Iron Metabolism in the Belgrade Rat

By John Edwards, Helmut Huebers, Christoph Kunzler, and Clement Finch

Iron metabolism in the Belgrade rat was examined in the intact animal and in the reticulocyte suspensions. The plasma iron turnover was increased. However, when allowance was made for the effect of the elevated plasma iron concentration, erythroid marrow capacity for iron uptake was at basal levels. Numbers of erythroid cells in marrow and spleen measured by the radioiron dilution technique were increased. Thus iron uptake was not proportionate to the erythroid hyperplasia in the b/b rat, despite a more than adequate plasma iron supply. This relative deficiency in iron uptake was reflected in a severe microcytosis and elevated red cell protoporphyrin.

Reticulocyte incubation studies demonstrated an unimpaired uptake of the transferrin-iron-receptor complex but a marked reduction in iron accumulation. The different transferrin molecule, when it did give up iron within the cell, released both of its iron atoms so that only apotransferrin was returned to the media. In contrast to the nearly complete release of iron within the normal reticulocyte, the major portion of iron taken up by the Belgrade reticulocyte was returned to the plasma. The release mechanism that can be impaired in iron-deficient reticulocytes by EDTA or cadmium was shown to be affected by lower concentrations of these substances in the Belgrade reticulocyte. It is concluded that the Belgrade rat has an abnormality of iron release within the absorptive vacuole that is responsible for a state of intracellular iron deficiency, involving the erythron and other body tissues.

THE ANEMIA of the Belgrade rat is an autosomal recessive trait, determined by a radiation-induced mutation. It is characterized morphologically by red cell hypochromia, microcytosis, and reticulocytosis. There is a modest increase in total iron-binding capacity of the serum with a proportionately greater increase in the serum iron concentration. Despite the hyperferremia, however, there is an absence of stainable iron in the body tissues and a lack of sideroblasts in the marrow. Studies in a small number of animals suggest that iron utilization for hemoglobin synthesis is reduced. Parenteral iron treatment fails to cure the anemia, even though it results in a gross iron overload of spleen and liver.

More detailed studies of the behavior of immature erythroid cells have shown an impaired iron uptake of transferrin iron. Globin chain synthesis in reticulocytes, as measured by the incorporation of 3H-L-leucine in vitro, is decreased but balanced. Incubation of reticulocytes with exogenous hemin partially corrects the defect in globin synthesis but has little effect on iron uptake. When reticulocytes are exposed to 59Fe, 125I-labeled transferrin, there is an unimpaired uptake of transferrin but a decreased uptake of iron.6 The examination of 125I-labeled cell membrane in acrylamide gel does not disclose an obvious difference in receptor sites for transferrin. In summary, past studies have shown a block in the acquisition of iron by body tissues, the nature of which has not been precisely defined. The purpose of this study was to provide more quantitative dimensions to the iron uptake abnormality and to define the nature of the abnormality.

MATERIALS AND METHODS

Anemic Belgrade laboratory male rats (b/b) of a mixed genetic background were mated with normal female Long Evans rats to provide obligatory heterozygotes. These heterozygotes were intercrossed to provide anemic (b/b) and nonanemic (−/b, −/−) control animals for studies of erythropoiesis. Because it was not possible to distinguish between −/+ carriers and −/− normal homozygotes, the expression −/+ will be used to denote the nonanemic control animals. Although heterozygous (+/b) litter mates are not normal from the genotypic point of view, they are normal phenotypically. Other workers and ourselves have not been able to demonstrate any phenotypic differences between +/+ and +/b animals. In view of the high reticulocyte count of b/b animals, normal animals rendered iron deficient by bleeding and low iron diet with comparable reticulocyte counts were used in incubation studies as a control.

