Proliferation of Megaloblasts in Pernicious Anemia as Observed from Nucleic Acid Metabolism

By Yataro Yoshida, Akio Todo, Shigeru Shirakawa, Gyöichirô Wakisaka and Haruto Uchino

Megaloblastic anemia is due chiefly to deficiency of vitamin B12 (B12), of folic acid, or of both. Recent studies3,4,12,22 have been focused on the view that the metabolic defect leading to megaloblastosis may, in some way, be related to nucleic acid metabolism. However, the role of B12 coenzymes in mammalian nucleic acid metabolism has not been elucidated, nor are there exact data on the nature of the defect in the proliferative capacity or mitotic cycle of megaloblasts.1,4,15,17,18,20,22,24-26

The present study was undertaken (1) to clarify the cytokinetic patterns of DNA, RNA, and protein synthesis in the megaloblasts in cases of B12 deficiency, using microphotometric DNA measurement and in vitro autoradiography (2), to obtain further information on DNA synthesis in individual megaloblasts by means of the combined method of microphotometry and autoradiography (3), to elucidate the effect of specific B12 therapy on these patterns of synthesis, and (4) to relate the findings to current concepts of megaloblastic hemopoiesis.

Materials and Methods

Marrow was aspirated from the sternum in four patients with pernicious anemia (PA), before the institution of specific therapy, and after treatment when the hemoglobin had reached a level of over 10 gm./100 ml. and the megaloblastic marrow had been transformed to the normoblastic state. Marrow samples from five hematologically normal subjects were used as controls. Table 1 summarizes the clinical and hematologic data of these patients.

Microphotometric Estimation of the Amount of DNA

Bone marrow smears fixed in formaldehyde vapor were stained by the Feulgen procedure. The amount of staining of individual cells was measured by an absorption analyzing TV-Microspectrophotometer (Neutronics Research Co., Waltham, Mass., ME-101a) designed by...
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Zworykin. The apparatus, consisting of a closed-circuit television microscope (RCA TK-25A in conjunction with a Bausch & Lomb grating monochromator 33-86-40 and a Bausch & Lomb microscope RCL 88), and a line-analyzing oscilloscope (Tektronix 524AD), was revised in part so as to secure a rapid line-scanning system in the visible light area. The DNA content per cell in arbitrary units, was calculated according to Caspersson’s scheme. Repeated measurements in duplicate were made of approximately 150 to 200 erythroblasts in each case.

In Vitro Autoradiography

Marrow samples were suspended in a mixture of 1 part autologous serum and 2 parts Tyrode’s solution containing a few drops of heparin. The cell count was adjusted to 10^4 cells per cmm. H3-thymidine* (SpA 5.0c./mM), H3-uridine* (SpA 2.18c./mM), and H3-leucine* (SpA 215.1mc./mM), as labeled precursors for DNA, RNA, and protein, respectively, were added to the incubation mixtures to give a final concentration of 1 µc./ml. for H3-thymidine and of 5 µc/ml. for both H3-uridine and H3-leucine. The suspensions were incubated in siliconized test-tubes for 1 hour at 37 C. Following brief centrifugation, smears were made from the sediment, air dried, and fixed in methanol. The slides were coated with FUJI ET-2E stripping films (FUJI Photo Film Co., Ltd., Tokyo), exposed for 14 days in a refrigerator, developed, fixed, and stained with Ciemsa. Cells with 5 or more grains over the evaluated area (the nucleus in H3-thymidine and H3-uridine autoradiographs, and the whole cell in H3-leucine autoradiographs) were scored as labeled.

Consecutive Microphotometry and Autoradiography

The technique employed was similar to that described by Hale et al. Smears were prepared from cell suspensions to which H3-thymidine had been added. After Feulgen staining, suitable fields were selected and photographed. The DNA content of individual cells on the photographic map was measured. Subsequently, autoradiographs of the same slides were prepared as stated above except that the time of exposure was 4 weeks. Identification of cell types was based on counterstaining with Giemsa.

