measurements of each cell suspension. Serial measurements of the circulating radioactivity were made by collecting 0.5 ml. of tail blood in liquid heparin. Hematocrits were determined in duplicate on each sample, and the Cr\textsuperscript{51} counts were expressed per 1 ml. of cells. The survival curves were corrected for the amount of radioactivity removed by sampling.

Cohort labeling was introduced by injection of 3 &micro;g. of Fe\textsuperscript{59} in each of the donor rats. The blood was collected 4 days later, and 2 ml. of the washed cell suspension was injected into the tail vein of normal rats (350 Gm. body weight). Four recipients were used for each donor blood. To prevent unphysiologic increases in the red cell mass of recipients, 1.5 ml. of their blood was removed immediately before cell injection. Each recipient received 50 mg. of nonradioactive iron (Inferon) on the day before the cell injection and 5 mg. i.m. every third day throughout the experiment in order to minimize reutilization of the iron label.

RESULTS

Reticulocytes and Erythroid Marrow

Figure 1 shows the rapid drop in reticulocytes after placing rats on a protein-free diet, and their gradual reappearance after 4 weeks of protein starvation. The daily injection of 1.8 units of rabbit erythropoietin prevented the reticulocyte drop and sustained reticulocytes at levels within the range found in normal controls. The daily injection of 5 units induced a reticulocytosis above the normal levels, whereas daily injections of 1 unit resulted in reticulocyte levels in the order of 2 per cent (not shown in Fig. 1). No significant fluctuations in the reticulocyte response of either group were seen throughout the 32 days of protein starvation. Differential counts on the erythroid marrow were obtained at the end of the experiment and did not reveal significant differences between the normal rats and rats which had received 1.8 units of erythropoietin (Table 1).

Red Cell Mass

Red cell mass was measured on day 32 in the 4 groups of rats whose reticulocyte counts were presented in Figure 1, and on day 0 in 1 companion group of same age and weight. In reference to the latter (Table 2), the red cell mass in the untreated protein-starved group decreased from 4.0 to 2.5 ml., whereas the erythropoietin-treated groups had average red cell masses of 4.26 and 4.88 ml., respectively, in spite of an average weight loss of 40 Gm. The red
cell mass of the group which received 1.8 units was not significantly different (p > 0.1) from that in the normal control rats, which gained nearly 40 Gm. in weight and increased their cell mass to 4.52 ml. The hematocrit in the untreated protein-starved rats remained nearly unchanged, reflecting the concomitant shrinkage of the plasma volume. The hematocrit in the erythropoietin-injected rats rose to 54 and 60 per cent, respectively, indicating that the erythropoietin-induced increase in red cell mass was not accompanied by increases in plasma volume.

Properties of Red Cells Formed in Response to Erythropoietin: A comparison of red cell indices in normal rats (Group I) and in protein-starved rats after 30 days of erythropoietin injection (Group III) did not reveal differences in regard to volume or hemoglobin content of these cells (Table 3). It should be noted that more than 40 per cent of the cells present in the latter group were made in response to erythropoietin, and even moderate differences should thus be detectable. Price-Jones curves confirmed the identical cell size in the two groups and their osmotic fragility curves were virtually identical.

Red Cell Life Span: Blood cells obtained on day 32 from Groups I, II and III were incubated with Cr\textsuperscript{51} in vitro, and the survival curves of these randomly labeled cells in normal recipients are shown in Figure 2. The Cr\textsuperscript{51} survival of cells from untreated protein-starved rats was significantly shorter than that of cells from the two other groups. The experiment was repeated with nearly the same results, namely a Cr\textsuperscript{51} half-life of 17 days for cells from normal and from erythropoietin-treated rats versus 9 days for cells from untreated protein-starved rats.

