A Study of Erythropoiesis by Combined Morphologic, Quantitative Cytochemical and Autoradiographic Methods

Normal Human Bone Marrow, Vitamin B₁₂ Deficiency and Iron Deficiency Anemia

By S. N. Wickramasinghe, E. H. Cooper and D. G. Chalmers

The quantitative cytochemical measurement of nucleic acid and hemoglobin in single cells marked the beginning of a new approach to the study of erythropoiesis. This was soon overshadowed by the advent of tritiated thymidine (³H-TdR) as a specific marker for DNA and the subsequent development of various types of sophisticated kinetic analyses which have been extremely valuable in investigating the flow patterns of erythropoiesis in animals. The application of this approach to man although valuable has been limited by the necessity of multiple bone marrow biopsies and by the radiobiological hazard from in vivo ³H-TdR labeling. Previous experience has shown that a combination of ³H-TdR autoradiography and quantitative cytochemical measurements of the DNA content on the same individual cell can provide considerable information about the distribution of a population of cells in the various stages of interphase. Using this technique Menzies et al. have shown that a G₂ arrest and an arrest of cells in DNA synthesis occurs in the nucleated cells of the bone marrow in vitamin B₁₂ deficiency. They give no information about the stage in the erythrocyte maturation process at which the arrest occurred.

In the present study, this technique has been combined with a standard morphologic identification of each cell studied, prior to Feulgen staining. The purpose of this investigation has been twofold. Firstly, to investigate the relationship between the morphology of erythropoietic cells and their position in interphase, and secondly, to study the nature of the disturbance of erythropoiesis intrinsic in certain forms of anemia. The findings in normal human bone marrow, vitamin B₁₂ deficiency and iron deficiency anemia are reported in this paper.
STUDY OF ERYTHROPOIESIS

METHODS

0.5–2.0 ml. of sternal marrow was aspirated via a Salah needle into a siliconized glass syringe. The aspirate was immediately mixed with 4 ml. heparinized Hanks solution at 37 C. containing 2.5 µc. ³H-TdR per ml. (specific activity 5000 c/M). After 30 min. incubation at 37 C. marrow fragments were smeared on clean, grease-free glass slides. The smears were rapidly air dried and fixed in absolute methanol for 15 mins.

The fixed smears were stained with May-Grunwald-Giemsa (MGC) stain (pH 6.8) under controlled conditions. Cell trails from marrow fragments were selected where the spacing of the cells was suitable for microdensitometry and photographic maps made of these areas. The positions and morphologic features of the various erythropoietic cells were recorded on these maps. The position of a number of lymphocytes was also recorded at random throughout the mapped area to provide a standard for the diploid (2c) quantity of DNA.

A method of classification was devised in which the three main features of each erythroid cell were assessed independently. These were nuclear size, nuclear structure including the presence or absence of nucleoli and the appearance of the nuclear chromatin, and the tinctorial characteristics of the cytoplasm. On this basis, it was possible to separate erythroid cells into several morphologic categories. The different morphologic categories of polychromatic normoblasts were subsequently pooled into two major cell groups: early polychromatic cells with a nuclear diameter equal to or greater than 6 µ and late polychromatic cells with a smaller nucleus. In the megaloblastic series, a nuclear diameter of 7 µ was selected as the point of demarcation between the early and the late polychromatic megaloblasts.

The MGC stain was then leached out by treatment with 5 percent trichloroacetic acid at room temperature for 30 secs. followed by immersion in absolute methanol for 10–15 mins. The smears were then stained by the Feulgen method using a 10 minute hydrolysis in 1N. HC1 at 60 C. The absorbance of the Feulgen dye in individual nuclei was measured with a Deeley pattern integrating microdensitometer.

Autoradiographs were prepared with Ilford K5 emulsion, exposed for 7–14 days at 4 C. and developed with Kodak D19B at 16 C.

In some preparations, photographic negatives of Feulgen stained nuclei were projected to a constant magnification and the outline of the nucleus drawn on paper. A micrometer scale was photographed and projected at the same magnification. The area of each nucleus was calculated by comparing the weight of the paper enclosed by the image with that enclosed by 100 square microns obtained from the micrometer scale.

