Protein Metabolism and Erythropoiesis. II. Erythropoietin Formation and Erythroid Responsiveness in Protein-deprived Rats

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On the basis of findings presented in the preceding paper, two processes were considered as the possible cause of the marked depression of erythropoiesis in the protein-deprived animal: (1) a retardation of cytoplasmic protein synthesis in erythroid cells due to a suboptimal concentration of substrate; and (2) a depressed formation of erythropoietin. The latter can not be ascertained directly because present assay methods do not permit quantitative measurements of subnormal plasma levels of erythropoietin. An assessment of the former requires like information on the erythropoietin level because of a possible antagonism between the accelerating action of erythropoietin and the retarding effect of depressed cytoplasmic protein synthesis. The problem was approached by comparing the rate of red cell formation at equal erythropoietin levels in protein-starved rats with that of rats on a normal diet. At equal erythropoietin levels the remaining variable is the erythroid responsiveness, and a depression of it by rate-limiting effects of lowered substrate concentration should be demonstrable.

The following procedures were used to induce equal levels of (1) exogenous and (2) endogenous erythropoietin: (1) Rats on normal and non-protein diet were made polycythemic by hypertransfusion in order to abolish, or at least to suppress to a large extent, their endogenous erythropoietin formation. Identical amounts of erythropoietin, over a 20-fold dose range, were then injected into both dietary groups. The reproducible dose-response curves obtained in hypertransfused animals justifies the assumption that their erythropoietin levels depend in first order on the amount injected. (2) Endogenous erythropoietin formation was raised to measurable levels by exposing normal and protein-deprived rats to a graded lowering of atmospheric pressure. One-half of each group served for measurements of Fe incorporation. The plasma of the other half was collected and assayed for its erythropoietin concentration. These experiments were expected to provide information on the erythropoietin formation in the two dietary groups under the same environmental stimulus. Their primary purpose, however, was to induce, irrespective of the required degree of hypoxia, identical erythropoietin levels in the two dietary groups which would permit a comparison of their resulting rate of erythropoiesis.

From the Department of Medicine, University of Kansas Medical Center, Kansas City, Kan.
Supported by U. S. Public Health Service Grant HE-05502-03 (HEM).
Submitted June 28, 1963; accepted for publication Aug. 26, 1963.

Blood, Vol. 23, No. 2 (February), 1964
METHODS

Experimental animals, diets, erythropoietin preparation and isotope technics were as described.1 Polycythemia was induced in rats after 7 days of protein deprivation and in rats on normal diet by intraperitoneal injection of from 3 to 4 ml of washed red cells on two consecutive days. Erythropoietin was injected subcutaneously on days 3 and 4 after the second cell transfusion. Radioiron was given intravenously on day 5. Red cell mass (Cr51) and iron incorporation were determined 24 hours later.

In each of the hypoxia experiments, one group of 14 normal and one group of 14 protein-deprived rats (10 days) were exposed to lowered atmospheric pressure. The decompression chamber had a flow-through of about 10 liters of air per minute and the chamber pressure was kept constant within ±0.25 inches of Hg. The plasma of eight animals in each group was collected after an exposure of 30 hours. The remaining six animals in each group received radioiron after an exposure of 54 hours and their red cell mass and Fe59 incorporation were determined 18 hours later. The pooled plasma was assayed in female mice made polycythemic by exposure (18 hours daily, 3 weeks) to a barometric pressure of 380 mm. Hg.3 On days 3, 4 and 5, after removal from the chamber, each assay mouse received 1 cc. of plasma. Fe59 was given intravenously after the last plasma injection and heart blood obtained for counts 48 hours later. At least four mice were used in the assay of each pooled plasma.

RESULTS

(1) Hypertransfusion Experiments

The hematocrits in the hypertransfused rats ranged at the end of the experiments from 68 to 76 per cent in the protein-deprived, and from 64 to 71 in the normal diet groups. Eight animals in each of the two dietary groups received saline injections instead of erythropoietin. Their 24-hour Fe59 incorporation was between 0 and 1.6 (mean 0.6) per cent in the protein-deprived, and between 2.7 and 6.4 (mean 4.2) per cent in the normal diet group. The iron incorporations after erythropoietin injections are presented in figure 1. The slightly higher incorporations in the normal diet animals after injection of 0.5 and 1.0 units are attributed to their higher baseline values which probably reflect a less complete suppression of their endogenous erythropoietin formation. The dose-response curves are otherwise nearly identical, indicating that protein deprivation did not alter the responsiveness of the erythroid marrow.

