Characteristics of Vitamin B₁₂ Correction of the Abnormal Erythropoiesis of Pernicious Anemia

By ROBERT S. HILLMAN, JOHN ADAMSON AND EDWARD BURKA

The dramatic response of the pernicious anemia bone marrow to B₁₂ administration has intrigued many investigators and provides an ideal experimental situation for study of conversion of a defective erythropoiesis to normal. By morphological studies with serial bone marrows and in vitro marrow culture technics, investigators have described megaloblast reversion to normoblastic morphology. Others have failed to verify such findings and the question remains as to whether effective red cells can be produced from these abnormal precursors.

The following study employed detailed ferrokinetics throughout the immediate post-B₁₂ therapy period in patients with pernicious anemia to define the evolution of the conversion of ineffective erythropoiesis and megaloblast maturation to completely normal, effective erythropoiesis. During the conversion and throughout the reticulocyte peak response, marrow production levels were shown to remain relatively constant while iron utilization for hemoglobinization gradually returned to normal, implicating abnormalities of mitosis and maturation as the primary marrow defects. In addition, the initial reticulocytosis, its magnitude and cellular components, were analyzed to determine the reason for the extraordinary height of the maximum reticulocyte response and the effectiveness of these cells as precursors of adult red cells. These data indicated that reticulocytosis is the result of effective production at a level consistent with pretreatment marrow production levels. Due to a prolonged intracirculatory reticulocyte maturation time, at least two days of reticulocyte production are cumulatively counted to give the high reticulocyte peak.

Supported by Grant AM-09950 from the National Institutes of Health and Grant 11-0502—State of Washington Initiative 171 Funds for Research in Biology and Medicine.

First submitted June 14, 1967; accepted for publication November 1, 1967.

Address reprint requests to Dr. R. S. Hillman, King County Hospital, 325 Ninth Ave., Seattle, Washington.

Research Fellow—National Institutes of Health Grant No. SF2-AM-29, 857-02.

ROBERT S. HILLMAN, M.D.: Assistant Professor of Medicine, Chief of Hematology, King County Hospital, University of Washington Medical School, Seattle, Wash. JOHN ADAMSON, M.D.: Fellow National Institutes of Health, Bethesda, Md. EDWARD BURKA, M.D.: Assistant Professor of Medicine, Jefferson Medical College of Philadelphia, The Cardeza Foundation for Hematological Research, Philadelphia, Penna.
METHODS

Nine patients were studied either in Presbyterian Hospital, New York City or King County Hospital, Seattle, Washington. All were deficient in vitamin B₁₂; eight had classical pernicious anemia and one had non-tropical sprue. Intrinsic factor deficiency was confirmed by determination of gastric free acid after histamine stimulation and B₁₂ absorption tests using cobalt 57 vitamin B₁₂ with and without intrinsic factor as described by Nelp and associates.™ Gastro-intestinal bleeding, symptoms of congestive heart failure, or concomitant iron deficiency were considered contraindications to inclusion in the study. In all instances, the hematocrit, less than 25 percent prior to treatment, returned to normal range in response to vitamin B₁₂ therapy.

Ferrokinetic studies using both Fe⁵⁹ and Fe⁵⁵ were performed according to Bothwell and Finch.™ To insure transferrin binding, citrated Fe⁵⁹ or Fe⁵⁵ was first incubated with normal plasma for at least 30 minutes before injection. After injection, timed venous samples for plasma counting were obtained at five minute intervals for 30 minutes and at 15-minute intervals for the next two hours. The halftime plasma clearance was obtained from the semilogarithmic plot of these count rates. Plasma iron turnover (PIT) was determined from the halftime plasma clearance and the average of two serum iron measurements at zero time and 30 minutes. Marrow iron transit time (MIT) was measured by twice daily sampling and counting of whole blood. Both PIT and MIT were calculated according to Bothwell and Finch.™ The MIT was determined from the point of 50 percent plasma clearance of Fe⁵⁹ to 50 percent reappearance of the isotope in red blood cells, assuming the 7-day level as 100 percent reappearance. Normal MIT by this method was 3.3-3.5 days. In two patients, a PIT and MIT were performed seven days prior to therapy. In six, Fe⁵⁹ was given on the first day of therapy, 8-10 hours after the first intramuscular injection of 100-500 µg vitamin B₁₂. On three occasions, the administration of intravenous Fe⁵⁹ was delayed until 36-40 hours after the injection and finally, four patients received Fe⁵⁹ 5-7 days after B₁₂ administration. In some instances, the PIT was repeated on two or three occasions in the same patient, permitting a comparison at various points during therapy. All PIT measurements were performed at 9-10:00 a.m. in a fasting state. Once the PIT measurements were completed, most patients received 300 mgm. of ferrous gluconate q.i.d. to prevent iron deficiency during the response to therapy.

