Abnormalities of Megakaryocytes in W/W\textsuperscript{v} Mice

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Megakaryocytopenia was evaluated in genetically anemic mice of the W/W\textsuperscript{v} genotype and was found to be abnormal. Concentration and size of blood platelets were normal. Megakaryocytes were decreased in number in tibial marrow and spleen, and the size of mature megakaryocytes was increased.

MICE OF THE W/W\textsuperscript{v} genotype have hereditary macracytic anemia,\textsuperscript{1} and they have defective hematopoietic stem cells that do not proliferate and differentiate normally after transplantation.\textsuperscript{2,3} The anemia can be corrected by transplantation of functional stem cells into W/W\textsuperscript{v} mice,\textsuperscript{4-5} and splenic tissue from W/W\textsuperscript{v} mice supports colony formation by transplanted hematopoietic stem cells in irradiated hosts.\textsuperscript{6} For these reasons, it has been concluded that mice of the W series have a basic defect in the hematopoietic stem cell or its immediate descendants.

Origin of human megakaryocytes from a pluripotential stem cell common also to the erythroid and granulocytic cell lines was suggested by the distribution of the Philadelphia chromosome in marrow cells from patients with chronic granulocytic leukemia (CGL).\textsuperscript{7} Megakaryocyte participation in CGL has long been recognized,\textsuperscript{8} and intrinsic abnormalities of megakaryocytes are now also well documented. The megakaryocytes of CGL have been shown to be small in size\textsuperscript{9,10,11} and of reduced ploidy.\textsuperscript{12,13} Acute myeloblastic leukemia has also been thought to be basically a disorder of a pluripotential hematopoietic stem cell,\textsuperscript{14} and evidence for megakaryocytic abnormalities in this disorder or its preleukemic phase has also been reported.\textsuperscript{15,16,17} These clinical observations indicate that human megakaryocytes, granulocytes, and erythroid cells all derive from a common pluripotential stem cell. Transplantation studies suggest that the same is true in mice,\textsuperscript{18} and, specifically, that the stem cell is involved in megakaryocytogenesis.\textsuperscript{19}

If the anemia of W/W\textsuperscript{v} mice originates from a defect in this pluripotential stem cell, then it seems curious that these mice do not show overt abnormalities of platelets and granulocytes. Chervenick and Boggs, in fact, found that megakaryocytes and neutrophils were decreased in the marrow of W/W\textsuperscript{v} mice.\textsuperscript{20} Lewis et al.\textsuperscript{3} reported that splenic colonies that formed in irradiated recipients...
of W/W<sup>+</sup> stem cells were reduced in number and size by comparison to those formed from normal stem cells; colonies of megakaryocytic or myeloid cells, as well as erythroid cells, were small, thus suggesting that a restriction of proliferation affected all cell lines. Bennett et al. concluded that W/W<sup>+</sup> stem cells show a moderate deficiency in production of proliferating nonerythroid cells, and Sutherland et al. found that the W defect alters granulopoiesis to a modest degree.

The present studies were undertaken to evaluate megakaryocytic potential in this strain of mice in order to further clarify the relationship of the megakaryocytic cell system to hematopoietic stem cells.

**MATERIALS AND METHODS**

**Mice**

Female mice of the W/W<sup>+</sup> strain and their heterozygous and homozygous +/+ female littermates were obtained from the Jackson Laboratories, Bar Harbor, Me., at about 5 wk of age. They were kept on a diet of Charles River Rat, Mouse, Hamster Formula, and drinking water was supplemented with Neomycin and modified Drabkin's solution. Experiments were done when the mice were 12-14 wk of age. Body weight was measured for 42 mice with the following results: 14 W/W<sup>+</sup> had a mean weight of 19.9 g (range 16-24.5 g), 11 W/+ averaged 21.8 g (20-26 g), 6 W<sup>-</sup>W<sup>-</sup> 21.1 g (19-24 g), and 11 +/- 22.6 g (18-26 g). Mice were killed by cervical dislocation after cardiac puncture under ether anesthesia.

**Blood Counts**

Cardiac blood was obtained by closed-chest aspiration under ether anesthesia. It was anticoagulated with dry K<sub>2</sub>EDTA. Platelet counts were done by the method of Brecher and Cronkite. Hematocrits were done with a Drummond Microhematocrit machine, red cell counts by a Coulter Counter, model B, and reticulocytes by the method of Brecher and Schneiderman.