RESULTS

Microphotometric Estimation of the Amount of DNA

Figure 1 shows the composite data on DNA distribution in erythroblasts from a representative control and four cases of PA before and after treatment. In two out of the four cases examined (Cases 1 and 2), the DNA values of basophilic and polychromatic megaloblasts varied between 2c (postmitotic value) and 4c (premitotic value), exceeding 4c only occasionally. In the remaining two cases (Cases 3 and 4), the DNA values of basophilic and polychromatic megaloblasts showed a wide range of variation with a considerable number of cells falling around 4c. After B12 therapy, the DNA distribution in all cases showed a pattern similar to that in controls: in nearly all cells it ranged from 2c to 4c. In contrast, there were few differences in DNA value between orthochromatic megaloblasts and orthochromatic normoblasts, nearly all these having the 2c value (nuclei in the process of extrusion being excluded from Fig. 1).

In Vitro Autoradiography

The results are shown in Fig. 2. The percentage of cells labeled with H3-thymidine was lower in promegaloblasts, basophilic and early polychromatic megaloblasts than in the normoblastic series, while that of late polychromatic

megaloblasts was higher than normals. In the granulocytic series in cases of PA, though the labeling index was lower in the immature forms, the metamyelocytes, especially those of the giant variety, were labeled with H3-thymidine, while normal metamyelocytes were not labeled. Megaloblasts, in general, showed lower grain counts. On the other hand, the mean grain
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Fig. 2.—In vitro incorporation of H3-thymidine, H3-uridine, and H3-leucine into erythroblasts as measured by autoradiography. Each point marks the average of respective cases. Upper graph shows percentage of labeling and mean grain counts per cell as measured by H3-thymidine uptake. Lower graph shows mean grain counts of H3-uridine and H3-leucine autoradiographs. A total of 1000–2000 erythroblasts were counted in each case. ● = PA untreated, △ = PA after treatment, ○ = normal controls.

counts of H3-uridine and H3-leucine in the megaloblastic series were somewhat higher than normals. All these changes in the uptake of tritiated precursors by PA marrow cells returned to normal after B12 therapy, by an increase in the labeling index and the mean grain counts with H3-thymidine, and by a decrease in the mean grain counts in H3-uridine and H3-leucine labeled cells.

Consecutive Microphotometry and Autoradiography

In the two cases of PA (Cases 1 and 4) and 5 control subjects, 150–200 basophilic and polychromatic erythroblasts were examined by this method. In the normoblastic series, cells with DNA values between 2c and 4c were labeled with H3-thymidine, and cells with DNA values of 2c or 4c remained unlabeled (Fig. 3 left). The unlabeled 2c cells seemed to be in the postmitotic (G1) period, and the unlabeled 4c cells in the premitotic (G2) period. The absence of unlabeled cells lying between the 2c and 4c modes indicated that nearly all cells in the synthetic (S) period were labeled. In the megaloblastic series (Fig. 3 right, Case 4), the DNA distribution in the labeled cells exceeded the 4c value. On the other hand, there were many unlabeled cells with values between 2c and 4c. The presence of such unlabeled cells between 2c and 4c
Table 1.—Hematologic Data of Four Patients with PA before (A) and after B₁₂ Therapy (B)

<table>
<thead>
<tr>
<th>Case</th>
<th>Hb Gm. %</th>
<th>Hematocrit %</th>
<th>Retic. %</th>
<th>Bone Marrow</th>
<th>Urinary Excretion (mg./24 hrs.) of MMA*</th>
<th>Schilling Test (%)</th>
<th>Dose (μg.) of B₁₂</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>76</td>
<td>(A)</td>
<td>3.2</td>
<td>9.0</td>
<td>1.5</td>
<td>megaloblastic</td>
<td>197.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B)</td>
<td>10.3</td>
<td>31.0</td>
<td>2.6</td>
<td>normoblastic</td>
<td>37.8</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>(A)</td>
<td>5.6</td>
<td>16.0</td>
<td>0.6</td>
<td>megaloblastic</td>
<td>224.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B)</td>
<td>10.4</td>
<td>34.5</td>
<td>1.3</td>
<td>normoblastic</td>
<td>61.6</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>(A)</td>
<td>2.6</td>
<td>7.7</td>
<td>0.4</td>
<td>megaloblastic</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B)</td>
<td>10.6</td>
<td>33.0</td>
<td>0.9</td>
<td>normoblastic</td>
<td>1019.5</td>
</tr>
</tbody>
</table>

*MMA = methylmalonic acid, measured by silicic acid column chromatography; normal range below 20 mg./day.
†FICLU = formiminoglutamic acid, measured following oral loading with 15 g. of histidine; normal range below 30 mg./day.
‡2 USP. units of Bifacton, Organon Inc., Holland.
§Marrows were obtained 24 hrs. after 100 μg. of B₁₂ given.
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Fig. 3.—Histograms of DNA content distribution in the unlabeled (upper graphs) and labeled (lower graphs) populations. Consecutive determination of DNA content and H³-thymidine incorporation in individual basophilic and polychromatic erythroblasts from a normal marrow (left) and a PA marrow, (right, Case 4).