Subsequent percent utilization of iron was calculated at seven days using plasma volumes separately determined on the first and seventh days, to eliminate any error introduced by blood volume variations. Four patients had simultaneous red cell lifespan measurements. Fifty ml. of the patient’s blood in ACD solution was incubated with 50 µc of Cr⁵¹ and the red cells washed three times in saline prior to returning them to the patient. Venous samples were obtained daily for at least two weeks. The Cr⁵¹ halftime disappearance was determined from the count rate of 2 ml of whole blood without correction for hematocrit variation or elution. Cr⁵¹ lifespan of normal cells by this technic, expressed as Cr⁵¹ T½ clearance, was 28 days. The rate of pretreatment cell death (Fig. 1) was determined by comparing the uncorrected Cr⁵¹ T½ clearance rates to this normal value. Plasma volume was measured repetitively during the study from the Fe⁵⁹ plasma clearance or with T 1824 or I 131 labeled albumin. At least six venous samples were withdrawn over a 30-40 minute period and 2 ml of plasma counted for gamma activity or 4 ml of plasma containing T 1824 extracted for dye content measurement by the technic of Campbell et al.™ These values were then extrapolated to a zero time value. Both the amount injected and standard aliquots were measured by weighing the syringe before and after expulsion of its contents.™ Simultaneous plasma volumes in the same individual by these three technics agreed within a ± 2 percent error. A Nuclear Chicago gamma well counter was used for Cr⁵¹, I 131 and Fe⁵⁹ counting obtaining 10,000 counts above background for ± 1 percent accuracy. Fe⁵⁵ counting was done by the method of total digestion, electroplating to copper discs and counting with a gas flow end window Geiger Mueller tube.

Reticulocyte iron uptake studies were performed by the method of Walsh and co-workers™ employing in vitro incubation over a 2-hour period and measuring Fe⁵⁹ uptake. The serum iron was adjusted to a level of 200-250 µg. percent, 60-70 percent saturation of the total
VITAMIN B12 CORRECTION

Fig. 1.—The reticulocyte and hematocrit response data of patient (GH) are plotted for the full 10-day study period. The delay to reticulocytosis (interval-1) was calculated from the time of B12 administration to the first increase in reticulocyte count as extrapolated from the near linear rise over the next two days. Interval-2 represents the relatively linear accumulation of reticulocytes, corresponding to the delay in reticulocyte maturation.

Volume increments of red cells produced after vitamin B12 administration were calculated as the difference between the observed hematocrit (solid line) and the predicted fall in the number of red cells produced prior to vitamin B12 therapy (broken line). The latter line was calculated by comparing the Cr57 lifespan of the patient’s cells before treatment to a normal value of 28 days, i.e., 8.7/28 × 120 = 38 day lifespan. Thus, in a 10-day period, approximately 26 percent of the pretreatment cells, a hematocrit volume of 4.5 percent, should disappear.

Calculations. In the calculation of blood volume, the mean body hematocrit (0.91 × venous hematocrit) was employed.

