Megakaryocytogenesis in Rats
With Special Reference to Polyploidy

By T. T. Odell, Jr., C. W. Jackson and T. J. Friday

The distribution of megakaryocytes among ploidy classes has been determined in several species—including rabbits, guinea pigs, rats, and man—and some relationships between the development of polyploidy and the maturation process of megakaryocytes have been discussed.

In addition, the ploidy composition of megakaryocytes within morphologic maturation compartments has been reported. In the latter investigation, however, measurement of DNA content was made in a select population of cells, namely, in labeled megakaryocytes that were sampled at intervals after injection of tritiated thymidine (3HTdr).

The present studies were undertaken to determine the ploidy composition of megakaryocytes within morphologic compartments in an unselected population. 3HTdr was injected 30 minutes before the rats were killed; and the shapes of the distribution curves of cells among the 8N, 16N, and 32N ploidy classes were examined with and without the inclusion of DNA-synthesizing megakaryocytes. In addition, the relative sizes of megakaryocytes were determined and related to the ploidy class and morphologic stage. The ploidy distribution results and other information presented here provide clarification of our notions about the maturation process of recognizable megakaryocytes.

METHODS

Squash preparations of tibial bone marrow of two rats were prepared, and determinations were made of morphologic classification of megakaryocytes, of their 30-minute labeling index after injection of tritiated thymidine, of relative cell size of megakaryocytes and of relative quantity of DNA in their nuclei.

Young adult male albino CD rats obtained from Charles River Breeding Laboratories were used. 3HTdr was injected intravenously (leg), 1 μCi./gm. of body weight (specific activity 1.9 Ci./mM.).

Kodak NTB-2 liquid emulsion was used in preparing autoradiograms. After autoradiography, the marrow cells were stained through the emulsion with Giemsas. Megakaryocytes, both labeled and unlabeled, were classified into Types I, II, and III.

For sizing, the megakaryocytes on 35-mm. pictures were enlarged eight times on Fotorite rapid-process single-weight photographic paper RB-11 (contrast 4), cut out, and weighed to the nearest 0.01 gm. Each sheet of photographic paper was also weighed individually, and the results confirmed that the photographic paper is uniform within a small tolerance. This method of cell size measurement provides a one-plane value making use of the maximum...
dimensions of each cell; it is essentially the equivalent of taking a section through the center of each cell.

DNA of individual megakaryocytes was measured microspectrophotometrically on Feulgen stained preparations after removal of Giemsa stain and silver grains of the emulsion.\textsuperscript{3,5,7} The frequency distributions of the DNA values of all measured megakaryocytes and of megakaryocytes of each of the three morphologic types were compared. A mixture of normal (Gaussian) frequency distributions has been developed as a model for representing the frequency distribution of DNA values.\textsuperscript{3,9} The method of maximum likelihood,\textsuperscript{8,9} using the logs of the DNA values, was used to estimate population parameters in the model, namely, the mean ($\mu$), the variance ($\sigma^2$), and the number of cells for each ploidy class. An iterative method of computation was used.\textsuperscript{10} The means and variances in the DNA scale were estimated from the maximum likelihood estimates of the mean and variance in the log scale by\textsuperscript{11}:

\[
\text{mean} = 10^\mu + \frac{\sigma^2}{2}, \text{ and} \\
\text{variance} = 10^{2\mu} + 2\sigma^2 - 10\mu + \sigma^4.
\]

Earlier studies (unpublished) had shown that the maximum likelihood method was preferable to either the least-squares method or the chi-square method because it does not require the assumptions of exact doubling of successive means and of equal variance that were necessary with the other two methods to reduce the number of parameters so that convergence could be obtained with the iterative technic.

**RESULTS**

The average DNA values (arbitrary units) of megakaryocyte nuclei of the several polyploid classes are presented in Table 1, along with the average DNA value of diploid marrow cells (not megakaryocytes), to permit confirmation of the assignment of each DNA peak to the proper ploidy class. The diploid cells whose DNA was measured were not labeled, and therefore were not in DNA synthesis (S). Also exhibited in Table 1 are appropriate multiples of the next lower measured DNA value to demonstrate the degree to which the measured values conform to a $2^N$ series.