The circulating red cell does not participate in the internal protein exchange4 and the cell transfusion in itself thus does not constitute a protein supplementation. Protein precursors released after the breakdown of transfused cells, however, might have increased the substrate concentration and thereby invalidated the experiments. The red cell breakdown was therefore estimated from red cell volumes before and 6 days after transfusion, and from the amount of cells transfused. It amounted to 1.8 ± 0.2 cc. of cells, or about 1.4 cc. in excess of the breakdown in non-transfused rats. The effect of protein precursors derived from this volume of cells was tested in control experiments by injecting protein-deprived rats over a 6-day period with 0.2 and 0.3 cc. of hemolyzed cells daily. The cells were given intraperitoneally in two divided doses and did not cause hemoglobinuria. The results are presented
in table 1. The 0.3 cc. dose caused slight increases in iron incorporation, but the large difference which still exists between these animals and rats on a normal diet indicates that the protein released in breakdown of red cells did not materially affect the hypertransfusion experiments.

(2) Hypoxia Experiments

The temporal relations between exposure to lowered atmospheric pressure and rise in erythropoietin titer or Fe⁵⁹ incorporation were studied in preliminary experiments. The 18-hour Fe⁵⁹ incorporation rose to a maximum on the 3rd day of exposure and remained at that level during the following 2 days. The most consistent rise in erythropoietin titers was found after 24 to 48 hours of exposure. Plasma was therefore collected routinely after 30 hours. Radioiron was injected after an additional exposure of 24 hours in accordance with time lag between erythropoietin stimulation and the appearance of the corresponding crop of erythrocytes in the peripheral blood.⁵

The erythropoietin titers and iron incorporations in protein-deprived and normally fed rats at six different levels of atmospheric pressure are presented in figure 2. The erythropoietin titers, expressed as Fe⁵⁹ incorporations found in the assay mice, rose in both dietary groups with increasing severity of hypoxia, but at each level the titers in protein-starved animals were significantly lower than those in the normal diet groups. The iron incorporations showed a similar relationship with the exception of exposure at an atmospheric pressure of 11 inches of Hg. Six of the 14 normal, and three of the protein-deprived animals died during the exposure. The iron incorporations of the surviving rats were significantly lower than those observed at the 12.5 inches
Table 1.—Effect of Intraperitoneal Injection of Hemolyzed Cells on Fe\textsuperscript{59} Incorporation of Protein-deprived Rats (Mean and Range of Four Rats in Each Group)

<table>
<thead>
<tr>
<th></th>
<th>No Erythropoietin</th>
<th>1.2 Units Erythropoietin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-protein diet</td>
<td>3 ± 1</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>Non-protein + 0.2 cc. cells</td>
<td>6 ± 3</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>Non-protein + 0.3 cc. cells</td>
<td>11 ± 4</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Normal diet</td>
<td>34 ± 6</td>
<td>42 ± 5</td>
</tr>
</tbody>
</table>

pressure level, whereas the erythropoietin titers in both dietary groups showed a disproportionate increase.

At an atmospheric pressure of 12.5 inches of mercury, the erythropoietin titer of the protein-deprived rats was about equal to that found in the normal diet group at 15.5 inches of chamber pressure. The iron incorporations were 38 and 41 per cent, respectively, indicating that nearly equal endogenous erythropoietin concentrations resulted in nearly identical rates of erythropoiesis in the two dietary groups.

3) Effect of Realimentation on Erythropoietin Formation

Feeding of protein was found to raise within 3 days the depressed Fe\textsuperscript{59} incorporation in protein-starved rats to normal values. From the present findings it appears likely that this effect is attributable to an increase in erythropoietin formation, and, moreover, the erythropoietin increase must have occurred within the first 24 hours after re-proteinization. This was tested experimentally by measuring erythropoietin levels in three groups of rats after 30 hours exposure at an atmospheric pressure of 14 inches of Hg. Two of the groups had been on a protein-free diet for 10 days. The first group remained on this diet. Rats of the second group received 2 gm. of lactalbumin hydrolyzate by stomach tube immediately before exposure, and their diet was switched to Purina rat chow. The third group was on normal diet throughout. The plasma was collected after an exposure of 30 hours and was assayed in polycythemic mice. The erythropoietin titers, expressed in per cent Fe\textsuperscript{59} incorporation in the assay mice, were 3.6 per cent in the protein-deprived, 7.4 per cent in the re-proteinized, and 9.4 in the normal diet group. The results show that protein administration resulted within 30 hours in a significant increase in erythropoietin formation.

Discussion

The presented experiments were primarily concerned with the detection of a possible rate-limiting effect of protein deficiency on protein synthesis in erythroid precursors. Such a retardation would decrease the responsiveness of the erythroid marrow and would cause fewer red cells to be formed, per unit of time, in the protein-deprived than in the normally fed animal at the same erythropoietin level.

