Quantitative Assessment of Erythropoiesis and Functional Classification of Anemia Based on Measurements of Serum Transferrin Receptor and Erythropoietin

By Yves Beguin, Gisela K. Clemons, Pensri Pootrakul, and Georges Fillet

We evaluated the quantitative value of a simple model of erythropoiesis, based on the basic assumptions that the red blood cell (RBC) mass determines erythropoietin (Epo) production, which in turn stimulates erythropoietic activity. The RBC mass was quantitated by direct isotopic measurement (RCM), Epo production by serum Epo levels, and erythropoiesis by the ferrokinetic measurement of the erythron transferrin uptake (ETU), the serum transferrin receptor (TfR) level, and the reticulocyte (retic) index, and was completed by an evaluation of overall marrow erythron cellularity. We studied a total of 195 subjects, including 31 normal individuals, 38 patients with polycythemia, and 126 patients with various forms of anemia. Instead of only quantitating Epo and erythropoiesis in absolute terms, we also evaluated them in relation to the degree of anemia or polycythemia, and expressed the results as a ratio of observed values to values predicted from the regression equations between hematocrit (Hct) on the one hand, and Epo, TfR, and ETU on the other, obtained in a carefully selected subpopulation. The slope of the regression of TfR (as well as ETU) versus Hct was very similar to the slope of the regression of Epo versus Hct. Average EPO and TfR values predicted from the regression equations were quite comparable to observed values in most groups of subjects, with exceptions predictable from knowledge of the pathophysiology of these hematologic disorders. We identified four major patterns of erythropoiesis, ie, normal, hyperdestruction (with variants of hemolysis or ineffective erythropoiesis), intrinsic marrow hypoproliferation, and defective Epo production. Dissecting out groups of patients showed much greater heterogeneity than when patients were analyzed by group. This was particularly true in the case of a hypoproliferative component being combined with hyperdestruction, giving what we called a “mixed disorder of erythropoiesis.” We conclude that the pathophysiology of anemia can be assessed by a simple measurement of Hct, retic index, Epo, and TfR levels, with Epo and TfR being more informative when expressed in relation to the degree of anemia. The model is particularly useful for detecting the presence of multiple mechanisms of anemia in the same patient. However, it has limitations inherent to the relative invalidity of TfR in iron deficiency, the imprecision of a retic count, and the difficulty in distinguishing hemolysis from ineffective erythropoiesis in some patients and in recognizing a component of hyperdestruction in hypoproliferative anemia.

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Erythropoiesis depends on the proliferative capacity of erythroid progenitors in the bone marrow (BM) and their stimulation by erythropoietin (Epo). Epo production by the kidney is determined by the level of oxygen supply, which depends mostly on the red blood cell (RBC) mass. An inverse relationship has been shown between serum Epo levels and hematocrit (Hct). Epo stimulates RBC production by inducing the proliferation and differentiation of committed erythroid progenitors. In the presence of a normal marrow stem cell reserve, erythropoiesis will therefore increase in proportion to the degree of anemia. On the other hand, polycythemia can originate from an autonomous proliferation of marrow erythroid progenitors (polycythemia vera) or be secondary to increased Epo production.

The present study is based on a simple model of the regulation of erythropoiesis, ie, that the RBC mass determines Epo production, which in turn stimulates erythropoietic activity. Erythropoiesis was quantitated both by the ferrokinetic measurement of the erythron transferrin uptake (ETU) and the determination of plasma transferrin receptor (TfR) levels, which we recently showed to be a simple quantitative assay of total erythropoiesis in rats as well as in humans. This report is aimed at: (1) quantitating erythropoiesis relative to the Hct rather than simply in absolute values; (2) testing the validity of the model for the quantitative assessment of the erythropoietic response to Epo; (3) testing the appropriateness of a physiologic assessment of RBC disorders based on measurements of the Hct, retic index, serum Epo, and serum TfR in a single blood sample; and (4) examining the association between the pathogenesis of disordered erythropoiesis and clinical diagnosis.

Subjects and Methods

Subjects
A total of 195 subjects were studied. These included 31 normal subjects aged 18 to 76 years, 38 patients with polycythemia (PC), and 126 patients with anemia. The clinical diagnosis of PC was based on the isotopic measurement of an RBC mass (RCM) greater than 125% of predicted value, either currently or previously. Among polycythemic patients, 9 had polycythemia vera (PV), 12 secondary PC, 12 PC of uncertain origin, and 5 relative PC (no history of absolute PC and current RCM lower than 125% of predicted value in the presence of a reduced plasma volume).

Among anemic patients, 18 had a myeloproliferative disorder (MPD) (either chronic myelogenous leukemia [CML] or essential thrombocytopenia), 10 a myelodysplastic syndrome (MDS), 18 agnogenic myeloid metaplasia...

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Supported in part by Grants No. HL.22469 (G.K.C.) and HL.34408 (P.P.) from the National Institutes of Health, by Grant No. 3.4555.191 (Y.B. and G.F.) from the Fund for Medical Scientific Research (FRSM, Belgium), and by a grant (Y.B. and G.F.) from the University of Liège School of Medicine.

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Blood. Vol 81, No 4 (February 15), 1993: 1067-1076
(AMM), 11 hypoplastic/aplastic anemia, 15 chronic renal failure, 11
beta-thalassemia/hemoglobin E, 10 hemoglobin H (HbH) disease, 8 he-
ereditary spheroctysis, and 11 autoimmune hemolytic anemia
(AIHA). In addition, 5 patients with chronic renal failure were re-
ceiving recombinant human erythropoietin (rhEPO). In addition, 5 patients with chronic renal failure were re-
ceived an autologous (N = 4) or allogenic (N = 5) BM transplant (BMT)
6 to 12 months earlier. Informed consent was obtained from all sub-
jects. The vast majority of them underwent the procedure as part of
the routine investigation of their hematologic disorder. Except for
minor transplant patients, they were generally in steady state and
had not received blood transfusions in the preceding 3 months. None
of the patients was iron deficient, based on a serum ferritin level
higher than 12 ng/mL.