In order to study the lifespan of cohorts of equal cell age, radioiron was injected into the 3 groups and into 1 group of polycythemic rats (normal diet) which had received 2.5 units of erythropoietin. The curves presented in Figure 3 thus represent the survival of cells which were between 1 and 4
Table 1.—Erythroid Cell Counts (per 1000 Marrow Cells) in Normal Rats and after 32 days of Protein Starvation Plus Daily Injection of 1.8 Units of Erythropoietin (Mean of 4 Rats and Standard Error of Mean)

<table>
<thead>
<tr>
<th>Proerythroblasts</th>
<th>Pronormoblasts</th>
<th>Normoblasts Early</th>
<th>Normoblasts Late</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>2.1 ± 0.34</td>
<td>15.8 ± 1.5</td>
<td>61.1 ± 3.9</td>
<td>131.4 ± 7.9</td>
</tr>
<tr>
<td>Prot. Starv.</td>
<td>2.6 ± 1.1</td>
<td>19.5 ± 4.7</td>
<td>69.5 ± 6.8</td>
<td>124.6 ± 3.5</td>
</tr>
<tr>
<td>+ Erythrop.</td>
<td></td>
<td></td>
<td></td>
<td>220.6 ± 14.6</td>
</tr>
</tbody>
</table>

Table 2.—Red Cell Mass before and after 32 Days of Protein Starvation without and with Daily Injections of 1.8 and 5 Units of Erythropoietin (E)

<table>
<thead>
<tr>
<th>No. of Rats</th>
<th>Weight in Gm.</th>
<th>Total Red Cell Mass, mL†</th>
<th>Hematocrit Per Cent‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 32</td>
<td>Day 0</td>
</tr>
<tr>
<td>I. Complete diet</td>
<td>6</td>
<td>176 ± 3</td>
<td>214 ± 5</td>
</tr>
<tr>
<td>II. Protein starved, untreated</td>
<td>6</td>
<td>181 ± 3</td>
<td>131 ± 4</td>
</tr>
<tr>
<td>III. Protein starved + 1.8 U.E.</td>
<td>6</td>
<td>175 ± 6</td>
<td>126 ± 9</td>
</tr>
<tr>
<td>IV. Protein starved + 5 U.E.</td>
<td>6</td>
<td>172 ± 3</td>
<td>124 ± 3</td>
</tr>
</tbody>
</table>

*Mean and range.
†Mean and S.E.M.

Table 3.—Red Cell Indices of Blood from Protein-Starved Rats after 32 Days Injection with 1.8 Units Erythropoietin Daily (Mean and S.E.M. in 6 Rats)

<table>
<thead>
<tr>
<th>MCH</th>
<th>MCV</th>
<th>MCHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-starved + Erythrop.</td>
<td>20 ± 1.6</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>Normal rats</td>
<td>19 ± 2.4</td>
<td>58 ± 3</td>
</tr>
</tbody>
</table>

days of age at the time of their injection into normal recipients. The curves leveled off after 70 days, and the 10 to 15 per cent of radioactivity which remained in the circulating cells indicates the reutilization of the label. The survival curves of the 4 groups were not significantly different from each other, and the shorter survival of cells from untreated protein-starved rats, noted in the Cr51 survival curves, was absent.

DISCUSSION

The regulatory effect of erythropoietin has been largely studied in short-term experiments and an abundance of quantitative data has been obtained after single injections of exogenous erythropoietin into animals whose endogenous erythropoietin formation was suppressed by polycythemia. Their response, measured by reticulocyte counts or radioiron incorporation, reflects the magnitude of one finite wave of erythropoiesis induced by the injected erythropoietin. It is a valid comparative measurement of erythropoietic effect of the substance administered. The dose-response relation ascertained in such experiments, however, does not necessarily apply to steady states, and the observed erythropoietic stimulation cannot easily be interpreted in terms of quantity of...
cells or of hemoglobin synthesized. Our experiments were therefore designed to examine the role of erythropoietin in the physiologic range of steady state erythropoiesis. The use of protein-starved rats was based upon their proved depression of endogenous erythropoietin formation, and upon tentative evidence that their capability to synthesize normal red cells was not impaired by deficiencies in the proteinic building material.2 The results confirm the latter point. At equal reticulocyte levels, the erythropoietin-treated rats produced, as judged by their red cell mass, the same volume of red cells as the normal controls. No abnormalities were found in regard to cell size, cellular hemoglobin concentration or content, and the composition of the erythroid marrow gave no indication of an abnormal or ineffective erythropoiesis.