Relative production rates, based on reticulocyte counts and ferrokinetic data were expressed as production indices, i.e., the ratio of the observed value to the normal value, as previously used by Giblett and co-workers and Finch and co-workers. The normal values employed for these calculations were:

- Reticulocyte count = 1.0 reticulocyte/100 rbc
- Plasma iron turnover = 0.65 mgm./100 ml. whole blood/24 hr.

The indices were obtained by dividing the measured value by the appropriate normal value for example:

Reticulocyte production index = measured reticulocyte count corrected to hematocrit 44

PIT production index = measured plasma iron turnover

\[ \text{Reticulocyte production index} = \frac{\text{measured reticulocyte count corrected to hematocrit} \times 44}{1.0 \text{ (normal reticulocyte count)}} \]

\[ \text{PIT production index} = \frac{\text{measured plasma iron turnover}}{0.65 \text{ mgm./100 ml. 24 hrs.}} \]
Table 1.—Summary of Data Obtained in Nine Subjects Including Hematocrit Change, Blood Volumes, Plasma Iron Turnovers and Survival Measurements

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time to Encapsulate (h)</th>
<th>Time after Encapsulate (days)</th>
<th>Volumetric Change (g)</th>
<th>Hematocrit Change (g)</th>
<th>Serum Iron (mg/100 ml.)</th>
<th>Plasma Iron (mg/100 ml.)</th>
<th>Exchange of Iron over 24 Hr. (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK</td>
<td>20.0-29.5</td>
<td>2.1</td>
<td>10 hrs.</td>
<td>3400</td>
<td>35</td>
<td>25</td>
<td>35.0</td>
</tr>
<tr>
<td>LS</td>
<td>16.0-30.0</td>
<td>2.4</td>
<td>-7 days</td>
<td>4115</td>
<td>30</td>
<td>103</td>
<td>14.0</td>
</tr>
<tr>
<td>HR</td>
<td>17.0-26.0</td>
<td>2.5</td>
<td>10 hrs.</td>
<td>4300</td>
<td>16</td>
<td>100</td>
<td>27.5</td>
</tr>
<tr>
<td>AD</td>
<td>16.0-29.0</td>
<td>1.8</td>
<td>8 hrs.</td>
<td>4800</td>
<td>25</td>
<td>1.15</td>
<td>1.85</td>
</tr>
<tr>
<td>MP</td>
<td>16.5-29.0</td>
<td>2.5</td>
<td>10 hrs.</td>
<td>5100</td>
<td>16.5</td>
<td>1.85</td>
<td>2.85</td>
</tr>
<tr>
<td>GH</td>
<td>17.0-29.0</td>
<td>2.4</td>
<td>7 days</td>
<td>5100*</td>
<td>1.85</td>
<td>2.85</td>
<td>5.5</td>
</tr>
<tr>
<td>AF</td>
<td>18.0-25.0</td>
<td>2.5</td>
<td>8 hrs.</td>
<td>4500</td>
<td>22</td>
<td>1.7</td>
<td>4.7</td>
</tr>
<tr>
<td>HA</td>
<td>23.0-25.0</td>
<td>2.1</td>
<td>8 hrs.</td>
<td>4100</td>
<td>22</td>
<td>1.7</td>
<td>4.7</td>
</tr>
<tr>
<td>MR</td>
<td>12.0-25.0</td>
<td>2.4</td>
<td>7 days</td>
<td>4100</td>
<td>22</td>
<td>1.7</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Saline overload at time of measurement.
VITAMIN B<sub>12</sub> CORRECTION

Table 2.—Marrow Iron Transit Time (Days)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Before B&lt;sub&gt;12&lt;/sub&gt;</th>
<th>Time After B&lt;sub&gt;12&lt;/sub&gt; Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 Hours</td>
<td>36 Hours</td>
</tr>
<tr>
<td>Repetitive Studies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS</td>
<td>3.4</td>
<td>3.7</td>
</tr>
<tr>
<td>HK</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>3.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Single Studies</td>
<td>3.7</td>
<td>3.1, 3.6, 4.0</td>
</tr>
<tr>
<td>Mean</td>
<td>3.55</td>
<td>3.5</td>
</tr>
</tbody>
</table>