Blood Volume (BV) Measurement

Total BV was the sum of simultaneous measurements of RCM
and plasma volume. The patient's own plasma or a heparinized
and acquired immunodeficiency syndrome (AIDS)-free plasma from
a normal donor was used, depending on the patient's unbound iron-
binding capacity (UIBC). Ten milliliters of plasma was incubated for
30 minutes at 37°C with 2 to 3 µCi of 59FeCl3 previously mixed with
4% sodium citrate to ensure a molar ratio of citrate to iron of 50:1.
RBCs from 20 mL autologous whole blood were incubated for 30
minutes at 37°C with 10 to 20 µCi of Na251CrO4, washed twice, and
resuspended in normal saline. Weighed volumes of the labeled sol-
utions were set aside for the preparation of diluted standard solutions.
Weighed amounts of labeled RBCs and plasma were injected into a
forearm vein and samples were drawn from the other arm. Plasma
iron (PI) and total iron-binding capacity (TIBC) were measured by
standard procedures. 59Fe and 51Cr activities were counted in a liquid scintillation counter. Plasma vol-
ume and RCM were calculated as:

\[
\text{Plasma Volume or RCM (mL)} = \frac{I}{C \text{ (cpm/mL)}}
\]

where \( I \) is the total amount of radioactivity injected, and \( C \) is the
sample activity per milliliter of plasma extrapolated to 0 time or per
milliliter of RBCs in the 15- to 30-minute sample (60-minute sample
when delayed mixing was suspected). The observed/predicted (O/P)
ratio of RCM was calculated. 6

Ferrokinetic Studies

The plasma iron turnover (PIT) was calculated from a standard formula: 12

\[
\text{PIT (mg/dL whole blood per day)} = \frac{\text{Pl (µg/dL)} \times (100 - \text{Hct} \times 0.9)/100}{t^{-1/2} \text{ (min)}}
\]

where \( t^{-1/2} \) is the clearance half-time of transferrin-59Fe, and 0.9 the
correction for trapped plasma in the Hct (0.98) and to convert venous
Hct to whole body Hct (0.92).

Extravascular flux, a function of plasma iron concentration, 12 was
then subtracted to obtain tissue iron uptake (TIU):

\[
\text{TIU (mg/dL whole blood per day)} = \text{PIT} - \left( \frac{\text{Pl} \times (100 - \text{Hct} \times 0.9)/100 	imes 0.0015)}{100} \right)
\]

The effect of transferrin saturation (S) was removed by converting
TIU to iron-bearing transferrin uptake, and mononerythron uptake was
subtracted 13 to obtain ETU:

\[
\text{ETU} = \frac{\text{TIU} \times 10}{56} \times \frac{(200 + 2.2 S)}{(200 + 6.4 S) - 11}
\]

The derivation of this formula is described elsewhere. 2 ETU was also
corrected for abnormalities in blood volume by the ratio of measured
to predicted blood volume.

Epo Assay

Circulating Epo levels were measured by two different radioim-
unoassays (RIA). The first RIA is a modification of an assay previ-
ously described in detail, 2 and the second RIA is commercially
available (Innotest Corp, Stillwater, MN). Both use recombinant human
Epo (rhEpo) for tracer and standards. Samples are incubated with
rabbit anti-Epo serum for 3 days at 4°C (RIA #1) or 2 hours at room
temperature (RIA #2) before Epo tracer is added. After overnight
incubation, goat antirabbit serum is added. After centrifugation,
the unbound tracer is removed by decantation and the pellet is counted.
Several samples had to be diluted 1:10. Thirty samples, with Epo
concentrations ranging from 10 to 200 mU/mL, were measured by
the two RIAs and no significant difference was found. Twelve control
samples were run in each assay, with a between-assay coefficient of
variation ranging from 10.3% to 14.1%.

TfR Assay

Human placental receptor-transferrin complex was purified as de-
scribed elsewhere 14 and injected repeatedly into rabbits. Serum IgG
was isolated from rabbit serum 15 and transferrin antibodies were re-
moved by passing through a column of human dimeric transferrin
coupled to Affigel 15 (BioRad, Richmond, CA). Characterization of
plasma TfR and receptor antibody has been described elsewhere. 4,3
An enzyme-linked immunosorbent assay (ELISA) 4 with minor
modifications to measure serum levels of TfR was used. Immunoplates
were used. The aliquots of standards, and unknown samples
were added using a DigiTrep automatic pipetor (Micromedic System,
Philadelphia, PA). Standards were diluted to between 5 and 100 ng/
/mL and unknown sera were diluted 1:50 to 1:2,000 with 0.15 mol/L
phosphate-buffered saline (PBS; pH 7.4) containing 0.5% bovine
albumin and 0.05% Tween 20. After color development, dif-
erential absorbance was read in dual wave length mode at 492 and
690 in a TiterTek Multiskan MCC/340 plate reader (Flow Labora-
tories, Herts, UK). In drawing the calibration curve, allowance was
made for the molecular weight of the transferrin molecule included
in the receptor-transferrin complex by multiplying the protein content
of the standard by 0.65. Each sample was run in triplicate. The be-
tween-assay variability (coefficient of variation) was 7.2% when the
same control sample was measured in each plate.

