An Analysis of Megakaryocytogenesis in the C3H Mouse: An Animal Model Whose Megakaryocytes Have 32N as the Modal DNA Class

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The modal DNA content of normal marrow megakaryocytes from species so far examined usually has been reported to be 16N. In this report we describe an exception in the C3H mouse whose megakaryocytes have a modal DNA content of 32N. Female C3H/HEN mice had an average DNA content distribution of 14% 8N, 37% 16N, 43% 32N, and 6% 64N. Male C3H/HEN mice had somewhat higher proportions of 32N and 64N megakaryocytes (average DNA content distribution of 12% 8N, 29% 16N, 47% 32N, and 12% 64N) than females. All 11 other mouse strains examined had 16N as the modal megakaryocyte DNA content, although the proportions in the various polyploid DNA classes showed some strain variation. Megakaryocyte size was similar among all 12 strains evaluated, and mean platelet volume (MPV) of C3H/HEN mice differed from only 1 of the other 4 strains analyzed. Platelet counts of C3H/HEN mice were similar to those of six, and slightly but significantly lower than those of five other mouse strains examined. Compared with megakaryocyte concentrations of other mouse strains studied, that of C3H/HEN mice was similar to seven, somewhat higher than one, and slightly lower than three strains. Offspring from reciprocal matings of C57BL/6 and C3H/HEN mice had megakaryocyte DNA distributions intermediate between those of the parent strains, suggesting that a higher gene dosage of some component is responsible for the right-shifted megakaryocyte DNA content distribution phenotype of C3H mice. The proportions of 32N and 64N megakaryocytes increased in C3H/HEN mice in response to acute thrombocytopenia, as did those of CBA/CAJ mice used as a comparative strain. In summary, megakaryocytes of the C3H mouse have a higher average DNA content but similar platelet count, MPV, and megakaryocyte size and concentration as those of most other mouse strains. These results suggest that the number of platelets produced per unit of C3H megakaryocyte DNA is less than that for other mice.

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MEGAKARYOCYTES become polyploid before maturing to produce platelets. The degree of polyploidy of the maturing megakaryocyte dictates, in large part, the quantity of cytoplasm formed and presumably the number of platelets produced. Hence the level of polyploidy at which megakaryocytes mature provides one point of regulation of platelet production. Most reports indicate that in the mammalian species so far examined, the majority of megakaryocytes have a DNA content equivalent to a chromosomal complement of 16N, with lesser proportions having DNA contents of 8N and 32N and a few 64N. The megakaryocyte DNA content distribution can be experimentally altered by acutely increasing or decreasing the circulating platelet concentration. Although such experimental manipulation of platelet counts has been a valuable tool for understanding the effect of altered platelet demand on megakaryocyte polyploidy distribution, little has been learned about regulation of megakaryocyte polyploidy level in the steady state. For such studies animals with hereditary abnormalities of megakaryocyte polyploidy would be desirable; however, until now an animal model with a hereditary anomaly of megakaryocyte DNA distribution has not been reported. By chance we found that the C3H mouse has a modal megakaryocyte DNA content of 32N rather than 16N as found in other mouse strains. In this article, we have characterized and compared megakaryocytogenesis in the C3H mouse with that in other common laboratory mouse strains.

MATERIALS AND METHODS

C3H/HEN (8- to 24-week-old males and females), BALB/C (10- to 30-week-old females), C57BL/6 (11- to 24-week-old males and females), and DBA/2 mice (8-week-old females) were purchased from Charles River Breeding Laboratories (Wilmington, MA). A/J (17-week-old males), CBA/J (8-week-old males), CBA/NJ (8-week-old males), CBA/CAJ (12- to 19-week-old females), C3H/HeJ (7-week-old males), C3H/OuJ (9-week-old males), C3H/HeJ (7-week-old and 24-week-old males), DBA/1/J (8-week-old males), LP/J (7-week-old males), and SWR/J mice (15-week-old males) were obtained from The Jackson Laboratory (Bar Harbor, ME). The mouse age indicated is that at the time of study. Platelet counts were determined by phase-contrast microscopy after blood collection in platelet Unopettes (Becton-Dickinson, Rutherford, NJ). Hematocrits were determined using a microtechnique. Blood for platelet counts and hematocrits was obtained by puncturing a tail vein with a 27-gauge needle after vasodilation induced by warming mice under an examination lamp.

