Differences in Proliferative Activity between Normoblasts and Pernicious Anemia Megaloblasts

By E. G. Rondanelli, P. Gorini, E. Magliulo and C. P. Fiori

There are numerous conflicting concepts regarding the proliferation rate of pernicious anemia (p.a.) erythropoietic cells, compared with their normal counterparts; some authors claim that megaloblasts proliferate at a lower rate than normoblasts, while others affirm quite the opposite.

The proliferation rate of a cell population can be expressed as the frequency with which the cells enter mitosis per unit of time. Generally this frequency varies inversely with the generation time of the cellular population, the generation time being the sum of the intermitotic and mitotic times. Previously, estimation of the duration of mitosis in p.a. cells has yielded variable results, because of the different technics used (in vitro labelling, colchicine stathmokinetic method and others). Furthermore, absolute values for the duration or mitosis cannot be obtained from these data since these calculations lead only to relative expressions. We have made direct measurements of the chronology of mitosis by phase-contrast microscopic observations of in vitro cultured bone marrow cells, recorded by photography and time lapse cinematography. Although the in vitro technic implies separation of the cultured cells from homeostatic influences, it will still be valuable to compare the behavior of normal and pernicious anemia erythropoietic cells under the same environmental conditions.

Materials and Methods

Bone marrow was obtained by sternal puncture on 3 untreated pernicious anemia patients. Control bone marrow was obtained from three healthy subjects. Hanging drop cultures were prepared by quickly rinsing bone marrow tissue fragments in Tyrode's salt solution and explanting them on a cover glass, in a semisolid medium consisting of 1 part autologous plasma, 0.4 parts chicken plasma, 0.2 parts Tyrode's salt solution and 0.4 parts chick embryo extract. The cover-glass was then inverted on a well-slide and then, after incubation, inverted on a standard slide; the edges of the cover-glass were cemented down with melted paraffin.

Direct observation of the karyokinesis of erythropoietic cells was carried out by means of a Zeiss phase-contrast microscope placed inside a thermostatic (37 C.) chamber. For microphotography, a 6 x 6 Rada camera with panchromatic film of 15°Din sensitivity was used and, for microcinematography, a Zeiss-Siemens automatic camera loaded with 16 mm. reversible panchromatic film of 21°Din sensitivity running at about 8-12 frames per minute. The chronology of karyokinesis and its phases was recorded either directly or by analyzing the cine-records of a number of pooled mitoses from different cultures and different patients. A total of 34 mitoses in p.a. cells (9 promegaloblasts, 14 basophil megaloblasts, 11 polychromatophil megaloblasts) and 33 mitoses in normal erythroblasts (9 pronormoblasts, 12 basophil normoblasts, 12 polychromatophil normoblasts) were studied.

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The anaphase-telophase of the most immature p.a. megaloblasts is accompanied by cytotoxic phenomena. Cell diameters were chosen as follows:

- Promegablasts: 25–31 μm
- Basophil megaloblasts: 14–18 μm
- Polychromatophil megaloblasts: 10–14 μm
- Pronormoblasts: 20–25 μm
- Basophil normoblasts: 16–18 μm
- Polychromatophil normoblasts: 9–12 μm

Mitosis was subdivided as follows:

- Prophase - from the first nuclear changes to the breakdown of nuclear membrane (Fig. 1, frames 1–2);
- Metaphase - from the breakdown of the nuclear membrane to the disposition of chromosomes in an equatorial plate (Fig. 1, frames 3–9);
- Anaphase - poleward migration of sister chromosomes (Fig. 2, frames 1–2);
- Telophase - from the initial equatorial furrowing to the completion of the cleavage into two daughter cells and the reconstitution of interkinetic nuclei (Fig. 2, frames 1–6).

