The Hemolytic Anemia of Magnesium Deficiency in Adult Rats

By Sergio Piomelli, Valerie Jansen, and Joseph Dancis

The administration of a Mg-deficient diet to adult rats for 4–5 wk causes anemia. The RBC are slightly smaller and flatter, and have a reduced hemoglobin component and a decreased osmotic fragility. $^{51}$Cr survival time is shortened when the affected cells are infused into normal adult rats. In vitro synthesis of heme and globin, as measured with $^{14}$C-glycine was the same as in control rats with "reticulocytosis-rich" anemia produced by bleeding or immunologically. The activity of a series of enzymes associated with energy production also failed to yield findings specific for Mg deficiency. It is suggested that the anemia of Mg deficiency is hemolytic and results from the combination of a reversible extrinsic defect and of an irreversible structural defect in the RBC.

The effects of specific nutritional deficiencies in the pregnant rat have been systematically investigated in this laboratory. In the course of these studies, a microcytic anemia with erythroblastosis was observed in the offspring of Mg-deprived rats. Hemolysis was strongly suggested by the intense erythroblastosis. There was also an increased incorporation of radioactive iron into the fetal hemoglobin when $^{59}$Fe was administered to the Mg-deficient dam. However, it was not possible to define with certainty the nature of the anemia for several reasons. In the normal newborn rat nearly every circulating red cell is a reticulocyte, and accumulation of indirect bilirubin is prevented by transplacental clearance. Furthermore, the studies that could be undertaken were restricted because the offspring of Mg-deficient dams died shortly after birth.

It was observed that a more prolonged period of magnesium deprivation in the adult rat induces a similar anemia associated with marked reticulocytosis. This experimental model appeared to be much more suitable than the newborn rat for the study of the hematologic effects of Mg deprivation. Several experiments were therefore performed in the Mg-deprived adult rat. Because the anemia was accompanied by an intense reticulocytosis, the observations in the Mg-deprived animals were compared to measurements made on a group of rats in whom reticulocytosis had been induced by immune
hemolysis or by bleeding, in addition to a control group of animals on a Mg-repleted diet.

MATERIALS AND METHODS

Diet

The formulation was that described by us in a separate study of Mg deprivation.2 This was essentially the Na-deficient test diet described by Hartroft and Eisenstein,5 corrected by the addition of an appropriate salt mixture. MgSO₄ was omitted from the magnesium-deficient diet. Analyses in this laboratory demonstrated Mg content to vary between 0.50 and 0.53 meq/kg in the deficient diet and between 109 and 113 meq/kg in the control diet.

Animals

Nonpregnant adult female rats weighing approximately 200 g were obtained from Charles River Breeding Laboratories. Most of the studies were performed after 4–5 wk of test diet.

“Reticulocytosis Control” Animals

Reticulocytosis was induced in normal rats by production of either hemolytic or hemorrhagic anemia. An antiserum to rat RBC was prepared in rabbits by a series of three intravenous injections of 1 ml of rat blood on days 0, 3, and 6. Serum was harvested 1 wk later. The average antibody titer against rat RBC was 1:1000. One-fourth milliliter of this antiserum was injected intravenously into experimental rats, and the reticulocyte count was determined 2 days later. In some instances, a second injection was necessary to produce the desired level of reticulocytosis (13%–15%). The anemia produced by the antiserum resulted in marked acute hemolysis. Spherocytosis was apparent only in the first 24–48 hr. Intense reticulocytosis developed and persisted for 7–10 days. Reticulocyte-rich blood was, therefore, collected 1 wk after the last injection.

Hemorrhagic anemia was produced by bleeding rats from the tail vein of 2 ml of blood, twice weekly for 2 wk. Iron (25 mg in the form of Imferon) was given intraperitoneally at the time of the first bleeding, to prevent iron deficiency. Blood was collected at the end of the second week, at which time the reticulocytosis was usually between 12%–15%.

