Abnormal Megakaryocytopoiesis in the Belgrade Laboratory Rat

By Zoran Rolović, Nadežda Basara, Nevenka Stojanović, Nada Suvajdžić, and Vera Pavlović-Kentera

The Belgrade laboratory (b/b) rat has a hereditary hypochromic microcytic anemia because of defective transmembrane iron transport into erythroblasts. The present study was prompted by our previous work in which we showed that the b/b rat has hypomegakaryocytic thrombocytopenia associated with increased megakaryocyte size. To define the basic mechanism underlying this abnormality in the b/b rat we have studied both megakaryocytopoiesis and granulopoiesis in anemic b/b rats, chronically transfused b/b rats, iron-treated b/b rats, and controls. We have found decreased concentrations of megakaryocyte and granulocyte progenitors in the marrow of b/b rats. Full correction of the severe anemia by chronic transfusion resulted in normalization of megakaryocyte progenitors, small acetylcholinesterase positive cells, megakaryocyte size, and platelet counts, along with granulocyte progenitors. In contrast, the partial correction of anemia obtained by iron treatment resulted in improvement, but not normalization, of these parameters. These findings indicate that abnormal megakaryocytopoiesis in the b/b rat can be best interpreted as a consequence of hypoxia because of the severe anemia. Because we have recently shown that the number of erythroid progenitors in b/b rats is also low, we propose that abnormal megakaryocytopoiesis in this animal is a reflection of an acquired stem cell disorder induced by the prolonged hypoxia resulting from the severe anemia.

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MATERIALS AND METHODS

Animals. Female b/b rats from the new Belgrade colony of anemic rats were used at 10 to 12 weeks of age. Female nonanemic heterozygous (b/+)- or normal homozygous (+/+)-animals of the same genetic background were used as controls. The new Belgrade colony was raised from heterozygous b/+ male and female rats, which were kindly provided by Dr K. Kellar (Center for Disease Control, Atlanta, GA). The average hematocrit value (PCV) of 40 female b/b rats was 15 ± 3 and the hemoglobin (Hb) concentration was 2.9 ± 0.7 g/dL, while in 47 controls the PCV was 44 ± 3 and Hb was 15 ± 2 g/dL, showing that the animals in the new Belgrade colony of anemic b/b and nonanemic heterozygous rats have the same hematologic characteristics as the original colony produced in the “Boris Kidrić” Institute of Nuclear Science (Belgrade, Yugoslavia).

Experimental design. The following groups of female rats were studied: group 1, anemic b/b rats; group 2, chronically transfused b/b rats; group 3, partially transfused b/b rats; group 4, iron-treated b/b rats; and group 5, controls.

Chronically transfused b/b rats were administered intraperitoneally (i.p.) injections of syngeneic washed b/+ donors’ red blood cell (RBC) suspension (2.0 mL; PCV about 30%), three times a week for 4 weeks and were investigated 3 days after the last transfusion. The amount of iron administered in chronically transfused b/b rats was calculated to be 1.8 mg/wk. Partially transfused b/b rats were administered i.p. injections of syngeneic RBC suspension (1.0 mL PCV about 30%) once a week for 4 weeks and were investigated 5 to 7 days after the last transfusion. The amount of iron administered in partially transfused b/b rats was estimated to be 0.3 mg weekly.

Iron-treated anemic b/b rats were administered iron dextran complex (Ferrum, Lek, Ljubljana, Yugoslavia), 3.5 mg weekly. Treatment with iron was started at 30 days of age and lasted up to 3 months when the rats were studied. The rats tolerated injections of iron very well.

Peripheral blood counts. Platelet counts were performed by the method of Brecher et al on free-flowing blood from the tail vein of the rats. RBC and white blood cell (WBC) counts were made using a hemocytometer. PCVs were obtained from microhematocrit readings and blood Hb concentration by the cyanmethemoglobin technique.

O2 saturation was measured in arterial blood of experimental groups of rats by using oxymeter OSM9 (Radiometer Copenagen, Denmark), and arterial oxygen content was then calculated according to the formula: 1.36 × Hb × O2 saturation.

