Megakaryocytosis in the Rat

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(With the technical assistance of Janet Donovan and Donald Howard)

MEGAKARYOCYTES have been thought by some to make up a self-maintaining compartment.1-2 Observations of labeling with tritiated thymidine (H³ThdR), however, have indicated that morphologically recognizable megakaryocytes are not self-perpetuating but are dependent on influx from precursor cells.3,4 In the present studies, megakaryocytosis in the rat was further evaluated from the pattern of labeling with H³ThdR.

Originally, H³ThdR was believed to label only those cells which were synthesizing deoxyribonucleic acid (DNA) at the time of its injection.5,6 However, it has been demonstrated that tritium from DNA of labeled cell nuclei may be reutilized in the formation of new DNA. Megakaryocytes or their precursors apparently reutilize DNA-H³7 as do liver cells,8,9 bone marrow cells,10,11 fibroblasts and epidermal cells,12,13 sarcoma cells,14 and lymphocytes.15 Despite this, it has been possible to derive information about megakaryocytosis with H³ThdR.

MATERIALS AND METHODS

Seventy-eight female Sprague-Dawley rats, weighing from 138 to 184 Gm. were used. At time zero, each rat was injected, via tail vein, with H³ThdR,† 1 mc./Gm. body weight. At 30 minutes after injection and at intervals thereafter they were anesthetized with ether; blood for cell counts was obtained by cardiac puncture and anticoagulated with versene; they were killed with ether, and paint-brush smears of bone marrow made from split femora. After fixation of the smears in anhydrous methyl alcohol for 3-10 minutes, autoradiographs were made with Kodak NTB3 nuclear track emulsion. They were exposed in light-tight boxes for 28 or 30 days at -5° C. All slides for a given experiment were handled simultaneously so that autoradiographic and staining technics were identical for each experimental group of rats. After developing, the autoradiographs were stained either with Giemsa buffered at pH 5.8 or with a combination of Wright's and Giemsa stain.16

For each rat in experiment no. 2, 500 megakaryocytes were classified morphologically as recommended by Feinendegen et al.3 into stages I, II and III, and the number of nuclear grains for each cell counted. Since the results were essentially the same if only 250 cells were evaluated, for all other experiments (nos. 1, 4, 6 and 7), 250 megakaryocytes were observed for each rat. Background for each experiment was estimated from nomograms of the grain counts 30 minutes after injection of H³ThdR; between zero and

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†Schwarz BioResearch Inc., Orangeburg, New York, 1.9 c/mM, diluted with sterile saline to 500 μc./ml.

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MEGAKARYOCYTOPOIESIS IN THE RAT

background there was a decreasing frequency of “lightly labeled” cells. Background was considered to be the point at which (1) the number of “lightly labeled” cells reached zero or (2) the number of labeled cells began increasing, indicating true labeling. Background was estimated as 3, 5, 7, 9 and 12 grains per nucleus in these 5 experiments, and the values for mean grain count (MGC) were corrected for background.

In 4 experiments (nos. 1, 2, 4 and 6) one rat was sacrificed at each time after injection of H\textsuperscript{TdR}. In experiment no. 7, 5 rats were sacrificed at each of 5 time intervals to determine reproducibility of results.

RESULTS

Blood Counts

Platelet counts for the 78 rats included in these studies are given in table 1. Leukocyte counts for the entire group were 6,100-22,700/mm\textsuperscript{3} (average: 11,970); hematocrits were 32-48 per cent (average: 42 per cent). As can be seen in table 1, the average platelet count was lower for animals in the first experiment than in the other groups. These animals also had lower hematocrits (average: 40 per cent). The megakaryocyte differential and labeling characteristics, however, were similar to those of other experiments. The reason for the differences in peripheral blood values was not immediately apparent. It is noteworthy, however, that these animals were obtained from a different supplier than the others.