Platelet volume measurements. Mean platelet volumes (MPVs) were determined with a microcomputerized Elzone electronic particle counter (Particle Data Laboratories, Inc, Elmhurst, IL) equipped with a 48-μm orifice and a 128-channel analyzer. Data were accumulated on each platelet sample until a count of 4000 was collected in the peak channel. The samples were diluted so that the coincidence level was less than 1%. The volume distributions were analyzed as log-normal distributions. The data were expressed as MPVs. For platelet size analysis, blood (≈0.5 mL) was collected via cardiac puncture under general anesthesia after intraperitoneal

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injection of a heparin-Nembutal solution (50 U heparin and 2.5 mg
Nembutal) into plastic syringes containing 1 mL of 3.8% sodium
citrate. Platelet-rich plasma (PRP) was prepared by centrifugation
of the blood in 12 x 75-mm plastic tubes at 160g for 4.5 minutes at
22°C. The PRP samples were diluted 1:500 in Isoton (Coulter),
yielding a platelet concentration of 10,000 to 12,000 platelets/100
μL for size analysis.

Measurement of megakaryocyte DNA distribution. The DNA
distribution of megakaryocytes in unfractionated marrow was deter-

ined as previously described. Marrows from one tibia and one
femur were washed out and combined in 1 mL of CATCH medium
with 20 μg/mL of DNase. The megakaryocytes were specifically
labeled with a saturating concentration of rabbit antimouse platelet
antiserum (RAMPS) that had been absorbed with mouse and rat red
cells. The megakaryocyte-bound RAMPS was indirectly fluorescein-
ated with FITC-goat antirabbit IgG (Fab'), (Tago, Inc, Burlingame,
CA). Megakaryocyte recovery after the antibody labeling procedure
averaged 51%. Using an Epics 753 flow cytofluorometer (Coulter
Electronics, Inc, Hialeah, FL) and two-color flow cytometry, DNA
contents of all RAMPS-positive cells were measured (DNA contents
from 2N to 64N); however, for the purposes of this study, only those
cells with DNA contents ≥8N were considered, since the proportions
of 2N and 4N platelet-antibody-positive cells detected in unfraction-
ated marrow is very dependent on the selection of the green
fluorescence gate. The coefficient of variation for the modal polyploid
DNA peak routinely averaged less than 2%. The proportion of cells
in each ploidy class was determined by integrating the number of
cells under each DNA peak.

Bone marrow megakaryocyte concentration and diameter.
Megakaryocytes were recognized by their large size; large lobulated
nuclei with thick, clumped, deeply basophilic staining; and usually
abundant cytoplasm. The average number of megakaryocytes per
high power field (HPF; original magnification, 500x; field area
of 0.07 mm²) was determined in B-5-fixed, hematoxylin-eosin-stained,
ster nal marrow sections. One entire longitudinal section was evalu-
ated for each mouse. Megakaryocyte concentrations of animals with
increased average megakaryocyte diameter subsequent to induction
of acute thrombocytopenia were corrected for multiple counting
error by dividing the number of megakaryocytes per HPF by the
quotient of the average megakaryocyte diameter of the individual
thrombocytopenic mice divided by the average megakaryocyte
diameter of untreated control mice of the same strain. The diameters
of megakaryocytes (usually 50 per mouse) were determined in the
same sternal marrow sections with either an eyepiece micrometer at
a magnification of 1,250~ (diameter calculated as the square root
of the product of two diameter measurements made at right angles) or
a computer-assisted image analysis system (diameter calculated
from measurement of cell area assuming a spherical shape). The two
methods gave equivalent median diameters when performed on the
same specimens. All measurements were made by the same observer.
To examine the reproducibility of the diameter measurements,
average megakaryocyte diameter was determined on two different
occasions on sternums from the same four mice (not necessarily the
same megakaryocytes). The same average megakaryocyte diameter
was obtained from the two determinations for three of the four mice.
The two determinations on the fourth sample varied by 5%.