Reticulocyte (retic) Index

Blood smears were stained with new methylene blue and reticu-
locytes in 1,000 cells were counted. The retic index was calculated
after appropriate corrections for the subject's Hct and for reticulocyte
shift, as described elsewhere. 16 In the presence of anemia, a retic
index greater than 3 is indicative of hemolytic anemia, and a retic
index less than 2 of either a hypoproliferative or maturation abnor-
mality.

Statistical Methods

Log transformed Epo, TIR, and ETU values were used in statistical
analyses. Student's t-tests, with pooled or separated variances as ap-
propriate, were used to compare two groups. Analysis of variance
Fig 1. Relationship between EPO levels and Hct in groups of patients, each represented by its mean values. The regression line displays the relationship in reference subjects (see text). 1, β-thalassemia/HbE; 2, AMM; 3, AIHA; 4, spherocytosis; 5, hypoplastic anemia; 6, MDS; 7, HbH disease; 8, BMT; 9, MPD; 10, renal failure; 11, renal failure on rHuEpo; 12, secondary PC; 13, relative PC; 14, PC of uncertain origin; 15, PV; 16, normal subjects.

Fig 2. Relationship between TfR levels and Hct in groups of patients, each represented by its mean values. The regression line displays the relationship in reference subjects (see text). 1, β-thalassemia/HbE; 2, AMM; 3, AIHA; 4, spherocytosis; 5, hypoplastic anemia; 6, MDS; 7, HbH disease; 8, BMT; 9, MPD; 10, renal failure; 11, renal failure on rHuEpo; 12, secondary PC; 13, relative PC; 14, PC of uncertain origin; 15, PV; 16, normal subjects.

Results

Construction of Reference Regressions

To construct the reference curves representing the relationships between Hct on the one hand, and Epo, TfR, and ETU on the other, only a subset of the subjects were considered. Patients with polycythemia were excluded, because abnormal marrow proliferation and excessive Epo production are often part of the pathophysiology of the disease. Patients with renal failure or hypoplastic anemia (as well as BMT patients) were excluded because of likely impairment of Epo production or reduced erythroid activity (see clinical criteria below). Patients with AMM, MDS, or advanced CML were also excluded because of the possibility of a component of relative erythroid hypoproliferation (see definition below) in many of them. Therefore, only the groups of normal subjects and of patients with other MPDs, AIHA, spherocytosis, β-thalassemia, and HbH disease were included. Among these groups, 3 patients were later excluded because Epo (in 2 β-thalassemic patients) or TfR (in 1 AIHA patient) were well beyond 3 SD below the predicted value.

As shown in Fig 1, two least squares regression equations between Epo and Hct were computed, one for Hct less than 40% and the other for Hct greater than 40%. This cutoff Hct was chosen because it allowed for the best correlation for Epo data and because of literature data indicating that beyond such a Hct there is little modification of Epo levels. For Hct less than 40%, the following regression \( r = 0.83, P = 0.0000 \) was obtained between Epo (mU/mL) and Hct (%): log (Epo) = 3.420 - (0.056 Hct). For Hct greater than 40%, the regression equation \( r = 0.12, \text{NS} \) was: log (Epo) = 1.311 - (0.003 Hct). The reference regressions for TfR and ETU were calculated in the same subjects, using the same cutpoint Hct. The following regressions were obtained with TfR (μg/L): log (TfR) = 6.146 - (0.057 Hct), when Hct is less than 40% \( r = 0.75, P = 0.0000 \); and log (TfR) = 4.079 - (0.008 Hct), when Hct is greater than 40% \( r = 0.18, \text{NS} \). The following regressions were obtained with ETU (μmol/L/d): log (ETU) = 4.061 - (0.052 Hct), when Hct is less than 40% \( r = 0.66, P = 0.0000 \); and log (ETU) = 2.086 - (0.006 Hct), when Hct is greater than 40% \( r = -0.15, \text{NS} \). Thus the regression obtained for TfR and ETU had slopes very similar to those obtained with Epo \( r > 0.1 \) for differences in slope. Based on these formulas, predicted log (Epo), log (TfR), and log (ETU) values were derived for each sample and O/P ratios of log (Epo), log (TfR), or log (ETU) were calculated. Ninety-five percent confidence limits were calculated to define a range of reference values for individual O/P values. These limits were 0.80 to 1.22 for O/P Epo, 0.91 to 1.09 for O/P TfR, and 0.76 to 1.22 for O/P ETU.

Definition of Patterns of Erythropoiesis

The major patterns of erythropoiesis were defined experimentally, based on the Hct, O/P TfR, and O/P Epo. The
activity inadequately low for the degree of anemia (decreased TfR), relative (increased TfR), or intermediate activity inadequately low for the degree of anemia (decreased O/P TfR) because of inadequate Epo production (decreased O/P Epo). Defective Epo production could be simple (decreased TfR), composite (increased TfR), or intermediate (normal TfR). As with pattern C, the experimental diagnoses were Epo-dependent "hypoproliferative anemia" (pattern D1), and indeterminate (pattern D2). The corresponding erythroid marrow should be respectively: hypocellular, normocellular or hypercellular; and normocellular or hypocellular. The retic index was always less than 3 in this group.

"Experimental" Classification of Anemic Subjects

Analysis of groups of subjects. Hematologic measurements are presented in Table 1. Epo, TfR, and ETU levels of each group were compared with values predicted from their Hct according to the reference regression equations defined above, and ratios of O/P Epo, TfR, ETU, and RCM are shown in Table 2. The figures illustrate the relationship between mean Hct and mean Epo (Fig 1) or mean TfR (Fig 2) levels for the various groups of subjects, as compared with the predicted values.