Morphology of Megakaryocytes

Rat megakaryocytes were classified morphologically into 3 groups (I, II and III), and their appearance suggested that these were successive stages of maturation. These stages appeared to correspond to the megakaryoblast, basophil megakaryocyte and granular megakaryocyte as described by Besançon.\textsuperscript{17} No cell was classified as a megakaryocyte unless it was larger than myeloid and erythroid marrow cells. In group I megakaryocytes, the nucleus occupied most of the cell, it was not segmented, and the cytoplasm had a clear basophilic appearance. In group II, the nuclear-cytoplasmic ratio was lower, the nucleus was usually lobulated, and the cytoplasm had a foamy basophilic appearance. Some cells in group II had azurophilic granules in localized areas of the cytoplasm. Group III contained all other megakaryocytes including those which were breaking up into platelets; its distinguishing characteristic was diffuse azurophilic granulation of the cytoplasm. The nuclei of group III were either single, multiple or segmented (fig. 1).

In the 78 rats used in these studies, differential count of megakaryocytes showed 18.7 per cent stage I, 25.0 per cent stage II, and 56.4 per cent stage III. Each of the 5 groups of rats showed a similar distribution (table 1).

Mitotic figures were rarely seen and only in stage I megakaryocytes.

Labeling Index

Curves of the percentage of each stage of megakaryocytes which were labeled at various time intervals after injection of H\textsuperscript{TdR} are shown in
Table 1

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>No. of Rats</th>
<th>Platelets/mm.² (x 10⁶)</th>
<th>Megakaryocyte Differential</th>
<th>Ratio I:II:III</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>17</td>
<td>0.878 (0.485-1.370)</td>
<td>21.5 26.6 51.8</td>
<td>1:1.24:3.41</td>
</tr>
<tr>
<td>II</td>
<td>18</td>
<td>1.180 (1.015-1.398)</td>
<td>18.3 23.2 58.5</td>
<td>1:1.27:3.20</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>1.150 (0.922-1.470)</td>
<td>21.6 26.0 52.5</td>
<td>1:1.20:2.43</td>
</tr>
<tr>
<td>IV</td>
<td>8</td>
<td>1.272 (1.085-1.550)</td>
<td>20.5 26.8 52.7</td>
<td>1:1.31:2.57</td>
</tr>
<tr>
<td>V</td>
<td>25</td>
<td>1.148 (0.870-1.445)</td>
<td>15.4 25.2 59.3</td>
<td>1:1.64:3.85</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>1.107</td>
<td>18.7 25.0 56.4</td>
<td>1:1.34:3.02</td>
</tr>
</tbody>
</table>

figure 2. The first samples were taken at 30 minutes when 20-30 per cent of stage I were consistently labeled. Labeling indices of all stages progressively increased, beginning at 2-4 hours in stage I, at 4-8 hours in stage II and at 12-24 hours in stage III.

Variability of labeling indices was observed by sacrificing 5 rats at each of 5 time intervals (30 min., 2 and 8 hours, and 1 and 3 days) after injection of H²TdR. The results verified those of the previous 4 experiments (fig. 3, upper). It was apparent, however, that the range of values at any given time was wide enough that any single animal could alter the shape of the labeling curves. For this reason, the subsequent conclusions were based on the average curve (fig. 4).

Mean Grain Count

For comparison, mean grain counts (MGC) of labeled cells for each experiment were standardized by assigning the value 1.00 to the MGC of group I megakaryocytes at 30 minutes; the actual values were 25, 127, 180 and 210 in the 4 experiments. The actual value for every other MGC was then expressed as a proportion of the 30 minute value for stage I, thereby obtaining "relative" MGC.

Curves from the 4 studies are shown in figure 5. Variability in the MGC was considerable (fig. 3, lower), and frequently individual values did not reflect the general pattern. Therefore, the average curve (fig. 6) was considered more significant.

With frequent sampling, every 6 hours, there were fluctuations in the MGC but an over-all decline in activity of stage I megakaryocytes during the first 4 days which approached a first order curve. Thereafter, there was a change in slope and a slower decline in MGC. An estimate for grain count halving time (T/2) during the initial phase was 1.5-1.8 days.
Fig. 1.—Autoradiographs of rat megakaryocytes labeled with tritiated thymidine. Upper left, stage I; upper right, stage II; lower left, stage III; lower right, mitosis in stage I.