Ferrokinetic studies. Plasma iron turnover measurements were performed by injecting radioiron as FeSO4 intravenously into control animals and radioiron bound to normal plasma into homozygous animals. In the latter instance, the iron saturation of transferrin was set at that present in vivo, usually about 80%. Five samples of blood, each 0.2 mL, were withdrawn from the tail vein over the anticipated T½ disappearance time, and additional blood was drawn with the last sample for determination of plasma iron concentration and total iron-binding capacity. The plasma iron turnover (mg of iron/dL whole blood/d) was calculated according to the formula:

\[
\text{PIT} = \frac{\text{plasma iron (µg/dL)} \times \text{plasmatocrit}}{\text{T½ (min)} \times 100}
\]

In addition, a correction was made for the effect of plasma iron concentration on plasma iron turnover. In this correction, the PIT is converted to that which would be found at 100% transferrin saturation. The basis for this correction is the molecular advantage of 11.6 in iron delivery by diferric transferrin as compared with monoferric transferrin in the rat. The formula is:

\[
\text{PIT at 100% sat} = \frac{200}{s + 9.6} \times \frac{1}{11.6}
\]

where \( s \) = transferrin iron saturation.

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**Erythroid cell number.** Total marrow cellularity was determined in rats sacrificed six to eight hours after the injection of radioiron, which time plasma activity had decreased to <20% of the original level. Animals were exsanguinated and perfused with saline. Total marrow cellularity was determined in these animals by relating the cell count and radioactivity of an aliquot of marrow to the radioactivity of the total skeleton as previously described. Immediately after death, one hemurus was removed and its activity was determined. Then its marrow was flushed from the open end, using a syringe filled with Hanks’ buffered saline solution. The collected cells were refluxed with a syringe and 27-gauge needle until a uniform cell suspension was obtained. The number of cells and radioactivity of this suspension was determined. Then the suspension was centrifuged at 400 g and the supernate removed for determination of radioactivity. The animal carcass minus the viscera was then autoclaved and all bones removed for determination of total skeletal activity.

Marrow cellularity (MC) was determined in two ways. The first method calculated total erythroid cells of the marrow from the number of erythroid cells counted in the aliquot and the total activity of the aliquot (cells and supernate) as compared with skeletal activity. The second method calculated total erythroid marrow cellularity from the number of erythroid cells in the aliquot and the radioactivity of centrifuged cells alone, which was determined. If activity in the supernate of the aliquot represented shed cytoplasm but the cells were still countable, the first method would be the appropriate one. If the activity in the supernate of the aliquot represented broken cells that could not be counted, the second method would be proper. These two calculations are assumed to represent minimum and maximum figures, the true value lying somewhere between. The formulas used for these two calculations were as follows:

\[
MC \text{ (minimal)} = \frac{\text{aliquot erythroid cells}}{\text{total cell suspension}} \times \frac{\text{total skeletal activity}}{\text{total cell suspension activity}}
\]

\[
MC \text{ (maximal)} = \frac{\text{aliquot erythroid cells}}{\text{aliquot cell button activity}} \times \frac{\text{total skeletal activity}}{\text{aliquot cell button activity}}
\]

In addition, at the time the animal was killed, the spleen was removed and weighed and activity was determined. An aliquot of spleen was then weighed and torn apart so as to release its cell content into Hanks’ solution. This cell suspension was centrifuged, the supernate removed, and plasma substituted. It was then diluted to a known volume and shaken to a uniform suspension, and cells were counted. By relating the aliquot to the total spleen through weight ratio, total cell count was calculated.