For the 31 normal subjects, mean Hct was 45.9 ± 3.6% (range, 42.1 to 48.7), mean Epo was 9.7 ± 2.8 mU/mL (range, 5.7 to 13.7), mean TfR was 20.4 ± 4.7 µg/L (range, 12.1 to 32.5), mean ETU was 51.8 ± 10.1 pmol/L/d (range, 27.0 to 70.0), and mean Hb was 14.1 ± 2.3 g/dL (range, 11.9 to 17.3). The figures illustrate the relationship between mean Hct and mean Epo (Fig 1) or mean TfR (Fig 2) levels for the various groups of subjects, as compared with the predicted values.

Analysis of groups of subjects. Hematologic measurements are presented in Table 1. Epo, TfR, and ETU levels of each group were compared with values predicted from their Hct according to the reference regression equations defined above, and ratios of O/P Epo, TfR, ETU, and RCM are shown in Table 2. The figures illustrate the relationship between mean Hct and mean Epo (Fig 1) or mean TfR (Fig 2) levels for the various groups of subjects, as compared with the predicted values.

The 31 normal subjects had a mean (mean ± SD) Hct of 45.9% ± 3.6% (range, 40% to 52% in men and 37% to 47% in women), a mean Epo of 14.1 ± 2.8 mU/mL (range, 9.3 to 23.1), a mean TfR of 4,970 ± 1,030 µg/L (range, 2,700 to 7,650), and a mean ETU of 61 ± 15 µmol/L/d (range, 32 to 95). Their mean O/P Epo was 0.99 ± 0.07 (range, 0.85 to 1.17), their mean O/P TfR was 0.99 ± 0.03 (range, 0.91 to 1.05), and their mean O/P ETU was 0.98 ± 0.08 (range, 0.86 to 1.13).

Patients with renal failure had Epo levels normal in absolute value (Table 1), but inadequate for the degree of anemia, as illustrated in Table 2 (decreased O/P Epo) and Fig 1 (mean value well below the reference line). Those treated with recombinant Epo came closer to the line of prediction (Fig 1). All other groups of anemic subjects had increased Epo values as compared with normal subjects (Table 1). This increase was appropriate for the degree of anemia, as shown by the retic index and TfR served to separate major patterns into variants. A normal pattern of erythropoiesis (pattern A) was defined by normal Hct, O/P Epo, and O/P TfR. The TfR value helped differentiate a variant of compensated hemolysis (pattern A2) in which TfR was increased, with a corresponding clinical picture of increased erythroid marrow cellularity and decreased RBC life span.

The term "hyperdestruction" referred to an experimental diagnosis (pattern B) in which Hct was decreased and O/P Epo and O/P TfR were normal. The corresponding clinical diagnosis of increased RBC destruction and erythroid activity was based on the following criteria: (1) increased erythroid cellularity on marrow examination; (2) shortened RBC life span for a diagnosis of hemolysis. The retic index served to break this group down into two variants of "hemolysis" (pattern B1) and "ineffective erythropoiesis" (pattern B3). When the retic index was between 2 and 3, this distinction could not be readily made (pattern B2).

The term "intrinsic marrow hypoproliferation" referred to an experimental diagnosis (pattern C) defined by erythropoietic activity inadequately low for the degree of anemia (decreased O/P TfR) because of inadequate Epo production (decreased O/P Epo). Defective Epo production could be simple (decreased TfR), composite (increased TfR), or intermediate (normal TfR). As with pattern C, the experimental diagnoses were Epo-dependent "hypoproliferative anemia" (pattern D1), and indeterminate (pattern D2). The corresponding erythroid marrow should be respectively: hypocellular, normocellular or hypercellular; and normocellular or hypocellular. The retic index was always less than 3 in this group.