Discussion

Morphology and DNA Synthesis

Correlation between morphologic appearance of megakaryocytes and their stage of maturation was confirmed by the sequential appearance of H³TdR label in stages I, II and III. Of recognizable megakaryocytes, only the most immature, stage I, regularly appeared to synthesize DNA as indicated by heavy nuclear labeling 30 minutes after intravenous injection of H³TdR.
Fig. 2.—Curves of labeling indices of rat megakaryocytes after a single injection of H\(^3\)TdR. Each panel represents a separate group of rats; upper left, expt. no. 1; lower left, expt. no. 2; upper right, expt. no. 4; lower right, expt. no. 6. Each point represents a single animal.

The small number of II’s which were initially labeled had a low MGC suggesting either that background for these cells was higher than estimated for the entire slide or that these cells were misclassified and should have been late I’s. Early labeling of a few cells in stage III was quite often due to asymmetric labeling of a single nuclear lobe as had been noted previously by Cronkite et al.\(^4\)

**Loss of Labeled Cells**

In the 3 experiments in which samples were taken frequently during the first 2 hours after injection, the labeling index of stage I decreased without increase in labeling of other megakaryocytes. Apparent loss of labeled cells from stage I without their appearance in a more mature compartment could have been due to (1) influx of unlabeled cells without efflux of unlabeled cells, (2) loss of labeled DNA from cells, or (3) selective loss of labeled cells due to radiation damage or other mechanisms. The first possibility was unlikely since it implied changing of compartment size by influx not balanced by efflux. Since it is probable that DNA is not lost from cells,\(^18\) this finding implied selective destruction of labeled cells.

Smith et al.\(^19\) concluded that marrow kinetics in the mouse would not be altered by tracer doses of H\(^3\)TdR, and Painter et al.\(^20\) determined that pulse labeling of HeLa S3 cells with H\(^3\)TdR did not disturb normal metabolism.
or growth. Based on these observations it seemed unlikely that this dose of tritium produced enough radiation damage to cause cell death. However, a few stage I megakaryocytes could conceivably have been destroyed by irradiation; since the changes in MGC during this period were not consistent (fig. 3 and 6) it was not apparent whether the heavily labeled or lightly labeled cells were preferentially lost.

Alternatively, this disappearance of labeled cells may be indicative of normal megakaryocyte destruction, or ineffective megakaryocytopoiesis. If this
Fig. 4.—Average curves of labeling indices of rat megakaryocytes after a single injection of H³TdR. Each point is the average from 2-4 rats.

Fig. 5.—Curves of relative mean grain counts of labeled rat megakaryocytes after a single injection of H³TdR. The individual experiments are presented as in figure 1. Each point represents a single rat.
Fig. 6.—Average curve of relative mean grain counts of labeled rat megakaryocytes after a single injection of H\(^3\)TdR. Each point is the average from 2-4 rats.

If the case, stage I megakaryocytes would be rather selectively affected during or shortly after the period of DNA synthesis. With the present data, it is not possible to distinguish between these two possibilities.

Absence of Cellular Division

Using microcinematography of rabbit and rat bone marrow, Kinosita and Ohno\(^2\) described complete division of megakaryocytes followed, in some instances, by reunion of only the cytoplasm to produce multinuclear cells. Such a process could not be excluded in these rats. However, permanent cellular division did not occur in recognizable megakaryocytes since the peak values for MGC in stages II and III were as high as the maximum MGC of I. Nuclear “division” seemed likely since II’s and III’s frequently had lobulated nuclei while I’s had single nuclei and mitotic figures were occasionally seen.

Mean Transit Time of Megakaryocytes

The mean transit time for megakaryocytes could be estimated in several ways. Assuming that (1) unlabeled megakaryocytes were replaced by labeled
cells after the initial labeling of stage I megakaryocytes, and (2) tritium was not continuously available for labeling of cells, the rate of increase of label in either stage I or the entire pool of megakaryocytes would give an estimate of turnover time. In addition, a minimum value for mean transit time from stage I to II could be derived by considering the time for the initially labeled stage I cells to appear in stage II which was, for practical purposes, initially unlabeled. Given the time spent in any stage, time in the others could be estimated from the megakaryocyte differential (table 1); in the absence of division, the cell differential is determined by the relative amounts of time spent in each compartment.