Fig. 4.—Histograms of DNA content distribution in the unlabeled (upper graphs) and labeled (lower graphs) populations. Consecutive determination of DNA content and H³-thymidine incorporation in individual basophilic and polychromatic erythroblasts from a patient with PA (Case 4) during treatment. One dose (10 μg.) of coenzyme B₁₂ was given to the patient, and a second dose (1000 μg.) of cyanocobalamin was given 7 days later. Measurements were carried out 24 hours after each injection. For details of the clinical data and the therapeutic procedure, see ref. 29.
Table 2.—Differential Counts of Erythroblasts in the Bone Marrow of a Patient (Case 4) with PA during the Therapeutic Procedure

<table>
<thead>
<tr>
<th></th>
<th>Before Therapy</th>
<th>24 hrs. after FA 60 mg.</th>
<th>24 hrs. after Coenzyme B₁₂ 10µg.</th>
<th>24 hrs. after B₁₃ 1000µg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megaloblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promegaloblasts</td>
<td>3.6</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Basophilic</td>
<td>36.8</td>
<td>15.2</td>
<td>12.4</td>
<td>0</td>
</tr>
<tr>
<td>Early polychrom.</td>
<td>27.2</td>
<td>24.6</td>
<td>19.8</td>
<td>0</td>
</tr>
<tr>
<td>Late polychrom.</td>
<td>18.0</td>
<td>21.8</td>
<td>13.6</td>
<td>0</td>
</tr>
<tr>
<td>Orthochromatic</td>
<td>14.4</td>
<td>6.2</td>
<td>7.2</td>
<td>0</td>
</tr>
<tr>
<td>Intermediate Erythroblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basophilic</td>
<td>0</td>
<td>6.8</td>
<td>19.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Polychrom.</td>
<td>0</td>
<td>12.8</td>
<td>25.8</td>
<td>0</td>
</tr>
<tr>
<td>Orthochromatic</td>
<td>0</td>
<td>2.6</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>Normoblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basophilic</td>
<td>0</td>
<td>1.0</td>
<td>0.6</td>
<td>13.4</td>
</tr>
<tr>
<td>Polychrom.</td>
<td>0</td>
<td>4.2</td>
<td>0.2</td>
<td>78.4</td>
</tr>
<tr>
<td>Orthochromatic</td>
<td>0</td>
<td>3.6</td>
<td>0</td>
<td>9.0</td>
</tr>
<tr>
<td>E/M ratio</td>
<td>7/5</td>
<td>3/2</td>
<td>5/6</td>
<td>3/2</td>
</tr>
<tr>
<td>Mitotic Indices</td>
<td>25.0</td>
<td>44.0</td>
<td>29.5</td>
<td>40.0</td>
</tr>
</tbody>
</table>

A total of at least 500 erythroblasts were counted at each time. The frequency of each cell type is expressed as a percentage of all erythropoietic cells. The criteria for intermediate erythroblasts are those of Downey. Mitotic Index is expressed as the number of mitotic figures per 1000 erythroblasts.

was found in both cases examined by this method. The patterns of DNA distribution were improved 24 hours after the administration of 10 µg. of coenzyme B₁₂ or 1000 µg. of B₁₂ (Fig. 4). Cyanocobalamin, 1000 µg. was given to this patient (Case 4) 7 days after the administration of 10 µg. of coenzyme B₁₂. After each injection, most of the labeled cells fell between 2c and 4c, while all the unlabeled cells remained at 2c or 4c. This was also true of case 1 after treatment. However, the improvement by 10 µg. of coenzyme B₁₂ in case 4 might be due in part to the effect of folic acid given earlier; see below.