Mk number in the femoral marrow. The femoral marrow from rats was obtained as an intact column fixed in 6% formalin and embedded in paraffin. Sets of 30 longitudinally obtained bone marrow sections 4 μm thick were prepared and then stained with hematoylin and eosin. Sections were examined on a light microscope at a magnification of 1,000 ×, and a number of Mk profiles per cubic millimeter of section area (N,) was determined using the Weibel multigrade system. The number of Mks per cubic millimeter of bone marrow, corrected for errors owing to section thickness and optically lost cell profiles, was then calculated as previously described.

Megakaryocyte diameter was derived using an eyepiece micrometer. At least 100 section profile Mk diameters on bone marrow histologic sections were measured. When the Mks were not spherical, the diameter was expressed as the square root of the product of two measurements made at right angles.

Mean Mk diameter (D) was derived from the section profile diameter as already described. Before statistical analysis all D and N, values were corrected for the 20% tissue shrinkage that occurred during

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histologic processing. Shrinkage was estimated as the ratio of RBC diameter in tissue sections to the diameter measured in suspensions of whole rat blood.  

Quantitation of immature Mks using acetylcholinesterase staining (SACHE+ cells). Smears were made with a paint brush washed with normal rat serum from the marrow of split tibiae, air dried, and fixed in 3% glutaraldehyde for 10 minutes. These were stained for AChE activity according to the method described by Jackson.  

SACHE+ cells were located with bright field microscopy under a 100 x oil immersion objective and had to fit the criteria described by Jackson: diameter of less than 13 μm (measured with a calibrated micrometer), the presence of cytoplasmic AChE reaction product that appears diffusely golden brown, and a high nuclear/cytoplasmic ratio. After morphologic classification, at least 200 AChE containing Mks were sized and the ratio of SACHE+ Mks to AChE+ cells greater than 13 μm was determined. The total number (No.) of SACHE+ cells/mm³ of marrow was calculated using the formula proposed by McDonald et al:  

Total No. of SACHE+ Cells/mm³ of Marrow
= No of SACHE+ Cells on Smears
× No. of Recognizable Mks in Sections/mm³ / No. of >13 μm Diameter AChE+ Cells on Smears

CFU-Mk determination. The growth of Mk colonies that develop from Mk progenitors (CFU-Mk) of rat bone marrow was studied using the in vitro system previously described by Kellar et al. In brief, rat femoral marrow cultures were set at 2 x 10⁶ cells/mL in 35-mm Petri dishes (four replicative dishes for each rat) in McCoy's medium supplemented as described by Williams and Williams. The dishes before adding the cells in the agar medium in a volume of 0.025 to 0.20 mL. Briefly, the supernatant of normal rat spleen conditioned medium (SCM), prepared according to the procedure described by Kellar et al., was added to the dishes before adding the cells in the agar medium in a volume of 0.025 to 0.20 mL. Briefly, the supernatant of normal rat spleen cells incubated for 7 days in a medium with 5% FCS and pokeweed mitogen (5%, 1:15 dilution) was used for CFU-Mk growth. Cultures were incubated for 8 days at 37°C in a humidified atmosphere containing 5% CO₂. The number of colonies (50 cells or more) was scored using an inverted microscope.  

Bone marrow cell differential was determined on femoral bone marrow smears stained by the May-Grünwald-Giemsa method by differentiating 1,000 nucleated cells, respectively. Cells were divided into the following compartments: proliferative granulocytes (PG), metamyelocytes (META), granulocytes (GRAN), monocytes (MONO), lymphocytes (LYMPHO), erythroid cells, and others. Values are expressed both as absolute numbers per femur and as percentage of nucleated cells.

Statistics. Data were evaluated by use of Student's t-test and one-way analysis of variance.

RESULTS

The reduced platelet counts in b/b rats accompanied with extremely low blood Hb concentrations and decreased RBCs and PCV become normalized after chronic transfusion and correction of the anemia (Table 1). In iron-treated b/b rats as well as in partially transfused b/b rats the platelet count increased but was not normalized. It can be seen from Table 1 that blood Hb concentration in iron-treated b/b rats and partially transfused b/b rats was still significantly lower compared with the control and accompanied with persisting reticulocytosis, although the number of RBCs was higher than in controls. Typical RBC appearance, microcytosis, anisocytosis, and poikilocytosis on peripheral blood smears characteristic for anemic b/b rats did not change after iron treatment. It should be noted that body weight, which was significantly lower in anemic b/b rats, increased and was not different from controls after chronic transfusions, partial transfusions, and iron treatment.