Total megakaryocyte transit time was estimated from the curve of labeling index of all megakaryocytes (fig. 7). The slope of the curve indicated that about 40 per cent of megakaryocytes were replaced each day; thus, the transit time, or time for all of them to be replaced, was about 60 hours. From this value and the cell differential, stage I was estimated as 11 hours, stage II as 15 hours, and stage III as 34 hours.

The 8-hour delay before labeling index of stage II megakaryocytes increased gave an estimate of a minimum time for maturation of stage I megakaryocytes to stage II. From the ratio of I:II:III, a minimum estimate for stage II of 11 hours and stage III of 24 hours with a total minimum maturation time of 43 hours was derived, assuming that the same cells which pass rapidly through stage I also pass rapidly through stages II and III.

Between 2 and 4 hours after injection, few labeled I's were maturing to stage II, and the percentage of I's which were labeled increased due to influx from labeled precursor cells. The rate of increase of label was about 7 per cent per hour which gave an additional estimate of time in compartment I of 14 hours. From this, time in stage II would be estimated as 19 hours and stage III as 42 hours, for a total maturation time of 75 hours. These values probably represent maximum estimates of mean transit times, since it is unlikely that efflux from stage I consisted only of unlabeled cells or that influx consisted entirely of labeled cells.

Thus, the average total maturation time for rat megakaryocytes was in the range of 43 to 75 hours with 8–14 hours as stage I, 11–19 hours as stage II and 24–42 hours as stage III. Feinendegen et al.3 reported a 40 hour transit time for rat megakaryocytes, and Matter et al.21 observed a 2-day lag period after platelet depletion before the rate of platelet production increased.

Variability of Transit Times

It is probable that maturation time for each stage of megakaryocytes is not fixed within rigid limits but rather is a spectrum. Rapid maturation of some cells was shown by the steady increase of the MGC of stage II after 1 hour. Thus, the small percentage of II's labeled at 2 and 4 hours (2 and 5 per cent) were considered to represent rapidly maturing I's which were probably near completion of their DNA synthetic period at the time of injection, and so were lightly labeled. Between 4 and 18 hours after injection, 5–8 per cent of III's were labeled, suggesting that a small number of megakaryocytes pass through all stages of maturation more rapidly than indicated by the mean
MEGAKARYOCYTOPOIESIS IN THE RAT

Fig. 7.—Curve of labeling index of all rat megakaryocytes after a single injection of H3TdR. Each point is the average from 2–4 rats. The increase of about 40 percent per day implies a total time in the megakaryocyte compartment (TMK) of about 60 hours.

transit times. The increasing MGC in stage III without detectable increase in number of labeled cells during this period was due to influx of a small number of relatively highly labeled cells, so that interpretation of this portion of the MGC curve is subject to error. Slower maturation of some cells was implied by failure of stages II and III to achieve as high a labeling index as that seen in stage I. This was considered to be partially due to variability in time spent in the three compartments and to be further evidence that megakaryocytes do not move through the stages of maturation as exact cohorts.

Megakaryocyte Precursor Cells

Some information regarding the immediate megakaryocyte precursor could be derived from the early part of labeling curves of megakaryocytes. In the absence of prolonged availability of high levels of H3TdR after a single injection, increase of the labeling index without concomitant fall in grain count must have been due to influx from a labeled precursor compartment. The 2-hour lag period before the labeling index in stage I began to increase represented a minimum estimate of the postsynthetic maturation time for precursors. Following this, there was a 10-hour period during which the labeling index of stage I increased, accompanied by little change in MGC; this time represented the period of DNA synthesis for the immediate precursor cell. During this early period of increasing labeling index, the MGC in stage I was little different from that observed initially suggesting the possibility that maturation proceeds from DNA synthesis without mitosis of the precursor.