Plasma Mg, red blood cell hematologic indices, reticulocyte percentages, osmotic fragility, and microscopic studies were determined as previously described.3

Hemoglobin Analysis

Hemoglobin analysis was performed on cyanmethemoglobin solutions prepared from washed RBC by hemolysis with saponin in 0.05 M Tris buffer at pH 8.8, containing 0.02% K₃Fe(CN)₆ and 0.02% KCN. One part in ten of 10% NaCl was added after hemolysis. The stroma was removed by shaking with chloroform and by centrifuging at 30,000 rpm for 20 min. The hemoglobin solutions were studied by electrophoresis on starch block,° on starch gel with a discontinuous buffer system,7 and by alkali denaturation.8

RBC ⁵¹Cr Survival Time

RBC ⁵¹Cr survival time was measured in seven control and seven magnesium-deficient adult rats. The RBC were labeled with ⁵¹Cr and were then injected into normal adult rats on house diet. Radioactivity in cpm/ml was plotted against time on semilogarithmic paper, and the half-life (t½) was determined by the best fitting line.

Studies of Hemoglobin Synthesis

Studies of Hb synthesis were performed by incubation in vitro with either glycine-2-¹⁴C or ⁵⁹FeSO₄. In each experiment, pooled normal rat plasma was diluted 1:1 with 0.15 M sodium phosphate buffer, pH 7.4. To the buffered plasma was added either glycine-2-¹⁴C (0.7 μCi/ml) or ⁵⁹FeSO₄ (4.5 μCi/ml). The incubation medium was subdivided for use with control, Mg-deficient, or reticulocyte-rich RBCs. In the ⁵⁹Fe experiment, the total amount of elemental Fe in the medium never exceeded two-thirds of the Fe-binding
capacity. Washed RBCs were suspended in 9 volumes of medium and were incubated at 37°C for 3 hr. At the end of the experiment, the cells were washed five times with saline. A cyanmethemoglobin solution was prepared as described above and was dialyzed overnight against Tris 0.5 M pH 8.8. Radioactivity was determined in a well-type scintillation counter for estimation of 59Fe incorporation. Heme was crystallized after addition of carrier Hb and was counted as described by Murtz and Nair. Globin was precipitated from the hemoglobin solution with acetone, and the protein was measured according to Layne.

Glucose Consumption and Lactate Production

Glucose consumption and lactate production were measured in "pure red cell suspensions" obtained as described previously. The washed red cells were suspended to a hematocrit of 25%. The suspending buffer consisted of 9 volumes of Eagle’s solution and 1 volume of 0.1 M TES buffer, pH 7.4. The pH of the RBC suspension was adjusted to 7.4 by bubbling with CO2. Cross experiments were performed in which red cells from normal, Mg-deficient, or reticulocytosis control rats were incubated in plasma from either normal or Mg-deficient rats. For these experiments, heparinized blood was centrifuged at 1500 g; the plasma was separated, and the buffy coat was removed. The sedimented red cells were then reconstituted to a hematocrit of 25% in the designated plasma, and the resulting suspension returned to pH 7.4 by bubbling with CO2.

Glucose was measured enzymatically by the hexokinase reaction, and lactate was measured with beef lactic acid dehydrogenase utilizing acetylpyridine-NADP as substrate. Results were expressed as μmoles/hr/g Hb.

Activities of glycolytic enzymes and glucose-6-phosphate dehydrogenase (G6PD) in the RBC were measured with the techniques standard in this laboratory.

RESULTS

Clinical Observations

The experimental animals rapidly developed the characteristic hyperemia of ears and feet but otherwise remained vigorous and continued to gain weight for the 5-wk duration of the experiment. Plasma magnesium fell rapidly, and anemia developed more slowly (Fig. 1).