Arterial oxygen content was six times lower in b/b rats (2.96 mL/dL) than in controls (18.25 mL/dL). In chronically transfused animals arterial oxygen content was normalized (18.75 mL/dL), while in partially transfused animals it was still lower (10.77 mL/dL) and in iron-treated b/b rats the content was 14.54 mL/dL.

The results of the determination of the Mk number in the femoral marrow of anemic b/b rats, chronically transfused b/b rats, partially transfused b/b rats, iron-treated b/b rats, and controls are given in Table 2. It can be seen that the mean Mk number per cubic millimeter of marrow tissue

### Table 1. Peripheral Blood Values in Different Experimental Groups of Rats

<table>
<thead>
<tr>
<th>Group of Rats</th>
<th>Body Weight</th>
<th>Hb (g/dL)</th>
<th>PCV (L/L)</th>
<th>RBC (x10⁶/L)</th>
<th>WBC (x10⁶/L)</th>
<th>Platelet count (x10⁹/L)</th>
<th>Rtc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemic b/b rats (8)</td>
<td>44 ± 9*</td>
<td>2.62 ± 0.72*</td>
<td>14 ± 3*</td>
<td>3.26 ± 1.00*</td>
<td>15.50 ± 4.00*</td>
<td>560 ± 122*</td>
<td>46 ± 10*</td>
</tr>
<tr>
<td>b/b Chronically transfused (5)</td>
<td>140 ± 23</td>
<td>14.5 ± 1.25</td>
<td>47 ± 2</td>
<td>7.68 ± 1.1*</td>
<td>5.02 ± 0.93</td>
<td>892 ± 162</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>b/b Partially transfused (5)</td>
<td>100 ± 21</td>
<td>8.34 ± 0.88†</td>
<td>35 ± 6.7</td>
<td>5.2 ± 1.5</td>
<td>5.3 ± 1.35</td>
<td>730 ± 72†</td>
<td>ND</td>
</tr>
<tr>
<td>Iron-treated b/b rats (9)</td>
<td>142 ± 23</td>
<td>11.25 ± 1.04†</td>
<td>41 ± 2</td>
<td>10.4 ± 1.6*</td>
<td>5.03 ± 1.0</td>
<td>718 ± 109†</td>
<td>10.8 ± 5.45†</td>
</tr>
<tr>
<td>Controls (7)</td>
<td>123 ± 24</td>
<td>14.85 ± 1.4</td>
<td>44 ± 3</td>
<td>6.00 ± 0.78</td>
<td>5.56 ± 1.2</td>
<td>984 ± 75</td>
<td>3.1 ± 2.7</td>
</tr>
</tbody>
</table>

Values are mean ± SD. The number of rats is given in parentheses.

Abbreviations: ND, not determined; Rtc, reticulocytes.

Significance of the difference from controls: *P < .001; †P < .01; ‡P < .05.

§Lymphocytes × 10¹⁰.
from b/b rats of 2.14 ± 0.6 was significantly reduced when compared with the mean Mk number per cubic millimeter from control rats (4.66 ± 0.41). In chronically transfused b/b rats mean Mk number per cubic millimeter was normalized (4.89 ± 0.29) and not different from the mean Mk number per cubic millimeter found in controls, while in partially transfused b/b rats as well as in iron-treated b/b rats the Mk number per cubic millimeter was higher (3.31 ± 0.85 and 2.90 ± 0.16, respectively) than in anemic b/b rats, but still significantly lower than in controls.

Mean Mk diameter determined on histologic sections was significantly larger (Table 2) in anemic b/b rats than the mean Mk diameter in controls. After chronic transfusion, the mean Mk diameter returned to the control value. In partially transfused b/b rats and in iron-treated b/b rats the mean Mk diameter decreased but remained larger than in the controls.