The anaphase-telophase of the most immature p.a. megaloblasts is accompanied by cytotoxic phenomena.
plasmic activity such as bubbling and ameboid movements, which have never been seen in normoblastic mitoses.\textsuperscript{15,16}

The duration of mitosis and of its phases were measured and statistical analysis of the pooled chronologic data of each series was performed. The Variance Ratio test was first carried out to determine whether the two populations (megaloblasts and normoblasts) have significantly different variances, i.e., whether each population was reasonably homogenous with respect to the chronology of mitosis at every maturation stage. Analysis of variance was also carried out on the chronological data of each population in order to determine the significance of any change in mitotic time which could be produced by maturation. The "t" test was then applied.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Morphologic features of mitosis in a p.a. megaloblast. Same cell of the preceding figure. Frames 1–2: anaphase; frames 3–9: telophase.}
\end{figure}
Results

In table 1 the mitotic time in normoblasts and megaloblasts are reported. The duration of mitosis appears consistently shorter in megaloblasts than in normoblasts at any of the three maturation stages considered. Furthermore, the mitotic time appears to increase with maturation in both series. The significance of the chronologic differences was computed from the variance of each group of data and the ratio of variances of opposite groups (normoblastic versus megaloblastic groups). The differences in variance did not appear significant (table 2), implying that the two populations do not possess different degrees of homogeneity with respect to their morphologic and chronologic characterization. The observed values for the “t” test (table 2) indicate that the difference in mitotic time between megaloblasts and normoblasts is highly significant. The significance of this difference increases with more advanced maturation. The variance analysis of the chronologic data of

Fig. 3.—Morphologic features of mitosis in a p.a. megaloblast. Same cell of the preceding figures. Completion of cleavage and nuclear reconstitution.
Table 1.—Average Duration of Mitosis and Mitotic Phases in Normoblasts and Megaloblasts

<table>
<thead>
<tr>
<th>Maturation Stage</th>
<th>Determination Performed</th>
<th>Prophase</th>
<th>Metaphase</th>
<th>Anaphase</th>
<th>Telophase</th>
<th>Mitotic Time*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-</td>
<td>9</td>
<td>16'05&quot;</td>
<td>41'49&quot;</td>
<td>10'49&quot;</td>
<td>76'43&quot;</td>
<td>10'49&quot; ± 4'10&quot;</td>
</tr>
<tr>
<td>Basophil-</td>
<td>12</td>
<td>15'57&quot;</td>
<td>48'07&quot;</td>
<td>15'48&quot;</td>
<td>91'02&quot;</td>
<td>10'52&quot; ± 2'46&quot;</td>
</tr>
<tr>
<td>Polychromatophil-</td>
<td>12</td>
<td>16'54&quot;</td>
<td>55'01&quot;</td>
<td>19'29&quot;</td>
<td>105'38&quot;</td>
<td>13'12&quot; ± 1'11&quot;</td>
</tr>
<tr>
<td>Megaloblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-</td>
<td>9</td>
<td>11'20&quot;</td>
<td>32'25&quot;</td>
<td>6'20&quot;</td>
<td>55'47&quot;</td>
<td>8'20&quot; ± 3'56&quot;</td>
</tr>
<tr>
<td>Basophil-</td>
<td>14</td>
<td>13'21&quot;</td>
<td>40'15&quot;</td>
<td>9'26&quot;</td>
<td>71'52&quot;</td>
<td>10'53&quot; ± 2'04&quot;</td>
</tr>
<tr>
<td>Polychromatophil-</td>
<td>11</td>
<td>15'31&quot;</td>
<td>45'19&quot;</td>
<td>14'36&quot;</td>
<td>86'29&quot;</td>
<td>10'34&quot; ± 2'11&quot;</td>
</tr>
</tbody>
</table>

*±2 x s_m (s_m: standard error of the mean).

Table 2.—Statistical Comparison of Megaloblastic and Normoblastic Mitotic Times

<table>
<thead>
<tr>
<th>Maturation Stage</th>
<th>Variance</th>
<th>Standard Deviation*</th>
<th>Standard Error*</th>
<th>Variance Ratio F</th>
<th>Corresponding 5% Significance Level</th>
<th>Corresponding 1% Significance Level</th>
<th>Student's t</th>
<th>Corresponding 5% Significance Level</th>
<th>Corresponding 1% Significance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promegaloblasts</td>
<td>125.491</td>
<td>554.24</td>
<td>118.08</td>
<td>1.12</td>
<td>6.03</td>
<td>7.29</td>
<td>2.12</td>
<td>2.92</td>
<td></td>
</tr>
<tr>
<td>Pronormoblasts</td>
<td>141.063</td>
<td>375.58</td>
<td>126.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basophil megaloblasts</td>
<td>54.663</td>
<td>233.80</td>
<td>62.48</td>
<td>1.82</td>
<td>2.76</td>
<td>4.34</td>
<td>11.41</td>
<td>2.06</td>
<td>2.79</td>
</tr>
<tr>
<td>Basophil normoblasts</td>
<td>83.418</td>
<td>288.82</td>
<td>83.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polychromatophil megalobl.</td>
<td>100.784</td>
<td>317.46</td>
<td>96.71</td>
<td>1.49</td>
<td>2.88</td>
<td>4.60</td>
<td>30.05</td>
<td>2.98</td>
<td>2.83</td>
</tr>
<tr>
<td>Polychromatophil normoblasts</td>
<td>67.522</td>
<td>259.85</td>
<td>75.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*in m².