Fig 1. Polyploid megakaryocyte DNA content distributions of various mouse strains and substrains. Bars represent means ± 1 SD.
Numbers in parentheses indicate number of mice. From 289 to 2,267 (median, 1,1041 RAMPS-positive cells with DNA content ≥8N were
analyzed per mouse.
Assessment of marrow megakaryocyte maturation. Marrow was forced from a tibia or femur with air using a syringe, suspended with a few drops of 3.5% polyvinylpyrrolidone in Hanks' balanced salt solution and smeared on microscope slides. The smears were stained with a modified Wright's stain. Megakaryocytes were classified as immature or mature according to previously described morphological criteria. A range of 107 to 246 megakaryocytes were classified per mouse.

Induction of acute, severe thrombocytopenia. Acute, severe thrombocytopenia was induced in female C3H/HEN and CBA/CAJ mice by intravenous (IV) injection of RAMPs prepared as previously described. Female C57BL/6 mice were the first choice as a comparative strain for this experiment because their megakaryocyte ploidy response to acute thrombocytopenia had been described; however, their unavailability at the time prompted us to use the CBA/CAJ strain instead.

Statistical analyses. The Mann-Whitney rank sum test was used to test for statistical differences between groups and between megakaryocyte size distributions.

RESULTS

Polyploid megakaryocyte DNA distributions were examined in 12 commercially available laboratory mouse strains and substrains. The modal DNA content was 16N in all except C3H/HEN mice in which 32N was the modal DNA content (Fig 1). Male C3H/HEN mice had somewhat higher proportions (P < .001) of 32N and 64N megakaryocytes (average polyploid DNA content distribution of 12% 8N, 29% 16N, 47% 32N, and 12% 64N) than female mice (average polyploid DNA content of 14% 8N, 37% 16N, 43% 32N, and 6% 64N) of that strain.

Megakaryocyte DNA content distributions of males of other C3H mouse substrains were examined to ascertain whether all had 32N as the modal polyploid DNA peak. The modal polyploid DNA content was 32N in four of the five C3H substrains studied (Fig 2). In the exception, approximately equal proportions of 16N and 32N megakaryocytes were found in the C3H/OUJ strain. To be noted though is that while 32N was the modal megakaryocyte DNA content in older (24-week-old) C3H/HESNJ mice (Fig 2), megakaryocytes of young (7-week-old) C3H/HESNJ mice had a modal polyploid DNA content of 16N with a megakaryocyte DNA content distribution (19.0% ± 2.6% 8N, 47.3% ± 2.3% 16N, 31.0% ± 1.2% 32N, and 2.2% ± 0.6% 64N; data from five mice), which closely resembled that of CBA/CAJ mice. Average platelet counts of mice of the C3H/HEJ and C3H/HESNJ substrains were slightly lower (P = .02) than those of the other C3H substrains (Fig 2).

Average diameter of C3H/HEN mouse megakaryocytes in fixed marrow sections was similar to that of the other mouse strains (Table 1); cross-sectional area distributions of C3H/HEN mouse megakaryocytes were not significantly different from those of the comparison strains (C57BL/6 and BALB/C) illustrated in Fig 3.

Marrow megakaryocyte concentration of C3H/HEN mice also was similar to that of most of the mouse strains (Table 1).

The maturation profile of Wright's-stained C3H/HEN mouse megakaryocytes showed 39.8% ± 4.8% (six mice) with immature morphology, a value similar to that for C57BL/6 mouse megakaryocytes (39.2% ± 3.8% immature morphology, six mice). The ultrastructure of mature C3H/HEN megakaryocytes showed normal nuclear and cytoplasmic morphology with an extensive demarcation membrane system and could not be distinguished from that of C57BL/6 mice used as a comparative strain.