**Tibial Marrow**

Intact tibiae were removed, cut across just below the fibular junction, and the proximal epiphyseal cartilage was peeled off. A 25-gauge needle, attached to a syringe containing 1 ml of 1<sub>-</sub><sup>10</sup> Na<sub>2</sub>EDTA in saline, was inserted into the distal end of the marrow space. A suspension of marrow cells was prepared by repeatedly flushing the solution back and forth through the bone. An aliquot, 0.5 ml, of this suspension was stained by adding 0.05 ml of new methylene blue. Megakaryocytes were counted by hemocytometer without further dilution; 0.9 cu mm was counted in duplicate for each tibia. The remaining suspension was used for determination of total nucleated cell count in the Coulter Counter after dilution to 1:500 in Isoton (Coulter) and addition of 5 drops of 1<sub>-</sub><sup>10</sup> saponin in saline. Total nucleated cell and megakaryocyte counts of the two tibiae were averaged for each mouse.

**Femoral Marrow**

Smears were made with a 000 paint brush from the marrow of split femora, and stained with Wright's and Giemsa stain. Morphological criteria for classification of megakaryocytes into three stages of maturity were the same as used previously. Photomicrographs were taken of megakaryocytes with a Zeiss photomicroscope and Kodak Panatomic-X film. The developed negatives were projected on a photographic enlarger, and tracings were made of the projected cell images. The perimeter of the tracings was measured with a planimeter, thus giving a measure of cell area. The results were expressed as planimeter units (square inches × 100). By correcting for magnification of the microscope and the enlarger, it was found that 1000 planimeter units corresponded to 1612 sq µ of unmaginized cell area.
Platelet Sizing

For determination of platelet size distribution, cardiac blood was anticoagulated with ACD-A, 0.7 ml blood plus 0.1 ml ACD. The samples were immediately placed in a constant-temperature room at 37°C and transferred to siliconized (Siliclad) Wintrobe tubes. These tubes were centrifuged (International Model V) at 800 rpm (150 g) for 15 min at room temperature. The samples were returned to the warm room, where the platelet-rich plasma (PRP) was removed with a Pasteur pipette and mixed quickly in a spot plate. Three microliters of PRP was diluted in about 25 ml of Ringer's solution supplemented with 2.0 mM CaCl₂ and 0.1 volume of 0.13 M trisodium citrate. All glassware and solutions were kept at 37°C, and the diluted suspension was removed from the warm room to room temperature immediately before plotting the size distribution.

Size distribution was plotted with a Coulter model J plotter attached to a Coulter model B. A 50-μ aperture tube was used, and the machine settings were as follows: 1/amp, 3; 1/apc, 1; gain, 30; matching switch, 32H. The first two channels were disregarded because of background noise, and correction was made for background in the other channels by superimposing a tracing done with the suspending medium alone. The platelets of each mouse were plotted in duplicate or triplicate and the results averaged after normalizing to a modal value of 100.

The plotter was calibrated with suspensions of human or mouse red cells of known MCV in Isoton. For this purpose, the model B was set with 1/amp, 3 and 1/apc, 8. Correction for the different machine settings showed that the platelet plots, from channels 3-25, encompassed particle sizes of 1.56-19.50 μm, each threshold division being equal to 0.195 μm.

Spleens

Mouse spleens were fixed in Bouin's solution. Sections 12 μ thick were cut longitudinally. After five consecutive sections were cut and mounted on one slide, then approximately 20 sections were discarded before another set was mounted. This was continued through the entire spleen. The sections were stained with hematoxylin and eosin, examined microscopically for megakaryocytes, and the total number of megakaryocytes per section was recorded. To avoid duplicate counting of the same cells, megakaryocytes were not counted in adjacent sections. On each slide, every third section was counted, so that the sections in which megakaryocytes were counted were at least 24 μ apart. Morphological stage of megakaryocytes in splenic sections could not be determined, but most of those that were counted had abundant cytoplasm and thus appeared to be of the mature type. The average number of megakaryocytes per section was calculated for each spleen. The number of megakaryocytes per section was determined by both the concentration of megakaryocytes and the size of the section, the latter being determined by the size of the spleen. This determination, therefore, was proportional to the total splenic content of megakaryocytes.