Splenic erythroid cells

\[
= \frac{\text{splenic aliquot erythroid cells}}{\text{splenic weight}} \times \frac{\text{aliquot weight}}{\text{aliquot erythroid cells}}
\]

**Reticulocyte assay.** Reticulocyte-rich blood (reticulocyte count 28% to 38%) was obtained from Belgrade and genetically normal iron-deficient rats. The cells were washed and preincubated in Hanks’ balanced salt solution for 15 minutes to deplete the cells of prebound transferrin. Then cells were incubated in Hanks’ buffer with the transferrin preparations labeled with $^{55}$Fe or $^{59}$Fe or $^{55}$Fe and $^{59}$Fe (see Results) in Hanks’ buffer. In chaser experiments, the cells were incubated for ten minutes at 37°C with the tagged transferrin and 11 μg of cold diferric transferrin iron was added to start the chase. Populations of blood cells from iron-deficient control rats and Belgrade rats with the same reticulocyte count (28% to 33%) were prepared. Otherwise, the incubation mixture consisted of a transferrin concentration 1.0 mg/mL, dissolved in Hanks’ balanced salt solution, pH 7.4, hematocrit 20% to 25%. A typical total volume for a single uptake evaluation was 0.72 ml.

**Transferrin preparation.** Purification and iodination of rat diferric transferrin with 131I were done as described elsewhere. Transferrin was converted to apotransferrin by using deferoxamine. For the preparation of diferric transferrin, 30 μCi of $^{59}$Fe (13 to 22 μCi/μg of iron, dissolved in 0.5 mol/L HCl) (1 Ci = 3.7 x 10^10 Bq), was added to 50 mg of 131I-labeled apotransferrin (100 μCi of $^{131}$I) dissolved in 5 mL of 0.05 mol/L Tris HCl, pH 8.0/0.01 mol/L NaHCO₃. This was followed by the addition of sufficient iron as ferrous ammonium sulfate (273 μg of iron per milliliter in 0.01 mol/L HCl) to saturate exactly the free iron-binding sites present in the transferrin solution. This was achieved by a spectrophotometric titration technique in which the increase in color development at 465 nm was observed. At the point of saturation, the A465/A280 ratio of the transferrin solution was 0.046, characteristic of the diferric state of the transferrin molecule. Monoferric transferrin was prepared by column chromatography on Sephadex G50 at pH 5.1. Double-labeling of the purified transferrin was achieved by first labeling all sites with $^{59}$Fe, removing the iron from the acid labile site by chromatography at pH 5.1, and then saturating the open sites with $^{55}$FeSO₄. The preparation had a specificity of site binding of more than 80%. No site-to-site exchange of isotope occurred when stored at 4°C in 1 mmol/L bicarbonate-buffered saline (pH 7.4) for at least three weeks, as shown by repeating the Sephadex G-50 separation. In addition, the incubation of the selectively labeled diferric transferrin preparation in Hanks’ buffer at 37°C for two hours without or with washed reticulocytes failed to show any scrambling of the two isotopes.

**Miscellaneous methods.** Hemoglobin was determined by the cyanmethemoglobin method, hematocrit by the micromethod, and red cell count was done manually. Plasma iron and iron-binding capacity were determined according to standard methods. Red cell protoporphyrin was determined spectrophotometrically. Nonheme iron analyses were carried out by wet ashing and colorimetric analysis, as described in Bothwell et al. $^{59}$Fe was counted in a gamma counter, whereas samples for $^{55}$Fe analysis were wet ashed and counted in a scintillation counter. When $^{55}$Fe along with $^{59}$Fe were used, both isotopes were measured without extraction in an autogamma scintillation spectrometer (Packard, Model 5330). Simultaneous measurement of $^{59}$Fe and $^{55}$Fe activity was carried out after acid digestion, using the method of Eakins and Brown, in a liquid scintillation counter (Packard, Model 2405). Isoelectric focusing in polyacrylamide gel was carried out as described elsewhere. The methods of incubation, washing, wet ashing of the cells, counting radioactivity, chemical iron determination, and statistical evaluation were the same as previously described by this laboratory. Variations are expressed as ± 1 SD.