**DISCUSSION**

The present study revealed that megaloblasts in B₁₂ deficiency had a normal or substantially increased DNA content per cell, as well as decreased ability to synthesize DNA, as indicated by a reduced incorporation of H³-thymidine. These changes returned to normal after B₁₂ therapy. Reisner and Korson found no difference in the DNA value between megaloblasts and normoblasts of the same nuclear diameter, hence of the same stage of maturation. However, the relative predominance of immature large forms in PA marrows may possibly account for the finding that the DNA values in megaloblastic marrows are greater than in normal marrows, as far as the entire erythropoietic populations are concerned. Increased numbers of cells ranging around the 4c value, suggesting possible polyploidy formation, have been reported.\(^4\)\(^{20}\)
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By combining microphotometric and autoradiographic techniques, it is possible to relate the DNA content of individual cells to the uptake of DNA precursor by the very same cells. This might yield valuable information on the behavior of cells in the process of DNA replication. The results obtained by the combined method revealed a derangement in DNA synthesis in megaloblasts (Fig. 3). It is likely that many unlabeled cells between 2c and 4c had once started and then ceased to synthesize DNA during the 1 hour incubation period when H3-thymidine was available. Alternatively, an extreme slowing down of DNA synthesis might result in no detectable labeling in the course of experiment. Menzies et al. have recently reported similar observations on marrow cells as a whole in B12 and folate deficiency. Although loss of labeled material after the Feulgen procedure has been known, there was no major difference in the labeling index between Feulgen-treated and non-treated autoradiographs. In addition, preliminary observations with prolonged exposure up to 6 weeks showed the persistence of such unlabeled cells, suggesting that the result was not due necessarily to insufficient exposure. On the other hand, the presence of some labeled cells beyond the 4c value suggests that they continued to synthesize DNA even after their DNA had been synthesized up to the 4c value, which is usually the endpoint of DNA replication. All these findings apparently indicate the slowing down of the process of synthesis, with occasional arrests of DNA synthesis.

While the proliferation kinetics of megaloblasts has been the subject of long-term debates, the results presented here are in accord with those of others in view of the lengthening of the mean duration of the DNA synthetic period. Prolonged DNA synthesis may consequently cause a delay in entering mitosis as postulated by Reisner. Lajtha has suspected an elongation of the G2 period with a resultant increase of cells with 4c amount of DNA, RNA, and protein. The structural aberrations of chromosomes in megaloblastic bone marrow cells may be interpreted as an indirect evidence of abnormal DNA metabolism and of faulty cell division.

H3-leucine incorporation as measured by autoradiography can be regarded as representative of protein synthesis, while H3-uridine incorporation observed in this study may reflect primarily RNA synthesis. The present autoradiographic study using these precursors indicated active RNA and protein synthesis in immature erythroblasts in PA, as shown by high incorporation of these precursors. B12 therapy resulted in the reversion of these features to normal values. Thorell demonstrated a continued high concentration of cytoplasmic RNA in megaloblasts throughout the maturation process. Consistent with these changes at the cellular level is the observation that the RNA to DNA ratio, high in PA marrow in relapse, returns to normal after B12 therapy. The present results seem to indicate impaired DNA synthesis on one hand, and active RNA and protein synthesis on the other. Along the same line of inference, Schmid has suggested that the nuclear-cytoplasmic asynchronism characteristic of PA megaloblasts may be an indication that cytoplasmic development proceeds fairly normally in spite of the inhibition of nuclear maturation.
These cytokinetic patterns of DNA, RNA, and protein synthesis appear to support Beck's\(^3\) concept that megaloblasts display an unbalanced growth analogous to the elongated forms of B\(_{12}\) deficient *Lactobacillus leichmannii*. He suggests that the unbalanced growth of B\(_{12}\)-starved microorganisms is due to impaired DNA synthesis, inability to divide, and unimpaired RNA and protein synthesis. Though this concept as derived from microbiological studies may not directly apply to human megaloblasts, the present study on PA megaloblasts seems to afford very suggestive evidence for this view. On the basis of these observations, it would appear that the major defects leading to megaloblastic hemopoiesis consist in an aberrant synthesis of nucleoprotein, especially impaired DNA synthesis and defective cell division associated with active or unaffected RNA and protein synthesis. This would also explain the enlargement of both erythrocytic and granulocytic cells.