RESULTS

Morphology

Analysis of the detailed morphologic characterization of erythropoietic cells adopted in this study revealed no relationship between minor differences in nuclear structure and the position of a cell in interphase. In normal erythropoiesis, both pronormoblasts and basophilic normoblasts were observed in all stages of interphase. In addition it became apparent that although, in the normal, the majority of basophilic cells fell into the category of pronormoblast or basophilic normoblast, as defined in conventional hematology, there were many cells with intermediate characteristics, particularly in bone marrow from pathological states. For this reason, the results for the proerythroblasts and basophilic erythroblasts have been pooled together into a single basophilic erythropoietic cell group. The group of cells classified as early polychromatic cells was found to include cells in all stages of interphase, and those classified as late polychromatic cells had diploid contents of DNA, characteristic of a non-dividing somatic cell population.

In vitamin B₁₂ deficiency, karyorrhexis and Howell-Jolly body formation was
<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>CASE</th>
<th>Hb, G/100 ml</th>
<th>MCHC %</th>
<th>Percentages</th>
<th>S/G, U</th>
<th>Number of Nuclei Assessed</th>
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<tbody>
<tr>
<td>Iron Deficiency</td>
<td></td>
<td></td>
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<td>Untreated</td>
<td>G.P.</td>
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<td>22.5</td>
<td>31</td>
<td>61</td>
<td>8</td>
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<td>Part. Gastr., Untreated</td>
<td>G.H.</td>
<td>8.0</td>
<td>23.0</td>
<td>20</td>
<td>76</td>
<td>4</td>
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<tr>
<td>Untreated</td>
<td>R.F.</td>
<td>7.0</td>
<td>24.0</td>
<td>12</td>
<td>87</td>
<td>1</td>
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<td>Responding to iron therapy</td>
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<td>7.9</td>
<td>26.0</td>
<td>27</td>
<td>66</td>
<td>7</td>
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<tr>
<td>Vitamin B12 Deficiency</td>
<td>F.H.</td>
<td>7.6</td>
<td>26.0</td>
<td>26</td>
<td>69</td>
<td>6</td>
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<tr>
<td>Pernicious anemia</td>
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<td>30.0</td>
<td>10</td>
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<tr>
<td>Pernicious anemia</td>
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<td>28.0</td>
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<td>32.5</td>
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<td>Part. Gastr.</td>
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<td>27.0</td>
<td>13</td>
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<td>32.5</td>
<td>25</td>
<td>32</td>
<td>19</td>
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<td>29.0</td>
<td>16</td>
<td>73</td>
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<tr>
<td>Pernicious anemia</td>
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<td>32.0</td>
<td>10</td>
<td>83</td>
<td>7</td>
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<td>Normal Values (5 cases)</td>
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<td>32</td>
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<td>Mean</td>
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<td>24-36</td>
<td>58-70</td>
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seen predominantly in the polychromatic megaloblasts. Such abnormal nuclei were usually unlabeled and although the majority had a diploid DNA content, a number of karyorrhectic nuclei with tetraploid or intermediate DNA contents was seen.

**Cell Cycle Distribution**

Table 1 shows the relative distribution of basophilic erythropoietic cells in the different stages of the cell cycle in marrow from the three groups of patients studied. G₁ represents post-mitotic cells with a diploid (2c) content of DNA, S represents cells synthesising DNA and G₂ represents pre-mitotic cells following completion of DNA synthesis, with a tetraploid (4c) DNA content. Column U represents unlabeled cells with a DNA content lying clearly between the normal spread about the 2c and 4c modes. In the normal, among 831 nuclei of basophilic erythropoietic cells studied, only three cells belonging to this latter category were found. The vitamin B₁₂ deficient group, with the exception of a single patient (H.P.), showed a decreased percentage of cells in G₁ and a normal or increased percentage of cells in S. Several cases also showed an increased percentage of cells in category U, with or without a decreased S/G₂ ratio. One case (H.P.) showed a more pronounced build-up of cells in G₂ and arrest in DNA synthesis associated with a low labeling index. In the iron deficient group, the findings were only significantly different from the normal in two cases (G.H. and R.F.) where there was an increased percentage of cells in S with a decreased percentage in G₁.

Table 2 gives the data for the dividing polychromatic cells in the three groups studied. With the exception of the two patients with mild anemia (I.A. and E.D.), the vitamin B₁₂ deficient group showed a decrease in the S/G₂
Fig. 1.—The distribution of DNA in the labeled and unlabeled early polychromatic normoblasts in a normal marrow (case E.H.).

These changes are greater than those seen in the basophilic group of cells. Similar changes were not seen in the iron deficient group.

Figures 1 and 2 are histograms of the frequency distribution of the DNA contents of early polychromatic cells in a normal marrow, and in a patient with untreated pernicious anemia, respectively.