In situations of anemic stress the reticulocyte production index required further correction for reticulocyte maturation shift. When applied to the post-B<sub>12</sub> reticulocyte peak, a corrected formula was used (this will be discussed at greater length in the Result section):

\[
\text{Reticulocyte production index} = \frac{\text{retic. count corrected to hematocrit 44}}{\text{Maturation time} \times 1.0}
\]

For studies of the reticulocyte maturation time in four individuals, increments of new red cell production were calculated from the Cr<sup>51</sup> lifespan curve and the change in venous hematocrit at 6 hour intervals (see Figure 1):

\[
\text{New red cell increment} = \text{observed venous hematocrit} - \text{hematocrit value of pretreatment cells (derived from Cr<sup>51</sup> lifespan curve)}
\]

The reticulocyte volume increment was determined from the uncorrected reticulocyte count and the observed venous hematocrit:

\[
\text{Reticulocyte volume increment} = \text{uncorrected reticulocyte count} \times \text{observed venous hematocrit}
\]

RESULTS

Erythropoiesis Measurements

The results obtained from the ferrokinetic studies of the nine patients are summarized in Table 1. As seen from column 8, the plasma iron turnovers (expressed as mgs./100 mls. of whole blood/24 hrs.) ranged from 1.4 to 5.15, corresponding to production indices of 2–8 times normal. Despite variation within the group, sequential plasma iron turnovers during the period of B<sub>12</sub> response in six individuals demonstrated relatively constant marrow production levels. A consistent fall in serum iron and a shortening of the T<sub>1/2</sub> clearance rate of Fe<sup>59</sup> was observed after vitamin B<sub>12</sub> therapy.

In four cases, Cr<sup>51</sup> red cell survivals before therapy ranged from 5.5 to 13.4 days. Repeated blood volumes remained relatively constant in all but one of the patients, M.P., who was severely overloaded with saline at the time of her second blood volume measurement.

Time Lag to Reticulocytosis

The time lag until the reticulocyte response to vitamin B<sub>12</sub> therapy was computed from the time of B<sub>12</sub> injection to the first increase of the uncorrected reticulocyte count (Fig. 1.). An exact point of first increase of reticulocytes was derived by extrapolation of the slope of reticulocyte increase to the baseline reticulocyte level. In nine patients, the time lag was 1.8–2.5 days with a mean of 2.3 days. The time to peak reticulocytosis was approximately five
Table 3.—Fe59 Utilization (%)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Before B12</th>
<th>Time After B12 Therapy</th>
<th>10 Hours</th>
<th>36 Hours</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repetitive Studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS</td>
<td>38</td>
<td>76</td>
<td></td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>HK</td>
<td>75</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>65</td>
<td>82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single Studies</td>
<td>29</td>
<td>67, 69</td>
<td>65, 85</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>Mean</td>
<td>34</td>
<td>68.7</td>
<td>77.3</td>
<td></td>
<td>93</td>
</tr>
</tbody>
</table>

Fig. 2.—The reticulocyte response curve (solid dots) is compared to iron isotope reappearance curves (Fe55 solid line; Fe59 broken line) in patient (HR). The transit time of the Fe55 given 10 hours after vitamin B12, was 3.4 days, while the Fe59, given at 30 hours, was 1.8 days; the isotopes reappeared in the same population of reticulocytes. However, the utilization of the Fe55 was significantly less.

days. Delivery of reticulocytes to the circulation was also studied employing repeated marrow iron transit times throughout the response period. As shown in Table 2, the transit time was normal or slightly prolonged both before and at 10 hours after B12 administration. It was not until 36 hours after injection that a definite reduction in iron transit time to 2.0 days could be detected. At seven days, the mean iron transit time was still reduced, 2.4 days.