In an attempt to assess the effect of B\(_{12}\) on these cytokinetic patterns, 10 \(\mu\)g of coenzyme B\(_{12}\) was given to a patient (Case 4) 6 weeks after giving one dose of 60 mg of folic acid. Reversed autoradiographic changes, i.e., high thymidine, and low uridine and leucine uptake, were observed 24 hours after the folic acid or coenzyme B\(_{12}\) administration, as described elsewhere.\(^{29}\) Folate administration was followed by a decrease in immature megaloblasts and an increase in polychromatic megaloblasts and intermediate erythroblasts (Table 2). The arrest of DNA synthesis, however, was not corrected in 24 hours after the administration of 60 mg of folic acid. In 24 hours after the administration of 10 \(\mu\)g of coenzyme B\(_{12}\) it was corrected (Fig. 4), although it cannot be excluded that folic acid given earlier might, at least in part, be involved in this improvement. Whether the effect of folic acid had worn off prior to B\(_{12}\) administration, so that the marrow had reverted to its former state, remains uncertain. However, it is of interest that the deranged synthetic pattern could be corrected even when megaloblastic changes were still present in the marrow (Table 2, the third column). Further investigations along these lines are now under way.

Folic acid is known to participate in the conversion of deoxyuridylate to thymidylate, namely, in the biosynthesis of the thymine moiety of DNA. Though the possible biochemical role of B\(_{12}\) in the synthesis of DNA and its precursors is still unknown, it is suggested that B\(_{12}\) is involved in the biosynthesis of deoxyribosyls,\(^3\) or it is related also to DNA thymine synthesis via the regulation of folic acid constituents.\(^{11,30}\)

The present study also provides evidence that the deranged DNA synthesis in PA megablasts can be corrected shortly after B\(_{12}\) therapy. The finding\(^8\) that added B\(_{12}\) enhances incorporation of both purine and pyrimidine precursors into DNA of PA marrows in vitro is further evidence in favor of the definite effect of B\(_{12}\) on DNA synthesis. Though thymidine is not a normal precursor of DNA, cells synthesizing DNA incorporate it readily and specifically in vivo\(^6\) and in vitro.\(^{19}\) The intracellular pool which can dilute exogenous thymidine is considered so far to be extremely small,\(^{18}\) and exogenous thymidine is thought to be shunted into the pathway of DNA thymine synthesis, circumventing the endogenous formation of thymidylic acid.\(^8\) Further investigations will be necessary to evaluate the significance of low thymidine
incorporation into megaloblasts in the light of the activity of the enzymes involved in DNA synthesis and nucleotide triphosphate pools of these cells, as well as to delineate the biochemical role of B₁₂ in the pathway of DNA synthesis.

**SUMMARY**

In order to elucidate the nature of the megaloblastic lesion at the cellular level, DNA, RNA, and protein synthesis was studied in megaloblasts of pernicious anemia. While microphotometric estimation of DNA content showed an increase in cells with DNA values ranging around the 4c value, autoradiographic studies with H³-thymidine indicated, rather, a decreased ability to synthesize DNA. Combined microphotometric and autoradiographic studies suggested the impaired DNA synthesis with occasional arrests of synthesis, as well as the prolongation of the S period with or without the prolongation of the G₂ period. On the other hand, active incorporation of H³-uridine and H³-leucine indicated active or unaffected RNA and protein synthesis. Vitamin B₁₂ treatment rapidly corrected these aberrant patterns of synthesis. The significance of these findings has been discussed in relation to the mechanism of megaloblastic hemopoiesis.

**SUMMARIO IN INTERLINGUA**

Pro clarificar le natura del lesion megaloblastic al nivello cellular, le synthese de ADN, de ARN, e de proteina esseva studiate in megaloblastos de anemia perniciose. Ben que le estimation microphotometric del contento de ADN monstrava un augmento del cellulas con un contento de ADN circum le valor 4c, studios autoradiographic con tritiate thymidina indicava plus tosto un reducite potential pro le synthese de ADN. Combinate studios microphotometric e autoradiographic suggestionave le presentia de un lesionate synthese de ADN con arrestos occasional del synthese e etiam le prolongation del periodo S con o sin un prolongation del periodo G₂. Del altere latere, le active incorporation de tritiate uridina e de tritiate leucina indicava un active o non-afficite synthese de ARN e proteina. Tractamento a vitamina B₁₂ resultava rapidemente in le correction de iste aberrante modos de synthese. Le signification de iste constatationes es commentate con referentia al mecanismo del hematopoiese megaloblastic.

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