Figure 3 shows the relation between nuclear size and DNA content in the basophilic and dividing polychromatic cells in a normal marrow and in a patient with untreated pernicious anemia. Although there was a wide range of nuclear size for each DNA value, the nuclear sizes tended to be larger at higher DNA values.

DISCUSSION

The production of the erythrocyte is the result of a continuous process of cell differentiation accompanied by cell division. Although the classification of cells into major groups is relatively simple, the justification of the creation of further subgroups must depend on the demonstration of a clear distinction in maturation or function between them. On the evidence available so far, a clear distinction exists between the basophilic cell group, the dividing polychromatic cells and the non-dividing polychromatic cells. Classically the basophilic group contains two recognizable members, the pro-normoblast and the basophilic
normoblast. However, in a detailed study of individual marrow preparations from normal or particularly from pathological states, there is a gradual transition between these two cell types, which makes it impossible, by available techniques, to define accurately the boundary between them, and for this reason they have been considered as one functional compartment. Under well standardized conditions of staining, before autoradiography, the distinction between basophilic cells and polychromatic cells is reliable. The most difficult problem in classification is probably that of determining at what stage the polychromatic cell stops cyclic division. This is the point which will define the boundary between the early polychromatic and late polychromatic compartments. Clearly those cells which are labeled or contain a 4c content of DNA must by definition belong to the early polychromatic group, and are part of a dividing cell population. There is no absolute indication as to whether any individual polychromatic cell with a 2c content of DNA will proceed through a divisional cycle or will become a member of the non-dividing polychromatic cell group. As nuclear involution advances, the recognition of non-dividing polychromatic cells becomes simple but the early, post-divisional members of this group have no unique morphologic characteristics. For this reason, we have chosen, arbitrarily, to classify this compartment on the basis of nuclear size. A nuclear diameter of 6μ was chosen as the point of cut-off between the dividing

Fig. 2.—The distribution of DNA in the labeled and unlabeled early polychromatic megaloblasts in a patient with vitamin B₁₂ deficiency (case E.B.2).
and non-dividing polychromatic normoblasts in G1. Subsequent analysis of the results has shown that very few cells which had a nuclear diameter of less than 6μ were labeled, giving circumstantial support to the choice of size. In the megaloblastic series, because of the increase in the cell size throughout the
STUDY OF ERYTHROPOIESIS

series, a nuclear diameter of 7 μ was selected as the point of demarcation between the dividing and non-dividing polychromatic megaloblasts.

A comparison of the labeling indices in the two dividing cell compartments in normal erythropoiesis, with those published by other workers shows some discrepancy. Whereas the labeling index for the basophilic cells shows little deviation from previously published figures, the present system of classification gives a considerably higher labeling index in the early polychromatic cells than that of Bond and his co-workers. This discrepancy is probably related to the way in which the cells have been classified. The percentage of cells in each stage of interphase, within a particular compartment, is dependent on the accurate morphologic definition of that compartment. As the G₁ component of the early polychromatic compartment is dependent on an arbitrarily defined point of cut-off, minor variations in this point may considerably influence the percentages of these cells in the various stages of interphase. For this reason, we prefer to rely on the ratio of S/G₂ where the cells being studied are undoubtedly part of the dividing polychromatic cell group. It can be seen that this ratio is profoundly altered when there is a perturbation of normal cell kinetics.

It should be noted that a significant increase in nuclear size can occur as a cell moves through its cell cycle and that this is more marked in megaloblastic erythropoiesis. For this reason, caution is advised in the use of nuclear size as a basis for classification in the absence of information on DNA content.

In iron deficiency anemia, there was no gross abnormality in the distribution of erythropoietic cells in the various stages of the cell cycle, with no evidence of arrest of cells in DNA synthesis or build up of cells in G₂. The findings in the single case (F.H.) responding to iron therapy and in two of the four untreated cases, were essentially normal.

The findings in vitamin B₁₂ deficiency show three changes from those of the normal. Firstly, the increased numbers of cells which are found with a 4c content of DNA; secondly, the presence of a number of cells with an intermediate DNA content which are not in active DNA synthesis as detected by thymidine labeling; and thirdly, that although these defects are apparent in the basophilic group of cells, they are at their maximum in the dividing polychromatic cell group. These findings have been confirmed when an alternative DNA marker, tritiated deoxycytidine, is used.