It is concluded, therefore, that the injection of 1.8 units of erythropoietin in protein-starved rats over a period of 32 days induced a steady state erythropoiesis which could not be distinguished from that in the normal rat. It follows that erythropoietin, which was extracted from plasma of anemic rabbits, provided a complete substitution for the lacking endogenous erythropoietin. The formation of the latter was not completely abolished in our
Fig. 3.—Survival of Fe59 labeled cohorts of red cells in normal recipients (mean of 4 in each group). Cell donors as indicated.

experiments, and it is furthermore realized that the dose-response relation of a daily subcutaneous injection may differ from that of continuous endogenous secretion of the hormone. Nevertheless, it would seem reasonable to infer that the amount of erythropoietin produced in normal rats per 24 hours is in the same order as the substitution dose. From measurements of erythropoietin disappearance rates and distribution space, it has been estimated that approximately one-hundredth of the amount of erythropoietin formed per 24 hours in the normal rat can be expected to be present in one ml. of plasma. A daily formation of 1 to 2 units in the normal rat, as inferred from the present experiments, would thus result in plasma levels between 0.01 and 0.02 units per ml. Plasma concentrations of this order cannot be measured by current available assay methods, and the estimated daily erythropoietin formation is thus compatible with the failure to demonstrate erythropoietin unequivocally in the plasma of normal rats.
The effect of long-term injections of erythropoietin into normal animals has been studied by Van Dyke\textsuperscript{1} in monkeys and by Keighley et al.\textsuperscript{2} in rats and mice. Rather large daily doses were found to be required for the induction of erythremia. In Keighley's experiments, for instance, the daily injection of about 16 units (Standard A) of rabbit erythropoietin raised the hemoglobin concentration in 200-Gm. rats to 22 Gm. per cent in 49 days, and the same level was maintained during an additional injection period of 84 days. The disparity in the response-dose ratio between physiologic substitution dose and the dose which effects increments above the normal cell mass is clearly illustrated in our experiments. The daily injection of 1.8 units maintained a normal erythropoiesis and was responsible for the difference of 1.7 ml. in the red cell mass between these animals and the untreated group. The daily injection of 5 units resulted in red cell mass which was 2.3 ml. greater than that in the untreated group. The 3.2 units of erythropoietin which were given daily to these animals in excess of the substitution dose thus increased their cell mass only by an additional 0.6 ml. of cells.

Intense erythroid stimulation, induced by severe bleeding or by injection of large doses of erythropoietin, results in the formation of macrocytes whose lifespan is abnormally short.\textsuperscript{12} During more moderate acceleration of erythropoiesis—for instance, after lesser doses of erythropoietin or small phlebotomies—a shortened lifespan was no longer demonstrable.\textsuperscript{13-15} The discrepancy between persistent reticulocytosis and relative small increase in red cell mass noted in group IV (Table 2) may nevertheless be related to a shortened lifespan of some of the cells which were formed in response to 5 units daily. The matter was not pursued because the present study was concerned with the dose of rabbit erythropoietin capable of inducing a normal steady state erythropoiesis. Red cells, formed in response to such a physiologic substitution dose, were found by both random and cohort labeling to have a normal life span. The presented disappearance curves of normal rat cells are in agreement with those reported by Burwell, Brickley and Finch,\textsuperscript{8} who pioneered the Fe\textsuperscript{59} cohort labeling method, and by Forssberg and Tribukait,\textsuperscript{15} who used C\textsuperscript{14} glycine as a label. The shape of the curves indicates a combination of random destruction and removal of senescent cells as the physiologic pattern in the rat, and a finite lifespan can therefore not be assigned to the rat erythrocyte.