**Reagents for in vitro studies.** Ferrous ammonium sulfate [(NH₄)_2Fe(SO₄)₂·6H₂O] was purchased from Baker Chemical Co, Phillipsburg, NY. Disodium EDTA and cadmium chloride (Cd Cl₂·2½H₂O) were obtained from Sigma Chemical Co, St Louis, and from Analar BDH Chemicals Ltd, Poole, England, respectively. A 0.1 mol/L EDTA stock solution in saline was prepared and adjusted to pH 7.4 by the addition of 0.1 mol/L HCl. A 0.115 mol/L Cd Cl₂ stock solution in pH 2.0 saline was prepared and stored at this low pH and neutralized in the experimental mixture by the addition of 0.5 mol/L HEPES buffer (pH 7.4).

**RESULTS**

**Studies of erythropoiesis.** Hematologic data on four adult homozygous (b/b) animals and four adult control litter mates, either heterozygous or normal (−/−), reared under...
identical conditions were determined. Their respective mean hemoglobin concentration was 7.5 and 11.8 g/dL, mean cell volumes 34 and 63.4, and mean corpuscular hemoglobin concentration 19.0 and 27.8. Thus each b/b red cell had 0.37 the amount of hemoglobin as compared with the −/− red cell. Protoporphyrin values on four homozygotes were elevated to a mean value of 176 ± 74 as compared with a control value of 28 ± 6 g/dL RBC. Liver and spleen iron content was lower in b/b animals than in controls, i.e., 2.6 ± 2.0 vs 5.5 ± 1.4 mg of iron. Despite this evidence of iron deficiency in circulating red cells and other tissues, plasma iron and transferrin saturation of the b/b animals (Table 1) were elevated significantly above those of control animals.

Quantitative measurements of erythroid cellularity (Table 1) showed an increase of between two and three times controls in the nucleated red cell precursors within the skeleton and a still greater increase in nucleated cells in the enlarged spleen of the homozygous rats. The total number of erythroid cells (spleen and marrow) of b/b animals was increased to about four times that of −/− rats. While the two methods of calculating total erythroid precursor cellularity gave different values, the comparison between homozygote b/b and −/− was almost identical, i.e., 4.2 vs 4.1 x basal.

The plasma iron turnover of homozygous animals averaged 2.5 mg/dL per whole blood per day as compared with 2.0 mg in control animals. However, if a correction was made for the effect of the higher plasma iron concentration in the homozygous animals, the calculated plasma iron turnover of the two groups of animals at a transferrin saturation of 100% showed essentially no difference, i.e., 2.6 and 2.5 mg iron per deciliter of whole blood. Thus, despite a far greater number of red cell precursors, a better iron supply, and a lower hemoglobin, the homozygous animals showed no increase in erythroid iron uptake. Red cell utilization of radioiron was similar in the two groups, i.e., 81 ± 11 in the b/b vs 86 ± 7 in the −/− animals.

Reticulocyte iron uptake and release. Further studies were directed more specifically at the defective reticulocyte

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**Table 1. Erythropoiesis in the Belgrade Rat**

<table>
<thead>
<tr>
<th>Items</th>
<th>Homozygous</th>
<th>Heterozygous or Normal</th>
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<td>2</td>
</tr>
<tr>
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<td>BM Eryth (%)</td>
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<td>BM Eryth (x 10⁶/kg)</td>
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<td>11.5/19.3</td>
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<td>Spleen Eryth (%)</td>
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<tr>
<td>PI (µg/dL)</td>
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<tr>
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</tr>
<tr>
<td>PIT (100% sat)</td>
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</tr>
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</table>

*Significance of P < .05.

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**Fig 1. Uptake radioiron and ¹²⁵I-labeled transferrin by b/b reticulocytes (solid line) and iron-deficient reticulocytes (dashed line). Belgrade reticulocytes accumulated far less iron (a), whereas transferrin uptake appeared similar (b).**
iron uptake (Fig 1). When reticulocytes from the b/b and reticulocytes from iron-deficient genotypically normal rats were incubated with iron-saturated \(^{59}\text{Fe}\) and \(^{125}\text{I}\)-labeled transferrin (2 mg/mL at 37 °C), radioiron uptake was similar only during the first few minutes of incubation, and by 60 minutes, the uptake by b/b reticulocytes amounted to about one fourth that of the iron-deficient control reticulocytes. This occurred despite a similar initial \(^{125}\text{I}\) transferrin uptake.