RESULTS

Anemia

The results of hematocrit, MCV, and reticulocyte determinations are shown in Table 1. As compared to their +/+ and heterozygous littermate controls, W/Wv mice had anemia, macrocytosis, and mild reticulocytosis. Hematocrits for both the W/Wv and normals were lower than those reported by Bernstein et al. 28

Thrombocytopenia

Table 2 shows counts of platelets and megakaryocytes in W/Wv mice and their normal (+/+ and heterozygous littermates. Platelet counts were comparable in all groups. Megakaryocytes and nucleated cells were reduced in the tibial marrow of W/Wv mice when compared to +/+ controls (p < 0.001), and the percentage megakaryocytes was also reduced (p < 0.02). W/Wv splenic megakaryocytes were reduced to about 18% of those of +/+ controls.
(p < 0.001). Intermediate values were found in heterozygotes, but these were not significantly different from +/+ (p > 0.05 for W/+; p > 0.10 for W⁺/⁺).

Size distribution curves of stage III megakaryocytes (Fig. 1) showed that those from W/W⁺ mice were modestly larger than those from +/+ controls. The average size in W/W⁺ was 122±, that in +/+. Stage III megakaryocytes from W⁺/⁺ heterozygotes were not different from control; those from W/+ mice were shifted toward smaller sizes with the average size being 87±% of +/+ controls.

Two tests were applied to the megakaryocyte distribution curves to determine the level of significance of the observed differences from the +/+, or normal, controls (Table 3). First, the arithmetic mean cell size was determined for each genotype, and the Student's t test was applied to the difference between each W genotype and the +/+ value. Because cell sizes did not appear to follow a normal distribution, χ² analysis of each curve was also applied to test the probability that the megakaryocyte population in each W genotype was, in fact, identical to the population in the +/+ mice. The results of both tests indicated that W/W⁺ stage III megakaryocytes were significantly (p < 0.001) larger than normal. They also indicated that W/+ megakaryocytes were significantly smaller than normal. Thirty stage I megakaryocytes from 12 W/W⁺ mice showed no difference in size when compared to 52 stage I megakaryocytes from

Table 1. Results of Hematocrit, MCV, and Reticulocyte Determinations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hematocrit (%)</th>
<th>MCV (cu µ)</th>
<th>Reticulocytes (%)</th>
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<tbody>
<tr>
<td>W/W⁺</td>
<td>32.2 ± 1.75*</td>
<td>65.3 ± 3.47</td>
<td>3.9 ± 1.72</td>
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<tr>
<td>W/+</td>
<td>45.7 ± 0.46</td>
<td>45.4 ± 0.89</td>
<td>1.0 ± 0.33</td>
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<tr>
<td>W⁺/+</td>
<td>39.6 ± 2.32</td>
<td>51.2 ± 1.25</td>
<td>0.4 ± 0.25</td>
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<tr>
<td>+/+</td>
<td>44.2 ± 1.10</td>
<td>48.1 ± 0.82</td>
<td>0.7 ± 0.18</td>
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*Average ± SEM.
†Number of mice in parentheses.

Table 2. Counts of Platelets and Megakaryocytes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Blood Platelets/cu mm (x 10⁻⁶)</th>
<th>Megakaryocytes (x 10⁻²)</th>
<th>Nucleated Cells (x 10⁻⁶)</th>
<th>Per Cent Megakaryocytes</th>
<th>Spleen Megakaryocytes Per Section</th>
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<tr>
<td>W/W⁺</td>
<td>1.346 ± 0.064*</td>
<td>6.2 ± 0.8</td>
<td>10.644 ± 0.372</td>
<td>0.058</td>
<td>20.3 ± 7.30</td>
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<td>W/+</td>
<td>1.358 ± 0.072</td>
<td>12.7 ± 1.0</td>
<td>15.322 ± 0.504</td>
<td>0.083</td>
<td>77.1 ± 8.16</td>
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<tr>
<td>W⁺/+</td>
<td>1.214 ± 0.048</td>
<td>12.2 ± 0.8</td>
<td>11.395 ± 1.051</td>
<td>0.107</td>
<td>66.5 ± 19.32</td>
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<tr>
<td>+/+</td>
<td>1.322 ± 0.059</td>
<td>12.1 ± 0.8</td>
<td>14.650 ± 0.590</td>
<td>0.082</td>
<td>110.2 ± 16.07</td>
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*Average ± SEM.
†Number of mice in parentheses.
ABNORMALITIES OF MEGAKARYOCYTES IN W/W' MICE

Fig. 1. Size distribution curves for mature megakaryocytes from W/W' mice and their +/- controls. Size is expressed in planimeter units.