Hematologic Data

The results of the hematologic studies are summarized in Table 1. The Mg-deficient animals exhibited a mild anemia accompanied by intense reticulocytosis. The hemoglobin and hematocrit percentage were significantly decreased, but the number of RBC per unit volume was unchanged. Morphologically, the red cells appeared more deeply concave (Fig. 2). The red cell indices indicated moderate microcytosis, with decreased mean corpuscular hemoglobin and unchanged mean corpuscular hemoglobin concentration. These changes were minor when compared to normal rat red cells but were more obvious when compared to the cells of rats with a similar degree of reticulocytosis. The microcytosis of the red cells of Mg-deprived rats was paralleled by a markedly decreased mean osmotic fragility with a shift to the left of the entire curve. Red cells of control rats with a comparable degree of reticulocytosis had osmotic fragility curves identical to the controls. Serum Fe and total Fe-binding capacity were measured in six pairs of control and Mg-depleted rats. There was no significant difference.

51Cr Survival Studies

51Cr survival studies were performed by labeling the RBCs of seven pairs of control and Mg-deficient rats. When these were injected into normal rats...
on house diet, the average $t_{1/2}$ was $15 \pm 2$ days for RBCs from control rats and $11.7 \pm 2$ days for RBCs of Mg-deprived animals. The difference was highly significant by paired t test ($p < 0.001$).

**Qualitative Hemoglobin Studies**

The five electrophoretic bands previously described in normal rat hemoglobin were observed. Their relative concentrations were not different in the Mg-deprived animals. No differences were observed in the alkali denaturation rates of either the whole hemoglobin solution or the individual bands eluted from the starch block.

**In Vitro Hemoglobin Synthesis**

Average $^{59}$Fe incorporation was increased in the RBC of Mg-deprived rats (368 cpm) when compared to incorporation in control RBCs (64 cpm). There was no difference, however, when compared to the $^{59}$Fe incorporation into the RBCs of the reticulocytosis control animals (442 cpm).

Similar observations were obtained when Hb synthesis was studied by measuring glycine-2-$^{14}$C incorporation into isolated heme and globin.

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**Fig. 1.** Mg deprivation in adult rats. Changes in plasma Mg, hemoglobin concentration, and body weight. Mean values of four control and four Mg-deficient animals. Controls, solid line; Mg-deficient, dashed line.
HEMOLYTIC ANEMIA

Table 1. Plasma Mg and Hematologic Data in Control, Mg-deficient, and Reticulocytosis-rich Controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (11)</th>
<th>Mg-deficient (8)*</th>
<th>Reticulocytosis-rich (8)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
</tr>
<tr>
<td>Plasma Mg (meq/liter)</td>
<td>1.47 (0.03)</td>
<td>0.22 (0.03)</td>
<td></td>
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<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>13.6 (0.28)</td>
<td>11.7 (0.40)</td>
<td></td>
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<tr>
<td>Hematocrit (%)</td>
<td>43.9 (1.16)</td>
<td>38.7 (1.79)</td>
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</tr>
<tr>
<td>RBC (× 10⁶/ cu mm)</td>
<td>6.7 (0.32)</td>
<td>6.7 (0.46)</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>2.8 (0.31)</td>
<td>13.1 (2.10)</td>
<td>12.4 (1.22) &lt;0.001</td>
</tr>
<tr>
<td>MCV (μm)</td>
<td>66.4 (3.33)</td>
<td>60.1 (1.91)</td>
<td>82.1 (3.10) &lt;0.005</td>
</tr>
<tr>
<td>MCH (μg)</td>
<td>20.7 (1.15)</td>
<td>19.8 (0.84)</td>
<td>27.05 (1.02) &lt;0.005</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>31.1 (0.2)</td>
<td>30.2 (0.71)</td>
<td>32.9 (0.59) 0.2</td>
</tr>
<tr>
<td>MCF (=NaCl g/100 ml)</td>
<td>0.43 (0.1)</td>
<td>0.38 (0.01)</td>
<td>0.43 (0.01) 0.6</td>
</tr>
</tbody>
</table>

* Reticulocytosis induced by producing hemolytic anemia with rat RBC antiserum.
† After 5 wk of diet.
‡ For the difference between control and Mg-deficient animals.
§ For the difference between Mg-deficient and reticulocytosis groups.
|| For the difference between control and reticulocytosis groups.

normal rat RBC the mean radioactivity was 345 cpm/mg heme and 5700 cpm/mg globin; in Mg-deficient rat RBC the mean was 2750 cpm/mg heme and 5340 cpm/mg globin; in reticulocytosis control RBC the mean was 2780 cpm/mg heme and 6700 cpm/mg globin. These experiments indicated that the glycine-2-¹⁴C incorporation into heme was equally increased by Mg deficiency and by reticulocytosis induced by other methods. Incorporation into globin was unaffected by either reticulocytosis or Mg deficiency.