Iron release in the normal reticulocyte is known to involve complete removal of iron from transferrin. This aspect was examined by using a doubly-labeled rat transferrin carrying \(^{55}\text{Fe}\) on the acid stable site and \(^{59}\text{Fe}\) at the acid labile binding site. At incubation, the normalized uptake ratio (determined by the \(^{55}\text{Fe}^{59}\text{Fe}\) uptake in the reticulocyte divided by the \(^{55}\text{Fe}^{59}\text{Fe}\) ratio in the media) was 1.0 ± 0.01 (n = 5) for the Belgrade rat and 0.99 ± 0.01 (n = 5) for the iron-deficient control reticulocytes. There could still be a random removal of one iron from the two sites, resulting in exiting of monoferric transferrin from cells. To investigate this, \(^{59}\text{Fe}\)-tagged diferric transferrin was used in which the protein part of transferrin was labeled with \(^{125}\text{I}\). After a 60-minute incubation, during which about 60% of the radioiron was removed by the iron-deficient reticulocytes and 16% by the Belgrade reticulocytes, isoelectric focusing profiles were prepared on the media (Fig 2). No accumulation of monoferric transferrin was seen in either and the amount of apotransferrin was proportional to the iron uptake by reticulocytes in the two cell mixtures.

That the abnormality involved the iron release mechanism was shown by a chase study (Fig 3). After ten minutes of preincubation of reticulocytes at 37 °C with diferric \(^{55}\text{Fe}\), \(^{125}\text{I}\) rat transferrin, a 20-fold excess of nonradioactive diferric rat transferrin was added and the incubation continued for another 20 minutes. A rapid efflux of \(^{125}\text{I}\) out of the cells was observed, essentially the same with Belgrade and iron-deficient reticulocytes. On the other hand, the behavior of \(^{59}\text{Fe}\) was quite different (Fig 3B). No efflux of \(^{59}\text{Fe}\) from control reticulocytes was observed, while Belgrade reticulocytes lost about 70% of the \(^{59}\text{Fe}\) taken up during the preincubation period.

This abnormal iron release became more evident when reticulocytes were exposed to EDTA or cadmium (Fig 4). Both of these inhibitors reduced iron accumulation in cells when present in millimolar concentration. The effect on Belgrade reticulocytes, however, was much greater than on control reticulocytes from iron-deficient animals. No effect on iron delivery was observed when the cadmium EDTA complex (1 mmol/L Cd\(^{2+}\), 2 mmol/L EDTA) was incubated with reticulocytes of either experimental animal (data not shown).

**DISCUSSION**

Belgrade rats have an autosomal recessive trait that, in its homozygous form (b/b), leads to a microcytic hypochromic anemia. While erythroid cells have the features of iron deficiency, including a decrease in hemoglobin content and elevated red cell protoporphyrin, some features are quite different from those expected in iron deficiency. Most conspicuous is the ample plasma iron, whereas body tissues are iron depleted. These findings suggest an iron block involving both gastrointestinal absorption and iron uptake from transferrin by body tissues. Of interest is the demonstration that the block in erythroid uptake does not appear to interfere with erythroid proliferation but does interfere with hemoglobin synthesis. Measurements by the authors on iron-deficient animals with severe anemia (mean hematocrit 15) show a total erythrocyte cellularity of \(7.1 \times 10^{9}\)/kg as compared with the mildly anemic homozygous Belgrade rats, with an erythrocyte cellularity of \(18 \times 10^{9}\)/kg. This implies that the Belgrade defect is less limited in the early phase of red cell development involving stem cell proliferation as compared with the late stages, in which hemoglobin synthesis predominates.