The frequency of megakaryocytes of the total recognized population in the several ploidy classes is shown in the first row of Table 2, and the frequency within each morphologic maturation compartment (Types I, II, and III) in the last three rows of Table 2. The proportion of cells with an 8N complement of DNA is markedly reduced in the successive compartments (32 per cent → 15 per cent → seven per cent), whereas the relative proportion of 16N cells

| Table 1.—Relative DNA Values of Megakaryocytes Measured by Microspectrophotometry |
|-----------------------------------------------|---|---|---|---|
| Mean amount of DNA (S.D.) | 2 | 8 | 16 | 32 |
| Calculated values | 97 (9) | 466 (49) | 928 (82) | 1844 (199) |

* A haploid set of chromosomes.  
† Forty-six diploid marrow cells (not megakaryocytes).  
‡ A total of 1122 megakaryocytes from two rats was measured and the values, normalized at 16N between the two rats, were combined.  
§ Obtained by multiplying the next lower measured value by the number shown in parentheses.
Table 2.—Distribution of DNA Values of Various Groups of Megakaryocytes Among Ploidy Classes

<table>
<thead>
<tr>
<th>Megakaryocyte Type</th>
<th>8N  (per cent)</th>
<th>16N (per cent)</th>
<th>32N (per cent)</th>
<th>No. of Cells</th>
<th>Probability †</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (I, II, III)</td>
<td>19</td>
<td>66</td>
<td>15</td>
<td>1122</td>
<td>0.27</td>
</tr>
<tr>
<td>I unlabeled</td>
<td>23</td>
<td>66</td>
<td>11</td>
<td>228</td>
<td>0.11</td>
</tr>
<tr>
<td>I labeled</td>
<td>54</td>
<td>42</td>
<td>4</td>
<td>99</td>
<td>—</td>
</tr>
<tr>
<td>I labeled and unlabeled</td>
<td>32</td>
<td>59</td>
<td>9</td>
<td>327</td>
<td>0.04</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>67.5</td>
<td>17.5</td>
<td>759</td>
<td>0.26</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>76</td>
<td>15</td>
<td>41</td>
<td>— §</td>
</tr>
</tbody>
</table>

* Data of two rats were pooled, the values having been normalized on the 16N class since the mean DNA values of this class are estimated with the greatest precision. The maximum likelihood method was used except where noted.

† Tests whether the observed distributions and the calculated distributions are dissimilar.

1 Values in this row were obtained by subtracting the unlabeled from the total Type-I cells, not by the maximum likelihood method because of lack of convergence (see text).

§ The 8N class of Type III comprised only three cells whose inclusion prevented a good fit with the maximum likelihood method to three log normal distributions; omission of these three cells permitted a good fit (p = 0.40) to two distributions (16 and 32N).

increases. The subgroup having the largest percentage of 8N megakaryocytes is the labeled Type-I population (row three of Table 2).

It was not possible to fit the DNA values of Type-I labeled (in DNA synthesis) megakaryocytes (row three) to a series of log normal curves as with the other groupings of megakaryocytes because the DNA values representing the ploidy classes overlapped to the extent that they could not be sorted into different distribution peaks; iteration did not produce convergence. The values in the table were obtained by subtracting the unlabeled Type I from the total Type-I cells.

The Kolmogorov–Smirnov statistic12 was used to test the hypothesis that the distribution of DNA measurements of populations of different Types (I, II, III) of megakaryocytes are identical. The hypothesis was rejected at a 0.01 level of significance when comparing I unlabeled and II and at a 0.025 level of significance for I unlabeled and III. The comparison of II and III was not significant at a probability level of 0.1, but might well be with a larger number of Type-III cells; this analysis included 38 Type-III cells.

The percentage distribution of megakaryocytes by ploidy and maturation stage in the total population is shown in Table 3. These frequency values are affected by the time spent in each morphologic compartment.

Table 3.—Ploidy Composition of the Total Megakaryocyte Population

<table>
<thead>
<tr>
<th>N</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Per Cent</td>
<td>No.</td>
</tr>
<tr>
<td>8</td>
<td>105</td>
<td>9</td>
<td>114</td>
</tr>
<tr>
<td>16</td>
<td>193</td>
<td>17</td>
<td>512</td>
</tr>
<tr>
<td>32</td>
<td>29</td>
<td>3</td>
<td>133</td>
</tr>
</tbody>
</table>

* Values of two rats were combined.
To study the initial labeling index of ploidy classes (Table 4), injections of \(^3\)HTdr were made 30 minutes before taking marrow samples and therefore only Type-I megakaryocytes were labeled.\(^5\) Note that the percentage of labeled cells is high among 4N cells and declines as the ploidy increases. The labeling index of all Type I megakaryocytes as a group 30 minutes after injection of \(^3\)HTdr was 32 per cent.