The maturation stages within both normoblastic and megaloblastic series (table 3), yielded values of the ratio F (variance estimate between samples/variance estimate within samples) markedly higher than the corresponding values of the 1 per cent (5.39) and 5 per cent (3.32) significance level; it thus appears that the lengthening of mitotic time induced by maturation in both normal and p.a. erythropoietic cells is significant. In table 1 the durations of the phases of mitosis are reported, in order to ascertain which phases are responsible for the changes in total duration of mitosis. It appears that the lengthening of mitosis in more mature normoblasts is due to the elongation of metaphase, anaphase and telophase, while prophase does not seem significantly changed. In megaloblasts maturation induced slowing of all mitotic phases, but the telophase appears disproportionately lengthened.

In table 4 the differences (in per cent) in duration of the mitotic phases between normoblasts and megaloblasts are reported. All phases of mitosis appear shortened in megaloblasts; this shortening is most accentuated in the prophase and telophase of the more immature cells. The mature cells, these
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Table 3.—Analysis of Variance in Normoblasts and Megaloblasts

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>F* Observed</th>
<th>5% Level</th>
<th>1% Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoblastic</td>
<td>83.73</td>
<td>3.32</td>
<td>5.39</td>
</tr>
<tr>
<td>Megaloblastic</td>
<td>95.61</td>
<td>3.32</td>
<td>5.39</td>
</tr>
</tbody>
</table>

\[ \text{F} = \frac{\text{Variance estimate between samples}}{\text{Variance estimate within samples}} \]

Table 4.—Differences (in %) in Mitotic Phase Duration Between Normoblasts and Megaloblasts

<table>
<thead>
<tr>
<th></th>
<th>Prophase</th>
<th>Metaphase</th>
<th>Anaphase</th>
<th>Telophase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between promegablasts</td>
<td>29</td>
<td>22</td>
<td>22</td>
<td>45</td>
</tr>
<tr>
<td>and promegablasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between basophil normoblasts and megaloblasts</td>
<td>16</td>
<td>16</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td>Between polychromatophil normoblasts and megaloblasts</td>
<td>8</td>
<td>17</td>
<td>22</td>
<td>25</td>
</tr>
</tbody>
</table>

differences are less in all mitotic phases, except for anaphase in which the difference is approximately constant.

DISCUSSION

There is divergence of opinion concerning the proliferative activity of megaloblasts versus normoblasts. Japa\(^1\) from studies of mitotic indices and karyokinetic histograms states that the duration of both mitosis and interphase in megaloblasts is double that of normoblasts, corresponding to a proliferative activity in megaloblasts half that of the normoblasts. Castle\(^2\) claims that in p.a., due to the lack of factors acting on nucleic acid metabolism, many cells fail to divide normally, with lowered frequency of mitosis due to prolongation of the intermitotic time, especially in tissues presenting high cell turnover such as intestine and bone marrow. Cytoautoradiographic investigations of Salera, Tamburino and Magnanelli\(^5\) demonstrated that the intermitotic time of p.a. megaloblasts is elongated in both basophil and polychromatophil stages as compared with normoblasts. Mauri, Torelli and Grossi\(^6\) found low degrees of H\(^3\)-thymidine labelling in megaloblasts.

Reisner\(^3\) suggests that megaloblastic change per se could be due to a delay in entering mitosis, because of lengthening of the pre-mitotic DNA-synthesis period. Lajtha and Oliver\(^4\) also conceive of lengthening of the G\(_2\) period (premitotic gap following the synthesis of DNA in p.a.), with a resultant increase in cells with tetraploid amounts of DNA, RNA and protein. Sisken and Kinosita\(^1\) however, feel that these variations may be a function of the time between the preceding division and the beginning of DNA synthesis or the G\(_1\) period.