The injection of exogenous erythropoietin permitted the induction of equal erythropoietin levels over a wide range of concentrations. At each level, the
iron incorporation in the protein-deprived rats was nearly identical with that in rats on normal diet. The serum iron levels were not significantly different in the two dietary groups, and identical rates of iron incorporations were interpreted as identical rates of erythropoiesis. The induction of equal levels of endogenous erythropoietin in the two dietary groups by means of hypoxia was limited by the depression of erythropoietin formation in the protein-starved groups and by the degree of hypoxia which could be applied without impairing erythropoietic response. An overlapping of erythropoietin titers in the two dietary groups was achieved only at one level, but the nearly equal iron incorporation found at this level is of considerable significance because it affirms the normal responsiveness of the protein-deprived animal in respect to endogenous erythropoietin. It is concluded that protein deficiency does not affect the cytoplasmic protein synthesis in erythroid cells directly, and the depression of erythropoiesis is attributed to a diminished formation of erythropoietin. The latter can not be demonstrated by assay methods in the unchallenged animal, but it is supported by strong indirect evidence. At all tested levels of hypoxia, the protein-deprived rats were found to have considerably lower erythropoietin titers than their normally fed counterparts, and it seems reasonable to extrapolate this difference to the erythropoietin formation at normal atmospheric pressure. The daily injection of erythropoietin has been shown to prevent a decline in red cell mass during a period of 4 weeks of protein starvation, but it remained undecided whether this indicated a restor-
eration of normal erythropoietin levels or whether the injections raised the erythropoietin to abnormally high levels which compensated for a decreased erythroid responsiveness. The present findings eliminate the latter possibility and the prevention of the anemia of protein starvation by erythropoietin is regarded as confirmatory evidence of the diminished erythropoietin formation during protein deficiency.

The lower level erythropoietin formation provides a rational explanation of what has been referred to as the economical utilization of amino acids in red cell formation during protein deprivation, namely its depression in the absence of anemia and the considerable regeneration after the emergency of blood loss. The former is simply the result of diminished erythropoietin formation, and the latter is explained by the fact that the protein-deprived animal has neither lost its responsiveness to erythropoietin nor the ability to increase its erythropoietin formation in response to anemic or hypoxic stimuli. The utilization of amino acids in erythropoiesis is thus governed by the level of erythropoietin. This also explains another puzzling aspect of erythropoiesis in relation to protein deficiency; namely, the precedence of hemoglobin formation over the production of other proteins during the early stages of realimentation. An increased supply of protein precursors after protein repletion is available to all tissues, but the erythroid tissue responds faster because realimentation results within 30 hours in a significant increase in erythropoietin formation, and it is the latter and not the improved supply of the erythroid cells with building material which is responsible in the priority position of erythropoiesis.

The question as to how protein deprivation depresses erythropoietin formation can not be answered at this time. The oxygen tension in erythropoietin producing tissue is generally accepted as the principal regulator. Hypometabolic states are known to be associated with a depressed erythropoiesis, and a decrease in oxygen consumption in the presence of a normal oxygen supply is thought to result in a relative hyperoxidosis and thereby in a decreased erythropoietin formation. The metabolic rate declines during protein deprivation, and an imbalance between oxygen supply and demand must therefore be considered as a possible cause. Aschkenasy has reported marked reticulocyte increases after administration of calorigenic hormones in protein-deprived rats, but he seems to deny a causal relationship between increase in oxygen consumption and reticulocytosis. An alternative possibility is related to the protein nature of erythropoietin. Decreases in tissue enzymes as well as in circulating enzymes have been demonstrated in protein-deficient animals and are attributed to rate-limiting effects of lowered substrate concentration on the synthesis of these proteins. It is attractive to speculate on a similar cause of the depressed erythropoietin level because it would link protein deficiency more directly to erythropoietin formation than the mentioned imbalance between oxygen consumption and supply.

SUMMARY

(1) Rats on a normal and non-protein diet were hypertransfused to suppress their endogenous erythropoietin formation. Injection of erythropoietin
elicited nearly identical increases in radioiron incorporation in the two dietary groups.

(2) Normal and protein-starved rats were exposed to lowered atmospheric pressure. At each level of hypoxia the erythropoietin titer in the plasma of protein-starved rats were significantly lower than those in normal diet groups.

(3) Re-proteinization resulted in significant increases in erythropoietin level.

(4) It is concluded that protein deficiency does not affect cytoplasmic protein synthesis in erythroid precursors directly, and the depression of erythropoiesis is attributed to a diminished formation of erythropoietin.

SUMMARY IN INTERLINGUA

1. Rattos recipiente un dieta normal o un dieta sin proteina esseeva subjicite a hypertransfusion pro supprimer lor formation endogene de erythropoietina. Le injection de erythropoietina evocava quasi identic augmentos in le incorporation de ferro radioactive in le duo grupplos dietari.

2. Rattos normal e rattos affamate pro proteina esseeva subjicite a conditiones de reducite pression atmospheric. A omne nivelbos de hypoxia, le titro de erythropoietina in le plasma del rattos private de proteina esseeva significative-mente plus basse que illo in le grupo a dieta normal.

3. Re-proteinisation resultava in significative augmentos del nivello de erythropoietina.

4. Es concludite que carentia de proteina non affice le synthese cytoplas-matic de proteina in le precursores erythroide directemente. Le depression del erythropoiesis es attribuite a un reducite formation de erythropoietina.

REFERENCES


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