Patterns of Fe59 utilization

As a measure of the effectiveness of erythropoiesis, percent utilization of Fe59 for adult red cell hemoglobinization was determined for each interval. As seen in Table 3, the pretreatment group showed very poor Fe59 utilization, 38 and 29 percent at seven days. The utilization of the group to whom Fe59 was given 10 hours post-vitamin B12 ranged from 65–76 with a mean of 68.7 percent. At 36 hours, the mean had risen to 77.3 percent, and by seven days utilization ranged from 86–100 percent with a mean of 93 percent. This suggestion of a gradual return to normal utilization was further illustrated by the study
Fig. 3.—Increments of red cells produced after vitamin B$_{12}$ therapy (solid line), as calculated in Fig. 1, are compared to the reticulocyte increments (broken line), i.e., reticulocyte count times hematocrit, during the initial reticulocytosis in four patients. Maturation delay is determined from the point of initial increase to the point of divergence of the two lines, as indicated by the arrows.

of patient H.R. (Fig. 2) where Fe$^{55}$ was given at 10 hours and Fe$^{59}$ at 30 hours after vitamin B$_{12}$ administration. Serum iron values at the times of isotope administration were 115 $\mu$g. percent at the time of injection of the Fe$^{55}$ and 65 $\mu$g. percent at the time of injection of the Fe$^{59}$ (percent saturations of 45 and 25). Plasma iron turnovers in the two studies were of similar magnitude, 3.3 and 3.1 mg./100 ml. whole blood /24 hrs., compatible with a relatively constant iron uptake by the erythroid precursors during the period of study. Moreover, the transit time of the iron tracer given at 10 hours was delayed so that both isotopes appeared in the same adult red cell population coincident with the reticulocyte peak. However, utilization of the isotope given at 30 hours was 82 percent whereas that given at 10 hours was only 65 percent.

Reticulocyte Maturation Time

Employing the Cr$^{51}$ lifespan data to predict the decline in hematocrit from death of red cells produced prior to vitamin B$_{12}$ therapy (broken line, Fig. 1), the increment of new cells was calculated for each 6-hour period during
Table 4.—Reticulocyte Counts, Maturation Times and Production Indices of Seven Subjects

<table>
<thead>
<tr>
<th>Corrected* Reticulocyte Count</th>
<th>Maturation† Time</th>
<th>Reticulocyte Production Index</th>
<th>Plasma Iron§ Turnover Production Index (× Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5</td>
<td>2.4</td>
<td>3.9</td>
<td>3.2</td>
</tr>
<tr>
<td>9.9</td>
<td>2.2</td>
<td>4.5</td>
<td>4.3</td>
</tr>
<tr>
<td>10.0</td>
<td>2.8</td>
<td>3.6</td>
<td>3.1</td>
</tr>
<tr>
<td>9.5</td>
<td>2.6</td>
<td>3.6</td>
<td>2.8</td>
</tr>
<tr>
<td>9.7</td>
<td>2.4</td>
<td>4.0</td>
<td>4.7</td>
</tr>
<tr>
<td>5.5</td>
<td>2.0</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td>15.0</td>
<td>2.2</td>
<td>6.8</td>
<td>7.9</td>
</tr>
<tr>
<td>Mean</td>
<td>2.3</td>
<td>4.2</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*Corrected to a hematocrit of 44 (retic count × venous hematocrit).
†Derived from increment plots of hematocrits and reticulocyte counts, Fig. 4.
‡Reticulocyte production index = \frac{\text{corrected reticulocyte peak}^*}{\text{maturation time}}
§PIT index = \frac{\text{PIT (mg./100 ml. blood/24 hr.)}}{0.65}

Correction of reticulocytosis. This increment of hematocrit increase was then compared to the corresponding reticulocyte volume increment calculated from the hematocrit and the uncorrected reticulocyte count at each time period. As shown in Fig. 3, the hematocrit and reticulocyte volume increment increases in four patients were identical for a period of 2.1–2.8 days. Subsequently, the increment curves diverged sharply. The hematocrit volume now exceeded the reticulocyte volume, indicating maturation of reticulocytes to adult red cells. This time period could also be estimated from the reticulocyte peak plot as the relatively linear increase phase of the initial reticulocytosis (interval 2 of Fig. 1).