Astaldi, Mauri and Allegri\(^7\) experimented with the stathmokinetic effect of colchicine on megaloblasts explanted in vitro; information on the average duration of mitosis was obtained by comparing the stathmokinetic index (the rate of arrested mitosis achieved in a given time) and the mitotic index. These
authors concluded that p.a. megaloblasts proliferate much more actively than normoblasts in both the basophil and polychromatophil stages, while the decrease in proliferation usually observed when passing from the basophil to the polychromatophil stage, is less marked than in normoblasts. Further elaboration of the stathmokinetic method enabled Astaldi and Mauri to estimate the mean duration of mitosis and its phases. Salera established a new index, called proliferative index, which is the rate of mitoses arrested by colchicine per unit of time (i.e., the number of cells entering mitosis per unit of time); the ratio mitotic index/proliferation index (assuming the mitotic index is constant throughout) expressed the duration of mitosis from which the duration of the single phases could be calculated in relation to their distribution in the karyokinetic histogram. According to both of these studies, mitosis in megaloblasts appeared definitely shorter than that of normoblasts. Our own direct measurements “in vitro” lead to conclusions roughly similar to those of these workers as regards a definite shortening of mitosis in megaloblasts.

The data presented in this report can be used to determine the proliferative capacity of megaloblastic cells. Leblond and Walker computed the generation time from the formula $t_0 = t_M/I_M$, where $t_0 = \text{generation time}$, $t_M = \text{duration of mitosis}$ and $I_M = \text{mitotic index (mitoses/1000 cells)}$. Johnson showed that this equation is applicable only when mitosis occurs at the end of the cytologic compartment. Killmann et al. demonstrated that the computation of $t_0$ from $t_M/I_M$ should be restricted to certain steady state proliferation schemes such as a stem cell compartment terminated by heteromorphogenic division (where the change of cytologic characteristics on which cell classification is based coincides in time with mitosis).

In our system, the compartment being initiated by stem cell differentiation, the last mitosis of polychromatophil erythroblasts gives rise to two cells which are no longer capable of mitosis and which rapidly mature into orthochromatic erythroblasts. The weighted average generation times of different cells thus computed would perforce be overestimated in this system, because the mitotable pool contains a number of polychromatophil erythroblasts which have gone through their last division, but which cannot yet be distinguished from the other polychromatophil erythroblasts which have not yet divided. If the fraction of non-dividing cells is small, however, the error in $t_0$ will also be small. (It is notable in p.a. that the proportion of polychromatophils in the erythron is strikingly smaller than in normal conditions.) In addition, for the application of the formula, the possibility of cell death within the compartment is disregarded. However, if the weighted average mitotic time is known, the weighted average generation time might be roughly computed, even if only for comparative purposes.

An approximate value for the weighted average mitotic time can be computed from the average duration of a given number of mitoses on the basis of their distribution in bone-marrow smears according to cell type and stage.
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of maturation. Killmann\(^{13}\) reports the relative frequency of mitoses to be 1.0 in pro-normoblasts, 4.1 in basophil normoblasts, and 9.5 in polychromatophil and large orthochromatic normoblasts. If our chronologic data are applied, of 14.6 mitoses 1.0 should have a duration of 76'43", 4.1 of 91'02" and 9.5 of 105'38" which would make the average duration of karyokinesis in a similar distribution of normoblastic mitoses correspond to about 99'. According to Schwarz,\(^{18}\) the normoblastic and megaloblastic cells differ essentially in their pattern of proliferation. While the pronormoblasts divide only once (the descendants are transformed into basophil normoblasts), the promegaloblastic generations are not limited and can indefinitely succeed one another without maturing into megaloblasts. He found the distributive ratio (the proportion of the proerythro- and erythroblasts in division in a collection of cells) to be approximately between 1.0:4.0 and 1.0:2.0, respectively, in normoblasts, and higher values were found in megaloblasts. This implies that the relative frequency of mitoses is increased in the less mature megaloblasts, and that the average mitotic time of the whole p.a. megaloblasts is nearer to the lowest value of the mean mitotic time, as found in promegaloblasts (55'47") rather than polychromatophil megaloblasts (86'29").