Cells from untreated protein-starved rats were found to have abnormally short Cr\textsuperscript{51} survival times in normal recipients. A similar observation has been reported by Delmonte et al.,\textsuperscript{16} who labeled cells in vitro with Cr\textsuperscript{51} after 50 days of protein starvation. These investigators attributed the observed shorter survival of these cells in normal recipients to a structural defect which renders cells from protein-starved rats more sensitive to in vivo hemolytic agents. This interpretation neglects the abnormal age distribution in the red cell population of the protein-starved rat as a possible cause of shortened Cr\textsuperscript{51} half-life. Our data show the severe curtailment of red cell formation during protein starvation, and the cell population which was present in these animals after 32 dietary days was thus considerably older than the population in normal or erythropoietin-treated rats. A shorter survival of the significantly older popula-
tion is to be expected. This interpretation is supported by the results of cohort labeling. In these experiments, the label (Fe\textsuperscript{59}) was introduced during the formation of cells, and the survival of cells was thus compared, which were between 1 and 4 days of age when injected into the recipients. The failure to detect any shortened lifespan of these cells (formed between the thirtieth and thirty-fourth day of protein starvation) does not preclude structural defects in cells after longer periods of protein starvation. Conclusions to this effect, however, cannot be drawn from Cr\textsuperscript{51} survival studies employing randomly labeled populations with significantly anomalous age distributions.

**Summary**

Protein starvation nearly arrested erythropoiesis in rats, and the red cell mass decreased during a period of 32 days from 4.0 to 2.5 ml. Protein-starved rats, injected daily with 1.8 units of rabbit erythropoietin, had reticulocyte counts within the normal range, and their cell mass increased during the same period to 4.26 ml., as compared to 4.52 ml. in normal controls. Red cell indices, Price-Jones curve and osmotic fragility were normal on blood obtained from erythropoietin-treated groups. Differential counts on their erythroid marrow composition were not significantly different from those in normal rats. It is concluded therefore that daily injection of 1.8 units of rabbit erythropoietin induced, over a period of 32 days, a steady state erythropoiesis which, on the basis of the parameters studied, could not be distinguished from that in normal rats. No evidence of a shortened lifespan of cells formed in response to erythropoietin was found after either random (Cr\textsuperscript{51}) or cohort (Fe\textsuperscript{59}) labeling. Random labeled (Cr\textsuperscript{51}) cells from untreated protein-starved rats had significantly shorter chromate survival times than cells from normal or erythropoietin-treated rats. The difference is attributed to the altered age distribution in their red cell population.

**Summario in Interlingua**

Affamation proteinic quasi arrestava erythropoiese in rattos, e le massa del erythrocytos declinava in le curso de un periodo de 32 dies ab 4,0 ad 2,5 ml. Rattos in affamation pro proteina, le quales recipeva diurne injectiones de 1,8 unitate de erythropoietina de conilio, habeva numerationes reticulocytic intra le limites del norma, e lor massa cellular montava durante le mesme periodo ad 4,26 ml, a comparar con 4,52 ml in normal animales controlo. Le indices erythrocytic, le curva de Price-Jones, e le fragilitate osmotic esseva normal in sanguine obtenite ab animales tractate con erythropoietina. Numerationes differential in specimen de lor medulla erythroide non differeva significative-mente ab illos in rattos normal. Per consequente, il es concludite que le injection diurne de 1,8 unitates de erythropoietina de conilio induceva in le curso de un periodo de 32 dies un erythropoiese de stato stabile le qual—a base del parametros studiate—non poteva esser distinguite ab illo in rattos normal. Nulle evidentia de un reduce longevitate del cellulias formate in responsa a erythropoietina esseva trovate post marcation aleatori a Cr\textsuperscript{51} o post marcation de cohorte a Fe\textsuperscript{59}. Cellulas marcate aleatorimente a Cr\textsuperscript{51} ab
nontractate rattos in affamation pro proteina habeva significativemente reduce temores de superviventia a chromato que cellulas ab rattos normal o ab rattos tractate con erythropoietina. Le differentia es attribuite al alterate distribution secundo le etates in lor populationes erythrocytic.

REFERENCES

Quantitative and Qualitative Aspects of Steady State Erythropoiesis Induced in Protein-Starved Rats by Long-Term Erythropoietin Injection

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