By using this time period as a measure of reticulocyte maturation delay, it was possible to calculate a corrected reticulocyte production index—reticulocyte production index [corrected] = \frac{\text{Retic. count corrected to hematocrit 44}}{\text{maturation time} \times 1.0}

When the corrected reticulocyte production index was compared to the PIT production index (Table 4) there was a close correlation of the two measurements in seven individuals.

As further support for prolongation of circulating reticulocyte maturation, the total daily reticulocyte increment, the area under the reticulocyte peak in Fig. 1, was compared to the total hematocrit rise over 10 days. In three patients, the sum total of the reticulocyte volume increments were 34, 24.5 and 24.7 while the 10-day hematocrit elevations were respectively 16, 13 and 12. This discrepancy would correspond to delays in maturation of 2.3, 1.9 and 2.0 days, values comparable to the previously calculated maturation times from Fig. 3 of 2.8, 2.6 and 2.4 days.
Fig. 4.—Iron uptake per reticulocyte (broken line) is compared to the observed reticulocyte peak after vitamin B\textsubscript{12} therapy (solid line) in a single patient. As reticulocytes accumulate iron uptake per reticulocyte falls rapidly, compatible with progressive dilution of young cells having a high activity for iron with more mature reticulocytes.

**Reticulocyte Studies**

The ability of circulating reticulocytes to take up radioiron was studied just prior to the reticulocyte response and on each day for five days during reticulocytosis in one patient. As seen in Fig. 4, there was no detectable iron uptake of reticulocytes produced prior to B\textsubscript{12} therapy. With the appearance of the earliest reticulocytes, the uptake per reticulocyte was extremely high. It then fell sharply as the reticulocyte rise continued, indicating either a dramatic change in the type of product coming from the marrow or the development of a mixed age population due to maturation of the earlier cells. A similar decline in reticulocyte ribosome content and protein synthesis during reticulocytosis was found by one of the authors.\textsuperscript{26}

**DISCUSSION**

Serial ferrokinetic measurements were performed in nine vitamin B\textsubscript{12}-deficient subjects undergoing therapy to characterize the conversion of megaloblastic to normoblastic erythropoiesis. The patients were carefully selected from a group of more than 30 individuals. Other illness, especially an infectious disease or cardiac decompensation, was taken as a contraindication for inclusion in the study. This selection may well have been responsible for the apparent uniformity of response and the absence of significant change in blood volumes with therapy. In each individual, the hematocrit returned to normal within one month.
As previously described by Finch and co-workers and Myhre, marrow production prior to vitamin B₁₂ therapy ranged from 2.0 to 7.9 times normal in the face of a low reticulocyte count and shortened red cell lifespan, by definition—ineffective erythropoiesis and adult red cell hemolysis. Although these abnormalities are known to disappear with vitamin B₁₂ administration, the pattern of reversion to normal has not been well defined. In the present studies, ferrokinetic measurements were made before therapy and throughout the post-therapy reticulocytosis period. Repeated PIT measurements in the same patient, before and during the initial seven days of treatment, demonstrated that erythroid marrow production remained relatively constant. Two patients, HK and MP, demonstrated reductions in PIT. This was associated with a fall in serum iron values to iron-deficient levels, similar to the two cases reported by Finch and co-workers and Myhre. When serum iron values remained above 60 μg per cent, the PIT did not change significantly. This suggests that as long as iron supply is sufficient, the level of marrow stimulation and proliferation are similar in the B₁₂ deficient state and for 7–10 days after therapy. While the proliferative level of the marrow remains unchanged, there is a cessation of abnormal megaloblastic maturation and conversion of ineffective to effective erythropoiesis.