Table 1. Hematologic Values (mean ± SD) in Groups of Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Hct (%)</th>
<th>Epo (mU/mL)</th>
<th>TfR (µg/L)</th>
<th>ETU (µmol/L/d)</th>
<th>S (%)</th>
<th>Ferritin (µg/L)</th>
<th>Retic (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>31</td>
<td>45.9 ± 3.6</td>
<td>14.1 ± 2.8</td>
<td>4,970 ± 1,030</td>
<td>61 ± 15</td>
<td>30 ± 8</td>
<td>95 ± 74</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Uncertain PC</td>
<td>12</td>
<td>51.8 ± 5.9</td>
<td>12.1 ± 4.2</td>
<td>7,280 ± 2,480</td>
<td>77 ± 20</td>
<td>32 ± 15</td>
<td>92 ± 81</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>PV</td>
<td>9</td>
<td>50.1 ± 7.8</td>
<td>10.1 ± 3.6</td>
<td>10,480 ± 5,870</td>
<td>97 ± 31</td>
<td>18 ± 9</td>
<td>40 ± 17</td>
<td>2.6 ± 1.6</td>
</tr>
<tr>
<td>Secondary PC</td>
<td>12</td>
<td>50.7 ± 4.2</td>
<td>52.9 ± 77.9</td>
<td>7,780 ± 3,910</td>
<td>80 ± 30</td>
<td>32 ± 18</td>
<td>162 ± 196</td>
<td>2.6 ± 3.0</td>
</tr>
<tr>
<td>Relative PC</td>
<td>5</td>
<td>52.7 ± 2.1</td>
<td>14.2 ± 3.9</td>
<td>4,840 ± 890</td>
<td>81 ± 21</td>
<td>35 ± 8</td>
<td>136 ± 103</td>
<td>1.8 ± 1.6</td>
</tr>
<tr>
<td>MPD</td>
<td>18</td>
<td>38.2 ± 3.6</td>
<td>21.6 ± 20.3</td>
<td>5,370 ± 1,140</td>
<td>76 ± 66</td>
<td>30 ± 18</td>
<td>510 ± 1,070</td>
<td>2.0 ± 1.3</td>
</tr>
<tr>
<td>MDS</td>
<td>10</td>
<td>34.6 ± 4.7</td>
<td>38.0 ± 20.3</td>
<td>7,980 ± 6,750</td>
<td>98 ± 105</td>
<td>47 ± 26</td>
<td>446 ± 708</td>
<td>3.3 ± 3.8</td>
</tr>
<tr>
<td>AMM</td>
<td>18</td>
<td>29.6 ± 5.0</td>
<td>74.5 ± 67.2</td>
<td>8,050 ± 2,270</td>
<td>133 ± 64</td>
<td>35 ± 21</td>
<td>276 ± 280</td>
<td>3.9 ± 2.6</td>
</tr>
<tr>
<td>Hypoplastic anemia</td>
<td>11</td>
<td>33.9 ± 6.1</td>
<td>88.9 ± 78.7</td>
<td>4,010 ± 1,410</td>
<td>50 ± 18</td>
<td>60 ± 23</td>
<td>550 ± 530</td>
<td>2.5 ± 2.4</td>
</tr>
<tr>
<td>Renal failure</td>
<td>15</td>
<td>21.6 ± 3.4</td>
<td>15.0 ± 5.0</td>
<td>2,610 ± 1,060</td>
<td>40 ± 28</td>
<td>36 ± 19</td>
<td>699 ± 770</td>
<td>2.1 ± 1.0</td>
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<tr>
<td>Renal failure on HluEpo</td>
<td>5</td>
<td>33.4 ± 3.6</td>
<td>12.7 ± 4.1</td>
<td>6,410 ± 860</td>
<td>71 ± 20</td>
<td>23 ± 6</td>
<td>189 ± 217</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>BMT</td>
<td>9</td>
<td>34.3 ± 4.8</td>
<td>51.8 ± 69.5</td>
<td>2,450 ± 1,000</td>
<td>63 ± 36</td>
<td>58 ± 23</td>
<td>2,925 ± 2,399</td>
<td>3.4 ± 1.5</td>
</tr>
<tr>
<td>β-Thalassemia/HbE</td>
<td>11</td>
<td>24.5 ± 5.0</td>
<td>80.4 ± 39.7</td>
<td>46,830 ± 11,730</td>
<td>562 ± 207</td>
<td>80 ± 26</td>
<td>1,686 ± 1,954</td>
<td>1.7 ± 1.3</td>
</tr>
<tr>
<td>HbH disease</td>
<td>10</td>
<td>33.0 ± 2.7</td>
<td>34.9 ± 15.1</td>
<td>23,250 ± 5,760</td>
<td>235 ± 106</td>
<td>57 ± 24</td>
<td>656 ± 809</td>
<td>2.0 ± 1.7</td>
</tr>
<tr>
<td>Spherocytosis</td>
<td>8</td>
<td>34.3 ± 7.0</td>
<td>44.6 ± 20.4</td>
<td>22,050 ± 11,040</td>
<td>506 ± 303</td>
<td>44 ± 11</td>
<td>923 ± 689</td>
<td>4.7 ± 1.5</td>
</tr>
<tr>
<td>AIHA</td>
<td>11</td>
<td>32.6 ± 7.0</td>
<td>97.5 ± 147.1</td>
<td>16,300 ± 7,270</td>
<td>235 ± 133</td>
<td>33 ± 13</td>
<td>535 ± 635</td>
<td>7.1 ± 3.4</td>
</tr>
</tbody>
</table>
in Fig 1 (all groups were near the line of prediction) and in Table 2 (O/P Epo close to 1).

Patients with AIHA, spherocytosis, \( \beta \)-thalassemia, and HbH disease had TfR and ETU values considerably elevated as compared with normal subjects (Table 1), indicating an increased erythroid marrow activity in response to an increased Epo stimulation. The distinction between hemolysis and ineffective erythropoiesis could be made by a retic index of greater than 3 and less than 2, respectively.

On the other hand, only the BMT, hypoplastic anemia, and renal failure groups had decreased TfR values (Table 1). They were also the ones that fell the farthest below the reference line (Fig 2) and had the lowest O/P TfR and O/P ETU (Table 2). Therefore, the anemia in these groups appeared to be a consequence of decreased erythroid activity, either because of reduced Epo production (renal failure) or intrinsic erythroid marrow hypoproliferation (BMT and hypoplastic anemia).

Patients with MDS or AMM had elevated TfR levels (Table 1). This indicated that, despite adequate Epo stimulation, there was a hypoproliferative component to their anemia. Hence, the pathophysiology of their anemia was mixed, i.e., a combination of hyperdestruction and hypoproliferation.

**Analysis of individual patients.** Individual patients were classified according to their experimental pattern of erythropoiesis. Table 3 discloses typical cases illustrating the various patterns, whereas Table 4 discourses each clinical group into experimental diagnostic groups. All normal subjects had a normal pattern of erythropoiesis (pattern A1). Of 11 patients with AIHA, 7 had typical hemolysis (pattern B1), 1 had an intermediate retic index (pattern B2), and 2 had the variant normal pattern with increased TfR (pattern A2), indicating that hemolysis was completely compensated by increased erythroid activity, and one had an important intrinsic marrow hypoproliferative component (pattern C1), due to metastatic breast cancer. Patients with spherocytosis had typical hemolysis (pattern B1), except for one who had returned to normal erythropoiesis (pattern A1) after splenectomy. Patients with \( \beta \)-thalassemia or HbH disease had typical ineffective erythropoiesis (pattern B3). However, in one HbH pa-