The percentages of megakaryocytes of the various ploidies that were in the generation cycle (GC) are also shown in Table 4; these percentages were calculated using the duration of S as 80 per cent of GC.\(^13-15\)

Comparisons of the number of grains over initially labeled megakaryocytes made with Student’s t-test showed that the grain count of 16N cells (average of 119 grains in 32 cells) was significantly greater (p < 0.05) than that of 8N cells (average of 80 grains in 35 cells).

The average sizes of megakaryocytes in each ploidy class within the Type-I and the Type-II maturation stages are presented in Table 5. Comparisons made with Student’s t-test showed that the average sizes differed between each pair of ploidy classes within a single maturation stage, and between maturation stages within a ploidy class (Types I and II) with p < 0.01.

**DISCUSSION**

It has already been shown that each morphologic maturation compartment of recognizable megakaryocytes includes cells with 8, 16 and 32N values of DNA.\(^5\) In addition, the proportional distribution of megakaryocytes in ploidy classes within these maturation stages was reported for a selected population, namely for labeled megakaryocytes examined at intervals after a single injection of \(^3\)HTdr. The question remained whether megakaryocytes in an un-

### Table 4.—Labeling Index of the Ploidy Classes of Type-I Megakaryocytes 30 Minutes After Injection of Tritiated Thymidine*  

<table>
<thead>
<tr>
<th>Ploidy Class</th>
<th>4N</th>
<th>8N</th>
<th>16N</th>
<th>32N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled Cells/Total Cells</td>
<td>8/11</td>
<td>53/105</td>
<td>42/193</td>
<td>4/29</td>
</tr>
<tr>
<td>Labeling Index (per cent)</td>
<td>73</td>
<td>50</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>Cells in GC (per cent)</td>
<td>91</td>
<td>62</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>(S = 80 per cent of GC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values of two rats were combined.

### Table 5.—Relative Sizes of Megakaryocytes  

| Type | Ploidy Class | Rat 1 | | | Rat 2 | | |
|------|--------------|-------|------|-----|-------|------|
|      | Cell Size (X) | No. of Cells | | | Cell Size (X) | No. of Cells | |
| Type I | 8N | 0.91 | 15 | | 1.06 | 68 | |
|        | 16N | 1.50 | 40 | | 1.78 | 169 | |
|        | 32N | 2.44 | 8 | | 2.74 | 18 | |
| Type II | 8N | 1.57 | 23 | | 1.57 | 83 | |
|        | 16N | 2.92 | 121 | | 2.74 | 389 | |
|        | 32N | 5.37 | 34 | | 4.70 | 9 | |

All comparisons between ploidy classes within a maturation stage, and between maturation stages within a ploidy class, showed a statistical difference (t-test) with p < 0.01.
selected population have a similar distribution of cells in ploidy classes within morphologic maturation stages. It is also known that only the most immature stage of recognizable megakaryocytes can synthesize DNA, whereas differentiation of the cytoplasm continues in the more mature megakaryocytes in the absence of nuclear replication.

The distribution of megakaryocytes in the ploidy classes within the Type II and III maturation stages was similar in this study to that reported for the selected group of labeled megakaryocytes. In the Type-I compartment, however, there were about 25 per cent fewer 8N cells and about 35 per cent more 16N cells in the present study than in the selected labeled population. This can be explained by the finding that a larger proportion of 8N cells of the Type-I compartment are in DNA synthesis than 16N cells (Table 2); therefore, when only labeled cells were examined, the relative proportion of 8N cells was greater and that of 16N cells was smaller than in the total Type-I population.

The finding that the proportion of 8N cells in the Type-II compartment is much smaller (about half) than in the Type-I compartment (Table 2) suggests that only part of the 8N I cells go to 8N II; the other 8N I cells presumably endoreduplicate (synthesize another complement of DNA without cell division) in I, becoming 16N cells. Most of the latter apparently then go to II as 16N cells (16N cells make up 59 per cent of I and 68 per cent of II), while a few undergo another endoreduplication and become 32N before moving into the Type-II compartment.

That there were fewer 8N than 16N cells in the Type-I compartment, even though most or all of the 16N cells are thought to be derived from recognizable 8N cells, indicates that the 16N cells remain in I longer than 8N cells. The Type-I stage apparently includes two subcompartments, one consisting of endoreduplicating cells and a second comprising cells in a post-DNA-synthesis phase after endoreduplication has permanently ceased. Most 16N cells pass through both of these subcompartments since few go to 32N I, whereas many 8N cells presumably endoreduplicate and go to 16N I without entering the second compartment of Type I. The 8N cells would therefore have a shorter turnover time, explaining at least in part how the frequency of 8N cells can be smaller than that of 16N cells in the Type-I stage; the relative length of time spent in a compartment influences the relative frequency.