An alternative explanation would be that the immediate precursor cell did divide before maturation; in that case, precursor cells should have been twice as heavily labeled initially as stage I megakaryocytes. Higher initial labeling of one cell type over another may be explained by (1) a more rapid
rate of DNA synthesis, (2) a larger surface area for uptake of H\textsuperscript{3}TdR, or (3) increased autoradiographic efficiency because of differences in cell geometry. None of these seem to apply in this case. (1) From the curve of labeling index, it appeared that 20–30 per cent of stage I were in DNA synthesis at any given time. With the transit time of 8–14 hours, a DNA synthetic period of about 2–4 hours was calculated, assuming that all stage I megakaryocytes pass through a period of DNA synthesis. This DNA synthesis period was shorter than the 5–8 hours generally observed for other mammalian cell systems. It is unlikely, therefore, that DNA synthesis in the precursor cells was even more rapid. (2) Identifiable megakaryocytes were, by definition, larger than other marrow cells, so had the largest area for absorption of H\textsuperscript{3}TdR. (3) Autoradiographic efficiency was relatively constant since the grain counts in question were all from the same type cell, the stage I megakaryocyte. Thus, the immediate precursor cell should not have been more heavily labeled than the stage I megakaryocyte.

The brevity of the calculated DNA synthetic period for stage I megakaryocytes and the relative constancy of their MGC for the first 12 hours suggested that initial labeling of stage I may be due to maturation of precursors during their own synthetic period rather than to DNA synthesis in recognizable megakaryocytes. With the present data one cannot distinguish between these two possibilities.

Between 12 and 18 hours after injection of H\textsuperscript{3}TdR the labeling index of I continued to increase, but the MGC dropped to about half its previous value. This reflects influx of precursors after a complete cell cycle and mitosis. By subtracting 2 hours for postsynthetic maturation from the 18 hours when the drop in grain count was seen, a generation time for precursor cells of about 16 hours was derived. The immediate megakaryocyte precursor may divide and be self-maintaining or may merely be another maturation compartment which, in turn, is being fed from a dividing cell compartment. In that case, one must postulate at least 2 megakaryocytic precursors which are not morphologically recognizable. A tentative scheme of thrombocytopoiesis combining these considerations is presented in figure 8.

Megakaryocytes did not divide, and the major part of the tritium label seen in them was derived from the maturation of labeled precursor cells. Therefore, the T/2 for stage I cells of approximately 1.5 days was a maximum estimate for generation time (Tg) of the most mature precursor cell which did divide. Maintenance of nearly 100 per cent labeling of stage I megakaryocytes for several days was interpreted by Feinendegen et al. as indicating that megakaryocytes arise from precursor cells which continuously synthesize DNA for 1–3 days, thus implying a Tg considerably longer than 1.5 days. This estimate (1.5 d.) of precursor cell Tg was longer than the value of about 16 hours discussed above, due, at least in part, to reutilization of tritium from H\textsuperscript{3}DNA in the synthesis of DNA by megakaryocytes or their precursor cells. In fact, the T/2 for stage I megakaryocytes was only 1.0 day when the amount of erythroid DNA available for reutilization was reduced by hypertransfusion with red cells. The labeling curves of megakaryocytes after injection of H\textsuperscript{3}TdR probably reflected true “pulse” labeling with H\textsuperscript{3}TdR for
the first few hours; later, relabeling of new DNA with breakdown products of H^3-DNA occurred, contributing to maintenance of the high labeling index of megakaryocytes. The gradual loss of label in megakaryocytes suggested loss by dilution as new DNA was made from both labeled DNA breakdown and unlabeled precursors.

Thus, recognizable megakaryocytes are not self-maintaining but are con-
tinuously fed from a precursor compartment. The morphologic nature of this immediate precursor is unknown. There is evidence for a totipotent stem cell, but it seems that the immediate megakaryocyte precursor is a more mature cell than this. In distinction to the immediate precursor, the bone marrow stem cell has been postulated to be in a state of no cell cycle or prolonged cell cycle during the normal steady state. A primitive mesenchymal cell which serves as a common precursor with more differentiated stem cells for each cell line has been postulated. The present studies support the concept of an immediate precursor which is already destined to be a megakaryocyte but which also may be self-maintaining; they do not clarify the problem of a common precursor cell.