### Table 2. Ratios of Observed to Predicted Values (mean ± SD) in Groups of Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>O/P RCM</th>
<th>O/P Epo</th>
<th>O/P TfR</th>
<th>O/P ETU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>31</td>
<td>1.03 ± 0.08</td>
<td>0.99 ± 0.07</td>
<td>0.99 ± 0.03</td>
<td>0.98 ± 0.08</td>
</tr>
<tr>
<td>Uncertain PC</td>
<td>12</td>
<td>1.34 ± 0.20t</td>
<td>0.93 ± 0.12</td>
<td>1.05 ± 0.051</td>
<td>1.05 ± 0.09*</td>
</tr>
<tr>
<td>PV</td>
<td>9</td>
<td>1.36 ± 0.28†</td>
<td>0.85 ± 0.11*</td>
<td>1.08 ± 0.09*</td>
<td>1.10 ± 0.11*</td>
</tr>
<tr>
<td>Secondary PC</td>
<td>12</td>
<td>1.37 ± 0.33*</td>
<td>1.31 ± 0.37†</td>
<td>1.05 ± 0.06*</td>
<td>1.07 ± 0.08*</td>
</tr>
<tr>
<td>Relative PC</td>
<td>5</td>
<td>1.17 ± 0.09*</td>
<td>1.01 ± 0.12</td>
<td>1.01 ± 0.02</td>
<td>1.09 ± 0.07*</td>
</tr>
<tr>
<td>MPD</td>
<td>18</td>
<td>0.92 ± 0.17†</td>
<td>1.00 ± 0.19</td>
<td>0.94 ± 0.051</td>
<td>0.86 ± 0.16*</td>
</tr>
<tr>
<td>MDS</td>
<td>10</td>
<td>0.79 ± 0.14t</td>
<td>1.00 ± 0.15</td>
<td>0.92 ± 0.041</td>
<td>0.80 ± 0.14t</td>
</tr>
<tr>
<td>AMM</td>
<td>18</td>
<td>0.85 ± 0.23†</td>
<td>0.93 ± 0.23</td>
<td>0.88 ± 0.051</td>
<td>0.83 ± 0.151</td>
</tr>
<tr>
<td>Hypoplastic anemia</td>
<td>11</td>
<td>0.75 ± 0.17†</td>
<td>1.02 ± 0.29</td>
<td>0.85 ± 0.061</td>
<td>0.75 ± 0.141</td>
</tr>
<tr>
<td>Renal failure</td>
<td>15</td>
<td>0.53 ± 0.09*</td>
<td>0.53 ± 0.10†</td>
<td>0.69 ± 0.041</td>
<td>0.51 ± 0.151</td>
</tr>
<tr>
<td>Renal failure on rHuEpo</td>
<td>5</td>
<td>0.80 ± 0.10†</td>
<td>0.72 ± 0.16†</td>
<td>0.90 ± 0.06*</td>
<td>0.80 ± 0.12†</td>
</tr>
<tr>
<td>BMT</td>
<td>9</td>
<td>0.64 ± 0.13†</td>
<td>1.01 ± 0.21</td>
<td>0.81 ± 0.061</td>
<td>0.78 ± 0.12†</td>
</tr>
<tr>
<td>( \beta )-Thalassemia/HbE</td>
<td>11</td>
<td>NA</td>
<td>0.94 ± 0.13</td>
<td>0.98 ± 0.06</td>
<td>0.97 ± 0.06</td>
</tr>
<tr>
<td>HbH disease</td>
<td>10</td>
<td>NA</td>
<td>1.03 ± 0.11</td>
<td>1.05 ± 0.04*</td>
<td>1.04 ± 0.09</td>
</tr>
<tr>
<td>Spherocytosis</td>
<td>8</td>
<td>0.91 ± 0.08</td>
<td>1.04 ± 0.12</td>
<td>1.01 ± 0.04</td>
<td>1.10 ± 0.08*</td>
</tr>
<tr>
<td>AIHA</td>
<td>11</td>
<td>0.84 ± 0.15*</td>
<td>1.05 ± 0.10</td>
<td>0.98 ± 0.09</td>
<td>1.01 ± 0.14</td>
</tr>
</tbody>
</table>

*P values are given for comparison with normal subjects. Abbreviation: NA, not available.

*P < .01.

†P < .001.

‡P < .05.

### Table 3. Some Typical Cases and Characteristic Exceptions to the Patterns of Erythropoiesis

<table>
<thead>
<tr>
<th>Pattern of Erythropoiesis</th>
<th>Clinical Diagnosis</th>
<th>Hct</th>
<th>O/P TfR</th>
<th>O/P Epo</th>
<th>TR</th>
<th>Retic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (A1)</td>
<td>Normal subject</td>
<td>48.8</td>
<td>1.00</td>
<td>1.04</td>
<td>4.670</td>
<td>2.1</td>
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<tr>
<td>Hemolysis (B1)</td>
<td>AIHA</td>
<td>31.1</td>
<td>1.00</td>
<td>0.94</td>
<td>24,000</td>
<td>4.1</td>
</tr>
<tr>
<td>Ineffective erythropoiesis (B3)</td>
<td>( \beta )-thalassemia</td>
<td>30.0</td>
<td>0.99</td>
<td>0.91</td>
<td>24,650</td>
<td>0.9</td>
</tr>
<tr>
<td>Intrinsic marrow hypoproliferation: hypoproliferative anemia (C3)</td>
<td>HbH disease</td>
<td>25.2</td>
<td>0.70</td>
<td>1.04</td>
<td>1.980</td>
<td>0.7</td>
</tr>
<tr>
<td>Intrinsic marrow hypoproliferation: mixed disorder of erythropoiesis (C1)</td>
<td>AMM</td>
<td>23.0</td>
<td>0.84</td>
<td>1.03</td>
<td>11,840</td>
<td>1.5</td>
</tr>
<tr>
<td>Defective Epo production: hypoproliferative anemia (D3)</td>
<td>Renal failure</td>
<td>20.6</td>
<td>0.68</td>
<td>0.51</td>
<td>2,500</td>
<td>1.0</td>
</tr>
<tr>
<td>Defective Epo production: mixed disorder of erythropoiesis (D1)</td>
<td>( \beta )-Thalassemia + infection</td>
<td>22.0</td>
<td>0.89</td>
<td>0.74</td>
<td>22,600</td>
<td>0.8</td>
</tr>
</tbody>
</table>