The return to a normal maturation pattern is not immediate but requires 2–3 days for completion. Effective reticulocytosis returned only after a delay of 1.8–2.5 days. Repeated measurements of marrow iron transit time and Fe₉ utilization for hemoglobinization of adult red cells also demonstrated a delay in the reversion to normal. Assuming the distribution of iron uptake in marrow precursors is the same in the vitamin B₁₂ deficient state as in the normal, the prolonged iron transit time prior to therapy must represent a prolonged intramarrow maturation time and/or recycling of the radioiron due to cell destruction. Once vitamin B₁₂ is administered, both defects should disappear; radioiron utilization should return to normal and the MIT should shorten appreciably to a less than normal value. Any delay in these corrections indicates either continued death of megaloblasts and their progeny; viz., a destruction of cells which are not normalized by therapy or a delayed release of the cells undergoing conversion.

Several lines of evidence indicate the former explanation is preferable. First, when two isotopes of iron were given to Patient HR in the first 30 hours, they eventually appeared in the same population of reticulocytes, coincident with the reticulocyte peak. Thus, in the 20 hours separating the two injections, only one population of precursors destined for release as viable reticulocytes underwent complete maturation and accepted the majority of the iron isotope for hemoglobin production. At the same time, the percent utilization of the earlier dose was significantly less. It seems unlikely that this was caused by variations in the initial uptake of the isotopes inasmuch as they were administered at comparable serum iron levels and PIT measurements were identical. Rather, the lower utilization of the iron isotope given just after vitamin B₁₂ therapy would seem to result from continued death of ineffective megaloblastic cells and recycling of iron with subsequent dilution of the isotope in reticulo-
endothelial cell and other non-heme iron pools. The alternate explanation, a delay in conversion of megaloblasts to normoblasts without cell death, should require either identical percent utilizations of Fe$^{55}$ and Fe$^{59}$ or better, a lower utilization of the second isotope. Since the more mature megaloblast will contain an excess of iron and hemoglobin for any subsequent conversion to a normoblast, the predicted change in iron uptake characteristics should be toward less iron uptake, a delayed MIT and a lower percent utilization of the second isotope during the initial reticulocytosis. This is opposite to that observed.

Second, if the large mass of megaloblasts present before therapy is capable of conversion to normoblasts by five days after therapy, when sufficient time has passed to permit an entirely new generation of normoblasts to mature from the stem cell level, the resultant reticulocytosis should far exceed the measured marrow production levels. Although this may seem an attractive explanation for the discrepancy between the height of the initial reticulocytosis and the PIT production index (Table 4), this is not supported by quantitation of actual new cell production. The PIT index and the hematocrit rise over ten days were in close agreement; while, reticulocyte production indices (not corrected for prolonged intracirculation maturation) of 5.5–15.0 times normal were not accompanied by a comparable rise in hematocrit. To explain this observation, it is necessary to postulate either death of at least one-half of the initial reticulocytes within a few days or to accept the concept of prolonged maturation of these reticulocytes. In the former case, abrupt death of 50 percent or more of the initial reticulocytes, between days 2 and 5, should have deformed the Fe$^{59}$ reappearance curve. This was not observed. Moreover, Kan and Gardner have been unable to show cell loss of this magnitude; they demonstrated at least a normal life span for these initial reticulocytes. Rather, the excessive reticulocytosis may better be explained as the result of premature delivery of marrow reticulocytes which because of a prolonged maturation period in circulation are cumulatively counted for 2–3 days. This is supported by the increment plot data of reticulocyte volumes versus new cell volumes during the initial reticulocyte peak (Fig. 3). For the first 2.1–2.8 days, all new cell production consisted entirely of circulating reticulocytes. Only after more than two days of reticulocyte maturation did some reticulocytes lose their reticulum and the total new cell volume exceeded the reticulocyte volume.

Further evidence for a maturation phenomenon was obtained from the changing pattern of iron uptake and globin production during the reticulocyte peak. Iron uptake per reticulocyte rose sharply with the initial reticulocytosis only to decrease despite continued accumulation of reticulocytes. This is also compatible with maturation of the first reticulocytes, which though cumulatively counted on the second and third day demonstrated a decrease in iron uptake.