The total number of SACH^{+} cells in anemic b/b rats (Table 2) was lower when compared with that of control rats. After chronic transfusion the total number of SACH^{+} cells in b/b rats was increased and did not differ from the total number of SACH^{+} cells found in controls. In partially transfused b/b rats and in iron-treated b/b rats, the total number of SACH^{+} cells increased as compared with anemic b/b rats, but was still lower than in controls.

The number of CFU-Mk-derived colonies was related to the amount of stimulator added to the cultures in b/b and control rats (Fig 1), the plateau dose of SCM being 0.1 and 0.2 mL, respectively. The number of Mk colonies per 2 × 10⁸ cells plated at the corresponding plateau dose of SCM was significantly reduced in b/b rats (35 ± 6) compared with controls (75 ± 18). In chronically transfused b/b rats the number of CFU-Mk-derived colonies was normalized (90 ± 17) while in partially transfused b/b rats and in iron-treated b/b rats Mk colony number increased, but was still lower (51 ± 22 and 42 ± 12, respectively) than in controls.

The number of CFU-GM-derived colonies per 0.5 × 10⁸ cells plated and 5% of colony-stimulating factor was significantly reduced in b/b rats compared with controls (Table 3). In chronically transfused b/b rats the number of CFU-GM colonies was not different compared with controls, while in partially transfused b/b rats and in iron-treated b/b rats CFU-GM-derived colonies were increased but still lower than in controls.

The number of nucleated cells per femur in b/b rats was three times less than in controls (Table 4). After chronic transfusion and iron treatment the number of nucleated cells per femur increased and was not different compared with controls.

A significantly lower absolute number and percentage of nonproliferative granulocytes (META + GRAN) and total number of granulocytic cells were found in anemic b/b rats' femoral bone marrow (Table 4).

**DISCUSSION**

The present study was prompted by our previous work in which we showed that the hereditary anemic b/b rat has hypomegakaryocytic thrombocytopenia associated with increased Mk size and an accelerated maturation rate of recognizable Msks. It was then postulated that these abnormalities may be the consequence of a primary genetic defect in the b/b rat or secondary to the severe anemia.

The results of this study show that hypomegakaryocytic thrombocytopenia in the anemic b/b rat is the consequence of a decreased number of Mk progenitors in the bone marrow. Furthermore, we have shown that the decreased number of Mk progenitors and accompanying changes in
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megakaryocytopoiesis in b/b rats are secondary to the severe anemia. Supporting this suggestion are the results obtained in a group of chronically transfused b/b rats in which Mk progenitors, SACHEx cells, Mk number, Mk size, and platelet counts returned to normal values.

The feature of abnormal megakaryocytopoiesis in the anemic b/b rat closely resembles the changes in Mk cell lineage observed in mice12,17 and rats subjected to prolonged hypoxia by others19 and by us.18 It was recently shown that prolonged hypoxia induces hypomegakaryocytic thrombocytopenia by reducing the pool of committed Mk progenitor cells17 along with the reduction of SACHEx cells.12 The extremely low arterial oxygen content as the best parameter mediated by hypoxia because the number of CFU-erythroid (E)-derived colonies returned to normal values. The effect of hypoxia on the behavior of hematopoietic stem cells and their descendents has been extensively studied under various experimental conditions,9,21 but none of these conditions can be applied to the b/b rat model. The fundamental difference between various experimental models and the b/b rat stems from the fact that the b/b rat exhibits a proliferative defect in the erythroid progenitor cells7 that prevents an orderly compensatory response of the erythron to the hypoxic stimulus.22,23

The absence of this response4 in combination with severe anemia because of the defect in transmembrane iron transport into erythroblasts3 produces the state of prolonged hypoxia. Therefore, it is possible to assume that prolonged hypoxia alters hematopoietic stem cell response,24 which further contributes to the numerous defects in hematopoiesis observed in b/b rats.