Platelet counts of C3H/HEN mice were similar to those of the majority of the mouse strains examined; however, the CBA/J, CBA/NJ, SWR/J, A/J, and CD-1 strains and C57BL/6 male mice had slightly higher values (P = .01) than those of C3H/HEN mice (Table 1). Mean platelet volumes of C3H/HEN mice were similar to all but the CD-1 strain, whose average MPV was slightly smaller (P = .013; Table 1).

The inheritance pattern of the 32N modal megakaryocyte DNA content phenotype was examined by reciprocal matings between C3H/HEN and C57BL/6 mice. C57BL/6 mice were chosen as the mating strain because their polyploid DNA content distribution had been previously characterized as having 16N as the modal DNA content. Megakaryocytes of offspring of these matings all had DNA content...
Table 1. Comparison of Megakaryocyte and Platelet Characteristics of C3H/HEN Mice With Those of Other Laboratory Mouse Strains

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Sex/No. of Mice</th>
<th>Platelet Count* x 10^7/µL</th>
<th>Mean Platelet Volume (fL)</th>
<th>MK/HPF</th>
<th>MK Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HEN</td>
<td>M/17</td>
<td>1,155 ± 168</td>
<td>3.99 ± 0.27 (9)</td>
<td>4.9 ± 0.4 (5)</td>
<td>20 ± 1.1 (5)</td>
</tr>
<tr>
<td></td>
<td>F/38</td>
<td>1,196 ± 177</td>
<td>4.2 ± 0.28 (12)</td>
<td>4.7 ± 0.8 (22)</td>
<td>20 ± 1.6 (22)</td>
</tr>
<tr>
<td>CBA/J</td>
<td>M/5</td>
<td>1,485 ± 41</td>
<td>—</td>
<td>6.4 ± 0.7</td>
<td>19 ± 0.5</td>
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<tr>
<td>CBA/CAJ</td>
<td>F/12</td>
<td>1,140 ± 126</td>
<td>—</td>
<td>4.3 ± 1.1 (9)</td>
<td>19 ± 1.2 (7)</td>
</tr>
<tr>
<td>CBA/NJ</td>
<td>M/5</td>
<td>1,388 ± 95</td>
<td>—</td>
<td>4.8 ± 0.8</td>
<td>19 ± 1.5</td>
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<td>SWR/J</td>
<td>M/5</td>
<td>1,421 ± 58</td>
<td>—</td>
<td>4.9 ± 0.6</td>
<td>20 ± 1.1</td>
</tr>
<tr>
<td>A/J</td>
<td>M/6</td>
<td>1,394 ± 89</td>
<td>—</td>
<td>5.2 ± 1.2</td>
<td>19 ± 0.8</td>
</tr>
<tr>
<td>LP/J</td>
<td>M/5</td>
<td>1,155 ± 129</td>
<td>—</td>
<td>4.7 ± 0.9</td>
<td>20 ± 0.8</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>M/5</td>
<td>1,362 ± 90</td>
<td>3.82 ± 0.35 (2)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>F/5</td>
<td>1,271 ± 162</td>
<td>4.01 ± 0.22 (3)</td>
<td>6.2 ± 1.0 (8)</td>
<td>19 ± 1.4 (8)</td>
</tr>
<tr>
<td>DBA/1J</td>
<td>M/5</td>
<td>1,245 ± 139</td>
<td>—</td>
<td>3.6 ± 0.4</td>
<td>19 ± 1.3</td>
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<tr>
<td>DBA/2</td>
<td>F/6</td>
<td>1,138 ± 40</td>
<td>4.11 ± 0.27 (5)</td>
<td>5.0 ± 0.6</td>
<td>19 ± 0.8</td>
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<tr>
<td>CD-1</td>
<td>F/6</td>
<td>1,672 ± 56</td>
<td>3.76 ± 0.07</td>
<td>6.1 ± 2.0</td>
<td>20 ± 0.4</td>
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<tr>
<td>BALB/C</td>
<td>F/4</td>
<td>1,128 ± 165</td>
<td>3.63 ± 0.03 (3)</td>
<td>6.5 ± 0.9 (6)</td>
<td>19 ± 0.8 (6)</td>
</tr>
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Numbers in parentheses indicate number of mice where different from number indicated in second column.