This sudden appearance of young, marrow reticulocytes requiring two or more days to lose their reticulum is to be expected in the vitamin B$_{12}$ deficient subject once therapy is initiated. As soon as the maturation delay within the marrow is corrected by vitamin B$_{12}$, the severe anemia and high erythropoietin
stimulation will demand delivery of these immature cells. This phenomenon has been well demonstrated in animals and normal man and should apply equally well to the treated vitamin B₁₂-deficient subject.²⁶,²⁹,³⁰ Acceptance of this mechanism as an important one in the responding B₁₂-deficient patient resolves the apparent discrepancies of various production measurements. By estimating the period of maturation delay for the first reticulocytes from the linear phase of accumulation (Fig. 1, period 2) or the increment plot data (Fig. 3), it is possible to calculate a corrected reticulocyte production index. This is an index corrected both for hematocrit and maturation delay. Such a corrected index is then in essential agreement with the PIT production index (Table 4) and observed hematocrit rise over ten days.

These studies and other data support therefore an initial response of completely effective reticulocytes which mature into viable adult red cells in amounts equal to measured marrow production. By nature of the delay to reticulocyte response and to reversion of the MIT and red cell utilization to normal these cells probably come from a new normoblast population. Conversion of the megaloblast population to a normoblastic one must be largely ineffective; cell death within the marrow or immediately after delivery to circulation must continue despite vitamin B₁₂. Otherwise the reticulocyte response and more important the initial hematocrit rise would far exceed the PIT production index as this extra pool of salvaged cells is delivered to circulation.

**SUMMARY**

Nine comparable patients with vitamin B₁₂ deficiency were studied during therapy by iron kinetic technic with special attention to the earliest phase of reticulocyte response, in order to define the fate of a megaloblast population and to explain the very high reticulocytosis typical of the pernicious anemia response. After B₁₂ therapy, there is a 2-2.5 day lag before reticulocytosis, during which ineffective erythropoiesis of the megaloblasts continues and repopulation by an entirely new normoblast series from the stem cell level occurs. Once reticulocytosis begins, it is characterized by delivery of very young reticulocytes from the bone marrow. These cells demonstrate a prolonged maturation phase, resulting in cumulative counting over several days and an apparent greater than expected reticulocyte level. If the reticulocyte peak is corrected for this maturation delay, a reticulocyte production index is obtained which correlates well with the measured plasma iron turnover production indices.

**SUMMARIO IN INTERLINGUA**

Novem comparabile patientes con carentia de vitamina B₁₂ esseva studiate durante le therapia con technicas ferrokinetic, con attention particular prestate al primissime phase del responsa reticulocytic, con le objectivo de definir le destino de un population de megaloblastos e de explicar le altissime reticulocytose que es typic del responsa de anemia perniciose.

Post le administration therapeutica de B₁₂, il occure un retardo de inter duo e duo dies e medie ante le reticulocytose. Durante iste intervallo un inefficace erythropoiese de megaloblastos continua, e il occurre repopulation per un integemente nove serie normo-
blastic ab le reservas de cellulas primordial. Quando le reticulocytose ha comenciate, illo es caracterisate per le liberation de juvenissime reticulocytos ab le medulla ossee. Iste cellulas ha un prolongate phase de maturation, lo que resulta in numerationes cumulative durante plure dies e in un numero de reticulocytos apparentemente plus alte que expectate. Post le correction del maximos de reticulocytos pro iste retardo del maturation, indices de production reticulocytic es obtenite que se trova in bon correlation con le mesure indices plasmatic del production de transitos de ferro.

REFERENCES

22. Morgan, E. H., and Carter, G.: Plasma iron and iron binding capacity levels in


Characteristics of Vitamin B₁₂ Correction of The Abnormal Erythropoiesis of Pernicious Anemia

ROBERT S. HILLMAN, JOHN ADAMSON and EDWARD BURKA