**Control of Platelet Production**

There are many observations which suggest that megakaryocytopoiesis and the rate of platelet production are under homeostatic control. Foremost among these is the constancy with which an individual's platelet count is maintained over a period of time. A negative feed-back from circulating platelets to megakaryocytes has been postulated as has a positive feed-back or humoral stimulant to platelet production. However, the stages of development on which these feed-back mechanisms exercise control is not clear. Cronkite et al. reported that the numbers of megakaryocytes were not altered by transfusion of platelets although platelet production was suppressed; they interpreted this to mean that the negative feed-back operated by halting maturation of megakaryocytes and their precursors. In irradiated rats, the same authors found that platelet transfusions inhibited repopulation of splenic megakaryocytes which may mean that the negative feed-back inhibited differentiation of precursor cells to megakaryocytes. After platelet depletion in dogs, Craddock et al. observed increases in number and immaturity of megakaryocytes as well as a 3–4 day period of persistent thrombocytopenia which implied a feed-back mechanism which acted by altering the rate of differentiation of immature cells. Matter et al. concluded from similar studies in rats that alteration in cytoplasmic production rather than number of megakaryocytes occurred in response to acute thrombocytopenia. With the present model of megakaryocytopoiesis, it is hoped that some aspects of control of platelet production can be clarified.

**Summary**

Labeling of rat megakaryocytes was observed after a single intravenous injection of tritiated thymidine, and the following conclusions were made.

1. Morphologic appearance of megakaryocytes corresponded to their stage of maturation.
2. Only the youngest recognizable megakaryocytes (stage I) appeared to have a DNA synthetic period.
3. Megakaryocytes did not undergo permanent cellular division.
4. There was probably some degree of ineffective megakaryocytopoiesis.
5. The average total maturation time of rat megakaryocytes was estimated...
to be 43–75 hours with 8–14 hours in stage I, 11–19 hours in stage II, and 24–42 hours in stage III.

6. There was continuous influx into the megakaryocyte compartment from a precursor compartment. The immediate precursor cell was morphologically unrecognizable, but it appeared to have a generation time of ~16 hours, a DNA synthesis period of ~10 hours and a post-synthetic maturation time of ~2 hours.

7. Reutilization of tritium probably accounted for maintenance of a high degree of labeling of megakaryocytes for a period of 1.5–4 days.

**Summary in Interlingua**

Le presentia de un marcation del megakaryocytos de rattos esseva observate post un sol injection intravenose de thymidina a tritium, e le sequente conclusiones es formulate.

1. Le apparentia morphologic del megakaryocytos corrispondeva con lor stadio de maturation.
2. Solmente le plus juvene megakaryocytos recognoscibile (stadio I) pareva haber un periodo de synthese de acido deoxyribonucleic.
3. Le megakaryocytos non esseva subjecte a un premanente division cellular.
4. Un certe grado de inefficace megakaryocytopoiese esseva probablemente presente.
5. Le valor medie del total tempore de maturation de magakaryocytos del ratto esseva estimate como 43 a 75 horas con 8 a 14 horas in stadio I, 11 a 19 horas in stadio II, e 24 a 42 horas in stadio III.
6. Esseva constatate nulle continue fluxo ad in le compartimento megakaryocytic ab un compartimento precursori. Le cellulas immediatamente precursori esseva morphologicamente non recognoscibile, sed illos pareva haber un tempore de generation de approximativemente 16 horas, un periodo de synthese de acido deoxyribonucleic de approximativemente 10 horas, e un tempore de maturation post-synthetic de approximativemente 2 horas.

7. Il es probable que un re-utilisation de tritium esseva le causa del mantenentia de un alte grado de marcation de megakaryocytes durante periodos de inter 1,5 e 4 dies.

**References**

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Megakaryocytopoiesis in the Rat

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