This explanation is supported by the higher initial labeling index among 8N than among 16N megakaryocytes. If the durations of GC and S are the same among megakaryocytes of all ploidies, as previously suggested, the percentage distribution among ploidy classes of initially labeled cells (8, 16, 32N) after injection of 3HTdr (Table 2, row two) is a measure of the percentages of cells of each ploidy that make up the actively endoreduplicating population. Since 54 per cent of these initially-labeled cells are 8N, they could supply the reduplicating 16N cells (42 per cent) and 32N cells (four per cent) as well as send some cells to 8N II.

The labeling index results also indicate that over 90 per cent and possibly all of the recognized 4N cells are in the generation cycle; that is, they are undergoing endoreduplication, since 73 per cent of 4N cells (8/11) were labeled and since the DNA-synthesis phase of the generation cycle makes up about 80 per
cent of the total GC of megakaryocytes. Similarly, by using 80 per cent of GC as the duration of S, about 62 per cent of 8N Type-I megakaryocytes were in the generation cycle, about 28 per cent of 16N cells, and about 18 per cent of 32N cells.

It should be kept in mind that in our determinations of ploidy class the 4N class includes cells in the latter part of S going from 2N to 4N and also cells in early S going from 4N to 8N. The same situation exists for the 8N and 16N ploidy classes, while in the case of 32N megakaryocytes only cells entering that ploidy class are recorded since very few cells go beyond 32N.

Since 32N I cells are presumed to pass through both subcompartments of Type I, times spent in each subcompartment can be calculated based on the proportions of cells in each subcompartment and the total time required to reach full labeling of the Type-I compartment after a single injection of 3HTdr. We estimate that about 82 per cent of the 32N I megakaryocytes are in the second subcompartment (not in S) and the total time to reach full labeling of the Type-I stage is about 24 hours; thus 19.7 hours is an estimate of the maximum time spent in the second subcompartment of I. This leaves about 4.3 hours for the first subcompartment, which is in good agreement with the 4.6 hours (half of one GC) that cells entering 32N I would be recognizable as having a ploidy of 32N. Since labeled megakaryocytes first begin to enter the Type-II compartment about 9.5 hours after injection of 3HTdr, the minimum time spent in the second subcompartment of I is about nine hours.

The full time spent in the Type-I compartment may be longer than 24 hours for some megakaryocytes since we are considering here only the last generation cycle plus the second subcompartment of I, whereas cells with higher ploidy values presumably pass through more than one generation cycle in I.

The size determinations showed a marked increase in size of megakaryocytes when moving either to a higher ploidy level within the Type-I compartment, or when moving at the same ploidy level from the Type-I to the Type-II compartment. Therefore, not only is it possible to separate megakaryocytes into size classes that correspond to their morphologic stage of maturation, as was shown by Ebbe and co-workers, but it is also possible to distinguish size groups corresponding to ploidy classes within a maturation stage. The differences in size between the ploidy classes within the Type-I maturation stage are doubtless related in large part to differences in size of the nuclei of cells of different ploidy; in general, the Type-I megakaryocytes have a relatively small amount of cytoplasm. The marked differences in size of megakaryocytes having the same ploidy but differing in maturation stage (I and II) are, however, due to changes in amount of cytoplasm. It is apparent, therefore, that megakaryocytes undergo a very active metabolic period between the time that DNA synthesis ceases and platelet formation takes place, and during this time the organelles and special structures are being laid down.
Summary

The morphologic stage of maturation, ploidy level, and cell size of individual megakaryocytes from the marrow of normal rats were determined; the initial labeling index of this population of megakaryocytes 30 minutes after injection of \(^{3}H\)TdR was also determined. The results suggested some inferences about the flow of cells through the various ploidy and differentiation compartments during the maturation of megakaryocytes. Most \(8N\) Type-I megakaryocytes endoreduplicate and become \(16N\) Type-I cells, while a few mature to \(8N\) Type-II megakaryocytes. Most \(16N\ I\) cells mature to \(16N\ II\) although a few endoreduplicate to become \(32N\ I\) cells; the \(32N\ I\) cells mature only to \(32N\ II\). The Type-I morphologic stage contains two subcompartments that we have not distinguished morphologically, an endoreduplicating subcompartment and a post-DNA-synthesis subcompartment. The time parameter of the latter ranges from about nine to 19 hours. The time parameter of the former depends on the number of endoreduplications undergone by an individual megakaryocyte. The size of megakaryocytes increases both with increases in ploidy level and with cell differentiation.

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References

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