The extremely high plasma iron and transferrin saturation...
Fig 3. A chase experiment in which there was a preliminary incubation of reticulocytes with transferrin labeled both with radioiron and \(^{125}\text{I}\) for a period of ten minutes. Thereafter, a large amount of nonradioactive diferric rat transferrin (7.9 mg containing 11 \(\mu\)g iron) was added to the mixture. Uptake by b/b reticulocytes is shown by solid line and that of iron-deficient reticulocytes is shown by dotted line. In the diagram on the left, the similar rate of release of transferrin from the reticulocyte mixture is seen. In the diagram on the right, a rapid release of iron is observed from b/b reticulocytes but not from normal reticulocytes.

suggest an activation of the iron procurement mechanism within the body. It has been shown that increasing the number of immature erythroid cells somehow results in an increased procurement of iron.\(^6\) In thalassemia, the effect of erythroid proliferation is to increase absorption and total body iron, leading to parenchymal iron overload. In these animals, it would appear that total body iron cannot be increased because of an absorptive abnormality, but that the gradient between iron-donating tissues and plasma iron is altered so as to increase plasma iron supply. Because the delivery of iron to the erythron is not increased, procurement cannot be simply a replacement of iron after its removal from transferrin as previously suggested, but to some other aspect of erythroid hyperplasia.\(^7\)

Previous studies have shown a marked shortening in red cell life span by the chromium-51 technique to seven days in b/b animals as compared with 19 days in controls.\(^2\) These studies, however, were done with younger animals that show a severer degree of anemia. In the present study, while the number of b/b red cell precursors was about four times normal, this would represent a hemoglobin production of only 1.3 times basal, since red cells contained only about one third the hemoglobin concentration of \(-/^-\) red cells. The basal turnover of plasma iron gives no indication of hemolysis and suggests, if anything, that part of the increase in erythroid cellularity in these animals might be due to a lengthened maturation time. Splenomegaly was accounted for by the marked increase in erythropoiesis within the spleen.

Attention then focuses on the series of events occurring when the iron is taken up by the red cell. If there were defective unloading of iron from transferrin, this would not be seen by ferrokinetic techniques, since the shunt in and out of the red cell occurs so rapidly. The currently accepted model for transferrin-mediated delivery of iron to the mammalian erythroid cell involves a number of steps.\(^8\) Diferric or monoferric transferrin binds to a receptor on the plasma membrane at neutral pH, and the complex is endocytosed into the cell cytoplasm. The endocytotic vesicle would be expected to contain an adenosine triphosphate-dependent proton pump, which acidifies its contents. Iron is released from transferrin in the acid environment of the vesicle and somehow transported across its membrane into the cytoplasm and ultimately to the mitochondria for incorporation into heme. The apotransferrin–receptor complex returns to the surface of the cell and fuses with the plasma membrane. On exposure to a higher pH, apotransferrin leaves the receptor and becomes available for reutilization.\(^9\)

In the Belgrade rat reticulocyte, the initial steps involving transferrin–receptor interaction and transferrin–iron–recepto
ior internalization are intact. However, the release of iron from transferrin in the cell interior is greatly diminished. The release mechanism, although quantitatively abnormal, appears to be qualitatively normal, because when iron is removed from the cell, only apotransferrin is returned to the medium.

The release of iron from transferrin within the genotypically normal iron-deficient reticulocyte is impaired by EDTA and cadmium ions. In this regard, Belgrade reticulocytes are much more sensitive than normal reticulocytes to the effect of EDTA and cadmium ions. Because EDTA can chelate calcium and magnesium, and because cadmium, due to its chemical similarity, can compete with calcium and magnesium, it is possible that the molecular lesion in the Belgrade rat involves a calcium-dependent enzyme system concerned in the release of iron from transferrin.

REFERENCES

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J Edwards, H Huebers, C Kunzler and C Finch