*Mean ± 1 SD.

distributions with 16N as the modal DNA peak (Fig 4) but with proportions of 16N and 32N megakaryocytes intermediate between those of the parent strains.

The thrombopoietic response to acute, severe thrombocytopenia was similar in both C3H/HEN and CBA/CAJ mice. Platelet counts 4 hours after RAMPs injection were reduced to an average of 3.9% and 3.1% respectively in C3H/HEN and CBA/CAJ mice.

Fig 3. Cross-sectional area distribution of female C3H/HEN mouse megakaryocytes compared with those of female C57BL/6 and BALB/C mouse megakaryocytes. The C3H/HEN distribution was derived from measurement of 250 megakaryocytes from five mice, while the BALB/C and C57BL/6 distributions represent 236 and 125 megakaryocytes each from six and four mice, respectively.

Fig 4. Polyploid megakaryocyte DNA content distributions of offspring of crosses between C3H/HEN and C57BL/6 mice. Bars represent means ± 1 SD. Numbers at upper right represent average platelet counts (mean ± 1 SD) in thousands per microliter of each group. Numbers in parentheses indicate number of mice. From 195 to 1,571 (median, 1,207) RAMPs-positive cells with DNA contents ≥8N were analyzed per mouse.
and CBA/CAJ mice (Fig 5). The rate of platelet recovery was similar in both mouse strains with the maximal rebound thrombocytosis occurring on day 5.

The modal megakaryocyte DNA content of CBA/CAJ mice shifted one DNA class to the right (from 16N to 32N) on days 2 and 3 post-RAMPS in accordance with an earlier report in C57BL/6 mice (Fig 6). In contrast, the modal megakaryocyte DNA content of C3H/HEN mice did not change (remained 32N), but the proportion of 64N megakaryocytes was significantly increased on day 1 ($P = .022$), and the proportions of both 32N and 64N cells were significantly increased on days 2 ($P < .001$), 3 ($P < .014$), and 4 ($P < .028$) post-RAMPS (Fig 6).

Megakaryocyte concentration showed no significant change following RAMPS. Megakaryocyte diameter distributions of normal C3H/HEN and CBA/CAJ mice were identical (Fig 7). The megakaryocyte diameter distributions of both strains were shifted to the right 2 and 3 days post-RAMPS ($P < .001$; Fig 7). Only CBA/CAJ mice showed a shift toward smaller megakaryocytes (on day 6 post-RAMPS, $P < .001$) in response to rebound thrombocytosis (Fig 7).

**DISCUSSION**

Polyploid nuclei are a characteristic of megakaryocytes. Most reports indicate that 16N is the modal DNA content of megakaryocytes in species thus far examined. In contrast,
we report here that 32N is the modal megakaryocyte DNA content in several substrains of the C3H mouse. However, the size distribution and concentration of C3H/HEN megakaryocytes and number and MPV of C3H/HEN platelets were similar to those of mouse strains having 16N as the modal megakaryocyte DNA content. Male C3H/HEN mice had somewhat higher proportions of 32N and 64N megakaryocytes than females. Matings of C3H/HEN and C57BL/6 mice yielded offspring with 16N as the modal megakaryocyte DNA content but with proportions of 16N and 32N megakaryocytes intermediate between those of the parent strains. Both C3H/HEN and CBA/CAJ mice responded to acute, severe thrombocytopenia by increasing the rate of platelet production, demonstrating rebound thrombocytosis, and increasing the proportions of 32N and 64N cells and megakaryocyte size.