One may also associate hypomegakaryocytic thrombocytopenia in the b/b rat with generalized iron depletion in body tissue that is known to exist in this animal,1,2,12 particularly regarding the fact that the influence of hypoxia on hematopoiesis besides the low intracellular iron content is difficult to examine. The role of iron as a regulator of thrombocytopoiesis has been controversial35,36 despite the fact that concomitant thrombocytopenia and megakaryocytopoiesis were observed in humans with severe iron-deficiency anemia35,37 and a decrease of Mk concentration was reported in experimentally induced severe iron-deficiency anemia.28

However, in a group of b/b rats treated with iron we have

Table 3. Bone Marrow CFU-GM-Derived Colonies With 5% Colony-Stimulating Factor in Different Experimental Groups of Rats

<table>
<thead>
<tr>
<th>Group of Rats (n)</th>
<th>No. of CFU-GM (0.5 x 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemic b/b (4)</td>
<td>7.1 ± 2.0*</td>
</tr>
<tr>
<td>b/b Chronically transfused (3)</td>
<td>36.6 ± 10.0</td>
</tr>
<tr>
<td>b/b Partially transfused (5)</td>
<td>25 ± 11†</td>
</tr>
<tr>
<td>Iron-treated b/b (4)</td>
<td>25.1 ± 12.0†</td>
</tr>
<tr>
<td>Controls (3)</td>
<td>59.5 ± 22.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD for groups of rats with two plates per rat.

The number of rats is given in parentheses. The same results were obtained in two separate experiments.

Significance of difference from controls: *P < .001, †P < .01.

Hypomegakaryocytic thrombocytopenia in the b/b rat can be explained by the deleterious effect of prolonged hypoxia because of severe anemia on the hematopoietic tissue. This suggestion is based on the findings that b/b rats have decreased numbers of CFU-Mk and CFU-GM along with CFU-E and burst-forming unit erythroid (BFU-E).4 After chronic transfusion, CFU-Mk- and CFU-GM-derived colonies as well as platelet and granulocyte counts returned to the normal values. The effect of hypoxia on the behavior of hematopoietic stem cells and their descendents has been extensively studied under various experimental conditions,9,21 but none of these conditions can be applied to the b/b rat model. The fundamental difference between various experimental models and the b/b rat stems from the fact that the b/b rat exhibits a proliferative defect in the erythroid progenitor cells7 that prevents an orderly compensatory response of the erythron to the hypoxic stimulus.22,23

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However, in a group of b/b rats treated with iron we have

Table 4. Number of Nucleated Cells and Morphologically Recognizable Bone Marrow Cells per Femur

<table>
<thead>
<tr>
<th>Group of Rats</th>
<th>Nucleated Cells/Femur x 10⁶</th>
<th>Total No. of Cells/Femur x 10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5)</td>
<td>72.5 ± 35</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>[100]</td>
<td>[2.2]</td>
</tr>
<tr>
<td>b/b (8)</td>
<td>28.5 ± 8*</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>[100]</td>
<td>[2.1]</td>
</tr>
<tr>
<td>b/b + Fe (5)</td>
<td>75.3 ± 20</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>[100]</td>
<td>[2.2]</td>
</tr>
<tr>
<td>b/b Chronic transf. (5)</td>
<td>56.2 ± 12</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>[100]</td>
<td>[1.9]</td>
</tr>
</tbody>
</table>

Number of rats given in parentheses. Values are mean ± SD. Numbers in brackets indicate percentage of nucleated cells per femur.

Abbreviations: See Materials and Methods.

Significance of difference from controls: *P < .05; †P < .01.
found improvement, but not normalization of megakaryocytopoiesis; ie, CFU-Mk, SACHbE+ cell, Mk number, Mk size, and platelet counts did not return to normal values. In addition, we observed that iron treatment of the b/b rat resulted in a significant increase of the originally decreased concentration of CFU-GM-derived colonies, but normalization was not achieved. Similarly, we have recently demonstrated that iron treatment of the b/b rat substantially increased the concentration of CFU-E-derived colonies and improved anemia, but the Hb and CFU-E concentrations were not completely normalized. Therefore, it appears that the feature of abnormal megakaryocytopoiesis as well as granulocyte and erythroid cell lineage still persists after iron treatment in b/b rats.

In conclusion, we believe that abnormalities of megakaryocytic cell lineage in b/b rats can be best interpreted as a consequence of an acquired disorder of the hematopoietic stem cell owing to the deleterious effect of prolonged hypoxia resulting from the severe anemia. The role of the prolonged hypoxia in producing an acquired hematopoietic defect may be further examined by applying a reliable assay to examine CFU-spleen in b/b rats under appropriate experimental conditions.

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