It must be noted that the measured mitotic times in vitro may not directly apply to in vivo situations. Our data showing that mitotic time is shorter in megaloblasts than in normoblasts is valid, if we assume that any change in the absolute value of mitotic time is imposed by the experimental conditions can reasonably be assumed to be equal for the two cell types. Since using the in vitro values for computing generation time in vivo is open to question, we have not tabulated absolute values for \(t_0\).

Our in vitro measurements of the mitotic time and the computed generation times suggest that:

1. proliferative activity in p.a. erythropoietic cells is greater than in normoblasts;
2. both mitosis and interphase are shortened in megaloblasts;
3. all the phases of mitosis are shorter in megaloblasts than in normoblasts at every stage of maturation, but the shortening is more marked in the most immature cells, and particularly in prophase and telophase.

Maturation up to the stage of basophil megaloblast induces a lesser degree of shortening in metaphase, telophase and particularly in prophase, while the shortening of anaphase remains unchanged. Between the basophil and polychromatophil megaloblast, the metaphase and anaphase degree of shortening is unchanged while the shortening of prophase is much less marked than in the preceding maturation stages. Telophase, even if less than in the preceding maturation stages, still appears to be the most shortened phase. Due to the increased proliferative capacity and morphologic immaturity of p.a. megaloblasts, one may thus conceive of p.a. megaloblastosis as a maturative rather than proliferative defect. Higher than normal marrow activity was found by the erythrokinetic studies of Finch, Coleman, Motulsky, Donohue,
Rondanelli, Corini, and Pecorari have demonstrated that mitosis in erythropoietic cells is shorter in the more immature dividing cell. In 1931 Introzzi hypothesized a direct origin of megaloblasts from histioid cells without any maturative passage through the hemocytoblasts. He termed p.a. as "megaloblastoid reticuloendotheliosis." The more marked shortening of telophase in promegaloblasts could possibly be related to the presence, during cleavage, of cytoplasmic bubbling and ameboid movements. This cytoplasmic activity can be found only in megaloblasts and never in normoblasts and appears similar to that seen in histioid cells, thus supporting Introzzi's hypothesis.

It is not known whether this cytoplasmic activity is a passive expression of a faster cleavage, or if it actively intervenes in speeding up daughter cell separation. The shortening of prophase in promegaloblasts could be due to an easier mobilization of the mitotic apparatus, possibly related to the more accentuated dispersion of nuclear chromatin in these cells.

**Summary**

The mitotic behavior of pernicious anemia and normal erythropoietic cells in vitro was studied by means of phase contrast microphotography and cinemaphotograph. Direct measurements showed that the duration of mitosis is significantly shorter in megaloblasts than in normoblasts at every stage of maturation. Maturation induces an elongation of mitosis in both the normal and the pathologic series. The mitotic index being practically the same in normoblasts and megaloblasts, the weighted average generation time in megaloblasts, calculated according to the formula \( t_0 = t_M/T_M \), resulted in a shorter time than in normoblasts. This indicates that there is a higher than normal proliferative activity of p.a. cells since the generation time appears inversely proportional to the frequency by which new mitoses are entered in a cell population. The role of each single mitotic phase in shortening the total duration of mitoses has been investigated. Possible alterations in the mitotic mechanisms involved have been discussed.

**Summario in Interlingua**

Le comportamento mitotic de cellulas erythropoietic normal e a anemia perniciose esseva studiate in vitro per medio de microphotographia a contrasto de phase e per cinemaphotographia. Mesurationes directe monstrava que le duration del mitose es significativemente plus breve in megaloblastos que in normoblastos a omne stadio del maturation. Maturation induce un prolongation del mitosis tanto in le serie normal como etiam in le serie pathologic. Viste que le indice mitotic es practicamente le mesme in normoblastos como in megaloblastos, le ponderate tempore medie de generation in megaloblastos, calculate per medio del formula \( t_0 = t_M/T_M \), esseva plus curte que in normoblastos. Isto indica que le cellulas a anemia perniciose ha un activitate proliferative plus alter que normal, proque le tempore de generation pare esser inversemente proportional al frequencia con que nove mitoses entra in un population cellular. Esseva investigate le rolo de cata-un del phases mitotic in
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reducer le duration total del mitoses. Es discutite possibile alterationes in le participante mechanismos mitotic.

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