Metabolic Studies

The activities of all glycolytic enzymes and of G6PD were measured in pure RBC suspensions from control, Mg-deprived, and reticulocytosis control rats. A slight increase in most enzyme levels was observed in the RBC from Mg-deprived rats and from reticulocytosis control rats. These increases were more marked and significant for hexokinase, aldolase, pyruvate kinase, and glucose-6-phosphate dehydrogenase. These enzymes are, at least in human RBCs, the most markedly age dependent. The increased enzymatic levels observed in Mg-deprived rats were consistent with a younger RBC population.

Glucose consumption and lactate production were studied next in cells suspended in buffer. Control RBCs utilized 12.6 ± 2.4 μmoles glucose/hr/g Hb and produced 23.7 ± 3.6 μmoles lactate/hr/g Hb (mean lactate/glucose ratio of 1.9 ± 0.1). Mg-deficient rat RBCs utilized 14.4 ± 2.4 μmoles glucose/
hr/g Hb and produced $25.3 \pm 5$ μmoles lactate/hr/g Hb (ratio $1.8 \pm 0.2$). Similarly, reticulocyte-rich RBCs utilized $14.8 \pm 3.3$ μmoles glucose/hr/g Hb and produced $26.3 \pm 6.1$ μmoles lactate/hr/g Hb (ratio $1.8 \pm 1.5$). Thus, in both Mg-deprived rats and reticulocytosis control rats, the RBCs exhibit a slight increase in glucose consumption accompanied by a parallel increase in lactate production. The lactate/glucose ratio remained unchanged. These findings were consistent with a younger mean RBC age in Mg-deficient rats.

Since the buffer used in these studies contained Mg, the glucose consumption, lactate production, and lactate:glucose ratio were also measured by incubating RBCs from control and Mg-deficient rats in plasma from either normal or Mg-deficient rats. The results of these experiments did not differ from those done with buffer as the incubation medium. The experiments were undertaken to investigate the possibility that Mg depletion, or other electrolyte changes that might occur in the Mg-deficient rat plasma, could affect RBC metabolism. No effect was detectable.

**DISCUSSION**

During our studies of anemia in the offspring of Mg-deficient dams, attempts were made to extend the experimental period by not interrupting pregnancy, but most newborns from the Mg-deficient mothers died soon after birth. A fortuitous observation was made during these experiments. The
mothers on the Mg-deficient diet of Ko et al. supplemented with Mg (control diet) became weak, fed poorly, and developed ruffled fur during the first week postpartum. These symptoms reversed within 24 hr when the animals were placed on a house diet. Similar experiments in which the control diet was based on the formula described by Hartroft and Eisenstein and was used previously in this laboratory to induce Mg deficiency produced no symptoms. It was evident that the formulation by Ko et al. even after repletion with Mg was inadequate to maintain rats under the stress of pregnancy and nursing.

There are obvious differences in the composition of the control diet described by Ko et al., and that of Hartroft and Eisenstein; for example, the published analyses indicate corn oil 150 g/kg vs. 70, calcium 3.7 g/kg vs. 8.0, Mg 0.74 g/kg vs. 1.6, and phosphate 11.7 g/kg vs. 15.3. To exclude the possibility of a relative Mg insufficiency, the Mg content of the diet of Ko et al. was raised to 1.6 g/kg, but this did not prevent the symptoms. No further attempts were made to determine the factor or factors responsible for the deficiencies of the diet of Ko et al. All subsequent experiments were done with diets based on the Hartroft and Eisenstein formula, as previously used in this laboratory.