An arrest after a period in DNA synthesis is only one of the possible explanations for cells which have been included in category “U” in this paper (U = Unknown). The possibility that the U cells may result from a re-arrangement of the histone/DNA relationship leading to an alteration in their Feulgen staining has been largely eliminated, firstly by the use of different hydrolysis times during Feulgen staining, and secondly by the use of gallocyanin chrome alum as an alternative DNA stain for quantitative cytochemistry. These results will be reported in detail elsewhere.

At present, there is no direct information regarding the metabolic activity of these cells which accumulate in G₂ or appear to be arrested in DNA synthesis. The fact that the classical morphologic changes of megaloblastic erythropoiesis

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can occur without a significant build-up of cells in $G_2$ or $U$, can be seen from the two cases of vitamin $B_{12}$ deficiency with mild anemia reported here. The relationship between anemia, megaloblastic change and the disturbances in the cell cycle reported here is not clear. The results in the iron deficient group of patients shows that anemia per se does not lead to a build-up of cells in $G_2$ or $U$.

Limited data are available from studies of patients with pernicious anemia, labeled with $^3$H-TdR in vivo. From such studies, Cronkite et al.\(^5\) have shown that the number of non-dividing polychromatic cells produced per 1000 dividing erythropoietic cells was significantly lower than normal in this disease. Fliedner et al.\(^15\) have demonstrated a disappearance of cells which is maximal in the dividing polychromatic cell compartment. It seems reasonable to assume that the disappearance of cells detected by these in vivo studies is related to the apparent arrest of cells in DNA synthesis and the build-up of cells in $G_2$ (probably due to a failure to enter mitosis). It seems likely that the death of these arrested cells is responsible for the ineffective erythropoiesis seen in vitamin $B_{12}$ deficiency.\(^16,17\)

**SUMMARY**

The relationship between the morphology of human erythropoietic cells and their position in interphase has been studied. In normal bone marrow, pronormoblasts, basophilic normoblasts and early polychromatic normoblasts were present in all stages of interphase. It has been shown that a significant increase in nuclear size occurs as a cell moves through its cell cycle, in both normal and megaloblastic erythropoiesis.

The relative distribution of the basophilic erythropoietic cells and the dividing polychromatic cells, in the various stages of interphase, has been determined in normal bone marrow, vitamin $B_{12}$ deficiency and iron deficiency anemia.

In vitamin $B_{12}$ deficiency, associated with moderate or severe anemia, there was an increased proportion of cells in $G_2$, and there were several cells with DNA contents that were between the $2c$ and $4c$ values, which were not in DNA synthesis as judged by $^3$H-TdR labeling. These abnormalities were most pronounced in the dividing polychromatic cell group. Similar abnormalities were not present in iron deficiency anemia, indicating that these disturbances were not produced by the presence of anemia per se. The possible relationship between these changes in the cell cycle and the ineffective erythropoiesis seen in anemia due to vitamin $B_{12}$ deficiency has been discussed.

**SUMMARIO IN INTERLINGUA**

Esseva studiate le relation inter le morphologia de cellulas erythropoietic human e lor position in le interphase. In normal medulla ossee, pronormoblastos, normoblastos basophilic, e precoce normoblastos polychromic esseva presente in omne le stadios del interphase. Ha essite demonstrate que un augmento significative del dimensiones nucleari occurre in le curso del progresso del cellula individual a transverso su cyclo cellular e que isto vale tanto pro le erythropoiese normal como etiam pro le erythropoiese megaloblastic.

Le relative distribution del basophilic cellulas erythropoietic e del cellulas polychromic in division esseva determinate pro le varie stadios del interphase in normal medulla ossee, in medulla ossee ab subjectos con anemia a carentia de vitamina $B_{12}$, e in medulla ossee ab subjectos con anemia a carentia de ferro.
STUDY OF ERYTHROPOIESIS

In carenia de vitamina B₁₂ associate con moderate o sever anemia, un augmento esseva constatate in le proportion del cellulas in C₂. Esseva etiam presente un numero de cellulas con contentos de ADN de inter le valores 2c e 4c le quales non esseva in synthese de ADN a judicar per observationes a TdR tritiate. Iste aberrationes esseva be plus marcate in le gruppo de cellulas polychronic in division. Nulle comparabile anormalitates esseva constatate in anemia a carentia de ferro. Isto indica que le disturbationes in question non esseva causate per le presentia de anemia per se. Es commentate be possibile relation inter iste alterationes del ciclo cellular e le inefficace erythropoiese incontreate in casos de anemia a carentia de vitamina B₁₂.

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