The higher modal DNA content but normal concentration and size distribution of C3H/HEN megakaryocytes together with normal platelet counts and MPV and normal platelet survival of these mice suggest that C3H/HEN megakaryocytes produce fewer platelets per unit of DNA than do other mouse strains. However, the validity of this conclusion is dependent in part on the ability of the methodology to detect differences in megakaryocyte size and concentration that might be associated with the higher modal DNA content of C3H/HEN mice. How much larger should C3H megakaryocytes be if they have the same cytoplasm-to-DNA content ratio as that of other mouse strains? One approximation of this difference in size might be the increase in average megakaryocyte diameter that occurs when megakaryocytes increase their DNA content in response to acute thrombocytopenia. In the acute thrombocytopenia experiment reported here, the polyploid megakaryocyte DNA content distribution of CBA/CAJ mice at 2 days after induction of acute thrombocytopenia had 32N as the modal DNA content and closely resembled that of normal C3H/HEN mice: average diameter of CBA/CAJ mouse megakaryocytes at 2 days was 10% larger ($P < .036$) than that of normal CBA/CAJ megakaryocytes whose modal DNA content was 16N. Thus we would predict that C3H megakaryocytes would have an average diameter about 10% larger than that of the other strains if the cytoplasm-to-DNA content ratio were the same in all strains. We did not detect such a difference with the methodology used here. Furthermore, size distributions of C3H/HEN megakaryocytes did not differ from those of megakaryocytes of the other mouse strains examined (Figs 3 and 7). Thus the megakaryocyte size and concentration measurements along with normal platelet count, platelet
survival, and MPV of C3H/HEN mice support the conclusion that the number of platelets produced per unit of megakaryocyte DNA is decreased in C3H/HEN mice compared with mouse strains having 16N as the modal megakaryocyte DNA content. The normal MPV and platelet number produced by the higher average ploidy megakaryocytes of C3H/HEN mice also suggests that platelet size is not a function of the DNA content of megakaryocytes.2

The slightly higher average megakaryocyte DNA content of male compared to female C3H/HEN mice was not found with C57BL/6 mice, suggesting that this sex-related difference may apply only to C3H mice. An average polyploid megakaryocyte DNA content of offspring of crosses between C3H/HEN and C57BL/6 mice intermediate between those of the parent strains suggests that the higher average polyploid megakaryocyte DNA content of C3H/HEN mice is due to a gene dosage effect; either C3H/HEN mice have a lower gene dosage of a component that influences megakaryocyte cytoplasmic volume or a higher gene dosage of a component that affects megakaryocyte DNA content. Conceivably such a component could be a growth factor receptor on the cell membrane or an enzyme involved in DNA replication or cytoplasmic growth.

Although the megakaryocyte and platelet responses of C3H/HEN mice to acute thrombocytopenia were similar to those of the control strain (CBA/CAJ), some subtle differences were observed. For instance, the proportions of 32N and 64N C3H/HEN megakaryocytes remained significantly elevated on day 4 post-RAMPS, while those of CBA/CAJ mice did not. In addition, the size distribution of C3H/HEN mouse megakaryocytes did not shift toward smaller sizes (in response to rebound thrombocytosis) on day 6 after RAMPS, while those of CBA/CAJ mice did. These differences suggest that either C3H/HEN mouse megakaryocytes are more sensitive to a given concentration of factors that stimulate polyploidy formation or that these megakaryocytes are less responsive to feedback regulation. One other interesting difference reported for C3H mice is that their platelets require more arachidonic acid for aggregation.19

In summary, several substrains of C3H mice have been found to have 32N (rather than 16N) as the modal megakaryocyte DNA content but normal megakaryocyte size. The inheritance pattern suggests that differences in gene dosage may be responsible for this phenotype. This and other mouse strain differences detected in megakaryocyte characteristics here may be useful models to further investigate regulation of megakaryocyte differentiation.

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An analysis of megakaryocytopoiesis in the C3H mouse: an animal model whose megakaryocytes have 32N as the modal DNA class

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