When preliminary studies demonstrated that anemia appeared in mature rats after 4 wk of Mg deprivation, it was decided to concentrate on this experimental model in an attempt to elucidate the mechanism of the hematologic effect of Mg deficiency. Intense reticulocytosis clearly indicated that the anemia was hemolytic in nature. The anemia of Mg-deficient adult rats was characterized, as in the fetus, by decreased hemoglobin and hematocrit concentration with normal RBC number per unit volume. These findings suggested that the cells were microcytic but not hypochromic. The changes were more clearly significant by comparison with the RBC of animals with a similar degree of reticulocytosis (reticulocytosis control). The cell volume and hemoglobin content appeared insignificantly smaller by comparison to RBC of normal age, but they were obviously decreased, when the degree of reticulocytosis was considered. (In the rat, at variance with other species, hemoglobin is lost through the process of aging. Rat reticulocytes, therefore, are not only larger, but also have an increased mean corpuscular hemoglobin.) The increased surface/volume ratio was also reflected in the microscopic appearance and in the grossly decreased osmotic fragility. The serum iron and unsaturated iron-binding capacity were not significantly different in the Mg-deficient and control animals, excluding iron deficiency anemia.

When hemoglobin synthesis was studied in vitro, the increased incorporation of both $^{59}$Fe and glycine-$^{14}$C into heme was found to reflect an increased percentage of reticulocytes and was not specifically due to Mg deficiency.

Magnesium is an element of great importance in cellular metabolism and is a necessary cofactor for several enzymes. This suggested the possibility that the hemolytic anemia resulted from disturbances in energy metabolism in the RBC. When the metabolic effects of Mg deprivation were studied in the RBC of adult rats, a slight increase in the rate of glucose utilization and increases in several Mg-dependent enzymatic activities were noticed. These
effects were most likely secondary to the younger red cell population, since they were also observed in the red cells of the reticulocytosis control animals. At least in vitro, therefore, the decreased Mg level was still adequate for normal (and slightly increased) metabolic activities.

These results conflict with the recent reports of Elin et al. and Oken et al., who have attributed the anemia in the Mg-deficient adult rat to ATP depletion and spherocytosis. Oken et al. also noted a decreased in vitro glucose utilization. Both groups of investigators used the diets formulated by Ko et al. The observations in our laboratory that nursing rats became sick on the control diet described by Ko indicate that this diet is marginal or unbalanced in some undefined nutrients. This might explain the grossly abnormal lactate:glucose ratio (2.47) reported by Oken for the RBC in their control rats. It is quite possible that the additional stress of Mg deficiency produced distinct metabolic abnormalities.

Observations in the human have demonstrated that deficiencies of essential cations might interfere with or exaggerate each other. Hypophosphatemia, alone, produces abnormalities in glycolysis without hemolysis. Jacobs and Amsden have reported a patient with severe hypophosphatemia accompanied by hypomagnesemia in whom hemolysis was noted. In view of the physiologic interactions of Mg and ATP, hypomagnesemia could exaggerate the effects of ATP depletion. It is, perhaps, significant that a hypophosphatemia was induced in the experiments of Oken et al.

It is clear from the present studies that a Mg depletion, more profound than that produced by either Elin et al. or Oken et al., will induce in the rat a severe hemolytic anemia in the absence of either marked spherocytosis or severe glycolytic defect. The Mg-deficient RBCs have a reduced survival even when infused into the circulation of normal rats. The reduction in lifespan of the Mg-deficient cell in the normal environment is relatively small by comparison with the intense reticulocytosis in the Mg-deficient animal itself. This discrepancy suggests that Mg deficiency caused both intrinsic and an extrinsic RBC defect. Only the extrinsic defect is corrected on return to an environment with normal Mg content, while the intrinsic defect is irreversible. The nature of the structural defect remains to be determined. Mg exists in the cell in free and complexed form, and it is probably the latter that is more significant to the integrity of the cell.

REFERENCES

7. Poulik, M. D.: Starch gel electrophore-
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