9 +/- controls, and statistical analysis (Table 3) confirmed this lack of difference.

Platelet size distribution in W/W' mice was identical to that from their +/- controls (Fig. 2). The same result was found for platelets from W'/+, and platelets from W/+ were not sized.

<table>
<thead>
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<th>Table 3. Analysis of Megakaryocyte Size Distribution Curves</th>
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<tr>
<td>Stage*</td>
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*Stage of megakaryocyte maturation (III is mature, I is immature).
†Number of mice.
§Number of megakaryocytes.
DISCUSSION

These observations showed that megakaryocytopoiesis was not normal in mice of the W/W' strain. These mice had normal platelet counts in spite of low numbers of tibial and splenic megakaryocytes. The mechanisms by which this was accomplished were not apparent. It could be proposed that the individual macromegakaryocytes of W/W' mice produced more than the normal number of platelets, thus, in part, compensating for the numerical deficiency of megakaryocytes. Clearly, the explanation is not this simple. Platelet counts in heterozygotes were also normal, as were megakaryocyte numbers. However, megakaryocytes in the W/+ heterozygotes appeared to be smaller than normal, thus emphasizing the lack of correlation between apparent megakaryocyte mass and platelet count. Megakaryocyte numbers were determined in two areas of hematopoiesis, the tibiae and the spleen; the possibility that there was expansion of hematopoietic tissue in W/W' mice, perhaps to areas of bone marrow that were not normally hematopoietic, must be considered before it can be concluded that the total body complement of megakaryocytes was decreased. The rate of turnover of megakaryocytes must also be known before megakaryocytopoiesis can be completely evaluated in these mice. That it may have been accelerated in W/W' mice was suggested by the decreased percentage of megakaryocytes in the marrow.
Platelet count alone also presents an incomplete evaluation of peripheral platelet kinetics. Total body mass of platelets, determined also by platelet size, blood volume, and size of the splenic platelet pool, and their rate of destruction, are more important measures of thrombocytopoiesis than cell concentration only. Platelet size was not abnormal in W/W* mice, but available information does not permit comment on blood volume, splenic sequestration, or rate of destruction of platelets in these mice.

Megakaryocyte size has been shown to be related to nuclear ploidy, so it could be proposed that the megakaryocyte population in W/W* mice tended to be of higher ploidy than normal. Macromegakaryocytosis has been observed to occur in response to peripheral thrombocytopenia, and to be associated with increased nuclear ploidy. Compensatory macrocytosis is demonstrable in both immature and mature megakaryocytes. The fact that only mature cells were found to be large in W/W* mice suggests that the macromegakaryocytosis occurred as a result of a mechanism different from that responsible for thrombocytopoietic stimulation in response to thrombocytopenia, and that, rather than increase in ploidy, these cells may have had a disturbance in cytoplasmic growth occurring after cessation of DNA replication. If the macromegakaryocytosis was, in fact, a compensatory change, then the stimulus for its production was not apparent. None of these mice was found to be thrombocytopenic, and the small standard error of the mean platelet count appeared to preclude the possibility that they had cyclical fluctuations of platelet count.

W/W* mice are extraordinarily sensitive to radiation, and the heterozygotes of the W strain have an intermediate LD to between those of the +/+ and W/W*. found that radioresistance was increased in W/W* mice and in W heterozygotes by transplantation of +/+ embryonic liver; this occurred along with correction of the anemia in the W/W*'s but without apparent change in erythropoiesis in the heterozygotes, suggesting that radiosensitivity may be determined, in part, by abnormalities other than those of the erythropoietic system. The present findings raise the possibility that abnormalities in megakaryocytopoiesis may contribute to the radiosensitivity.

These results demonstrate that megakaryocytes are not normal in mice of the W/W* strain. Others have shown that these mice have abnormal hematopoietic stem cells. The present findings are, therefore, consistent with the notion that normal megakaryocytopoiesis is dependent, in part, on normality of hematopoietic stem cells. The discrepancy between megakaryocyte and platelet numbers suggests that further studies of these mice may provide important clues about thrombocytopoietic regulatory mechanisms.

REFERENCES