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Table 4. Number of Individual Patients by Clinical Diagnosis in Each Pattern of Erythropoiesis

<table>
<thead>
<tr>
<th>Variant</th>
<th>A Normal</th>
<th>B Hyperdestruction</th>
<th>C Intrinsic Marrow Hypoproliferation</th>
<th>D Defective Epo Production</th>
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</thead>
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<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Hct</td>
<td>N</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>O/P TfR</td>
<td>N-(High)</td>
<td>N</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>O/P Epo</td>
<td>N-(High)</td>
<td>N</td>
<td>N</td>
<td>Low</td>
</tr>
<tr>
<td>TfR</td>
<td>N</td>
<td>High</td>
<td>&gt;3</td>
<td>2-3</td>
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<tr>
<td>Retic index</td>
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<tr>
<td>Erythroid cellularity</td>
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<td></td>
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<tr>
<td>Mean RBC life span</td>
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<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
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<th>B3</th>
<th>B3</th>
<th>B3</th>
<th>B3</th>
<th>B3</th>
<th>B3</th>
<th>B3</th>
<th>B3</th>
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<td></td>
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<tr>
<td>MPD</td>
<td>18</td>
<td>7</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
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<td>MDS</td>
<td>10</td>
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<td>1</td>
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<td>AMM</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>2</td>
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<td>Hypoplastic anemia</td>
<td>11</td>
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<tr>
<td>Renal failure</td>
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<td></td>
<td></td>
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<tr>
<td>Renal failure on rHuEpo</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>BMT</td>
<td>9</td>
<td>1</td>
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<td>P-Thalassemia/HbE</td>
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<td></td>
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<td>Spherocytosis</td>
<td>8</td>
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<td></td>
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</tr>
<tr>
<td>AIHA</td>
<td>11</td>
<td>2t</td>
<td>7</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*High O/P Epo.
†High O/P TfR.
Correlations Between Parameters of Erythropoiesis

There was an excellent correlation between Hct and O/P RCM ($r = .83$, $P < .0001$), as well as between ETU and TIR levels ($r = .82$, $P < .0001$). Expressing ETU values in micrograms per day and TIR in micrograms, by multiplying concentrations by blood or plasma volumes, respectively, did not alter the correlation ($r = .81$, $P < .0001$) between the two measurements of erythropoiesis. This is illustrated in Fig 3, in which each group of subjects is represented by its mean ETU and TIR values. In polycythemia patients, there was a positive correlation between TIR and Hct ($r = .42$, $P < .05$) as well as between TIR and RCM ($r = .63$, $P < .001$). In contrast to other groups of patients, there was a positive relationship between Epo levels and Hct in patients with renal failure ($r = .46$, $P < .05$). The overall correlations between Epo levels and TIR levels ($r = .45$, $P < .001$) or ETU ($r = .42$, $P < .001$) were relatively weak because they reflected many different patterns of erythropoiesis.

DISCUSSION

In the present study, we investigated the pathophysiology of RBC production in a large group of normal, anemic, or polycythemic subjects. The RCM was quantitated by direct isotopic measurement, Epo stimulation by serum Epo levels, and total marrow erythropoietic activity by the ferrokinetic measurement of the ETU, serum TIR levels, and retic index. Instead of only quantitating Epo levels and erythropoiesis in absolute terms, we also evaluated them in relation to the degree of anemia or PC, and expressed results as a ratio of observed to predicted values. These quantitative measurements were supplemented by the qualitative evaluation of erythroid cellularity on marrow examination. The study of anemic subjects is based on a simple model of the regulation of erythropoiesis, i.e., the RCM determines Epo production, which stimulates erythropoietic activity. If this model of erythropoiesis is valid from a quantitative point of view, then the relationship between Hct and erythropoiesis should be similar to the one between Hct and Epo. If this is the case, then (1) an inverse relationship should be observed between Epo and Hct, as well as between TIR and Hct; (2) the regression lines of log(Epo) versus Hct and of log(TIR) versus Hct should have similar slopes; and (3) Epo and TIR values predicted from the regressions should then not be different from observed values. These three conditions were indeed fulfilled. First, after excluding groups of patients whose anemia was known to be associated with renal failure and/or a hypoproliferative component, an excellent inverse correlation was observed between Hct and Epo on the one hand, and between Hct and TIR (as well as ETU) on the other. Second, the slope of the regression of TIR (as well as ETU) versus Hct was very similar to the slope of the regression of Epo versus Hct. Third, Epo and TIR (as well as ETU) values predicted from the regression equations were quite comparable to observed values in most groups of subjects (Table 2 and Figs 1 and 2). A predictable exception for Epo was the group of renal failure patients (O/P Epo 53% of predicted), whereas those treated with recombinant Epo came closer to the line of prediction (O/P Epo 72% of predicted) (Fig 1). For TIR, the groups of patients whose primary cause of anemia was decreased erythroid activity fell the farthest below prediction, whereas patients with MDS and AMM also had a hypoproliferative component to their anemia. All this is in keeping with our knowledge of the pathophysiology of these hematologic disorders.

When normal individuals are phlebotomized while iron supply is adequate, they are able to sustain an erythropoietic activity 3 to 5 times normal. This will translate into TIR levels increased in proportion to Epo stimulation. However, in the presence of erythroid hypoproliferation, TIR values will be lower than expected from the level of Epo stimulation. In the current study, only 20 patients had purely hypoproliferative anemia (decreased TIR), whereas all others had
normal or elevated TfR values. However, when examined relative to the degree of anemia, 42 additional patients were found to have varying degrees of erythroid marrow hypoproliferation, including 16 patients who actually had elevated serum TfR levels. Therefore, any measurement of erythropoiesis not related to the degree of anemia could grossly overestimate marrow proliferative capacity. Several attempts have been made previously to address this problem. Using sophisticated computer analysis of ferrokinetic data, a distinction could be made between anemic patients with high and low potential erythropoiesis on the basis of a value for total erythropoiesis higher or lower than 4 times basal, and a more precise rule could be derived by defining a decision boundary relative to the hemoglobin concentration.19

Examining serum Epo and TfR in relation to the degree of anemia had the further advantage of identifying patients with a “mixed disorder of erythropoiesis,” i.e., individuals with a hypoproliferative component combined with a component of hyperdestruction (hemolysis or ineffective erythropoiesis). Thus, besides typical patterns of erythropoiesis (Table 3), many patients had a composite pathophysiology. As shown in ferrokinetic studies,19 dissecting out groups of patients to examine individual patterns of erythropoiesis (Table 4) showed that the mechanism of anemia may be similar among patients with different diseases, but can also vary much among patients with the same clinical disorder. For instance, among patients with a clinical diagnosis of hemolytic anemia (spherocytosis or AIHA), 1 became normal after splenectomy, 2 compensated completely for the hyperdestruction, and 1 had a component of hypoproliferation. Besides patients with renal failure, defective Epo production was also observed in many patients, including some with β-thalassemia/HbE,20 AMM, MPD, and hypoplastic anemia or after BMT (Table 4).

Apparently aberrant patterns could also find an explanation. For instance, a patient with renal failure had normal erythropoietic activity despite inadequate endogenous Epo production because of administration of exogenous recombinant Epo, a BMT patient had normalized erythroid activity through excessive Epo stimulation induced by chronic hypoxia, and 2 patients had elevated TfR levels despite an otherwise normal pattern of erythropoiesis because they had fully compensated for a hemolytic process.

The identification of different pathophysiologic components could have important implications for the treatment of the anemia in individual patients with an otherwise well-defined clinical diagnosis. For instance, treatment with rHuEpo would probably be unsuccessful in the case of a purely hyperdestructive anemia, but would be more likely to be effective when a component of defective Epo production or a purely hypoproliferative anemia have been identified. In the case of diseases such as AMM, MDS, or MPD, in which the pathophysiology of the anemia is often composite, a careful analysis could separate patients into poor and good candidates for Epo therapy. This remains to be shown prospectively.

The proposed model of erythropoiesis has several limitations. First, the exactness of each measurement has its own limits; an apparent inadequacy of Epo production could in fact result from better tissue oxygenation with altered oxygen dissociation curves, iron deficiency could cause some elevation of TfR levels not related to erythropoiesis,21 and the retic count is hampered by imprecision and can be at best considered as a semiquantitative measurement. Second, while a mixed disorder of erythropoiesis can be readily identified in patients with low O/P TfR but increased TfR value, it is more difficult to distinguish a component of hyperdestruction from a situation of blunted marrow response to increased Epo stimulation without hyperdestruction when TfR level is normal. However, when we carefully analyzed patterns of erythropoiesis in relation to clinical diagnosis (Table 4), it became apparent that when TfR was normal and Epo production inadequate (decreased O/P Epo), blunted marrow responsiveness was by far more likely, whereas a component of hyperdestruction was more likely when O/P Epo was normal.

A third difficulty is encountered when one attempts to separate hemolysis from ineffective erythropoiesis, as both are characterized by a low Hct, elevated TfR, and normal O/P TfR and O/P Epo. After appropriate corrections are made,16 the retic index can be of great help in most cases. While the RBC life span can be evaluated by isotopic methods, there is no gold standard with which to measure ineffective erythropoiesis. Sophisticated computer analyses of ferrokinetic data have attempted to quantitate ineffective erythropoiesis, but some aspects of iron exchange remained impossible to resolve in these calculations, particularly the separation of wastage iron of erythropoiesis from iron taken up by parenchymal cells.22,23 Some cases presenting conditions defined as dyserythropoietic can also show a component of peripheral hemolysis, while so-called hemolytic anemias can be characterized by varying degrees of ineffective erythropoiesis.24,26 Therefore, the erythropoietic pattern may depend on the stage of erythroid development at which destruction occurs and a clear distinction between ineffective erythropoiesis and peripheral hemolysis may not always be possible.

Finally, the model does not contribute much to the approach to patients with PC. The RCM correlated positively with TfR much better than with the Hct, suggesting a causative and quantitatively valid link between the RCM and erythropoiesis as measured by the TfR level. However, a typical pattern of increased O/P TfR with (secondary PC) or without (PV) increased O/P Epo was not frequently observed. Therefore, a correct diagnosis of PC should be based on an RCM determination (and not a Hct) and other standard criteria.7 Serum Epo could be useful in identifying patients with secondary PC, although many of them have normal values,2,27-29 and serum TfR in making a diagnosis of PV in some patients with PC of uncertain origin, as illustrated in two of our patients.

Given these limitations of a model incorporating the Hct, serum Epo, serum TfR, and retic index, it could be possible to substitute other measurements. The isotopic measurement of the RCM gives a better idea of the total number of RBCs than the Hct, particularly in the case of splenomegaly or altered plasma volume. Although a direct evaluation of the
RCM is indispensable in the diagnosis of PC, the Hct remains sufficient for most practical purposes in a situation of anemia. While we do not have any equivalent tool to replace serum Epo, erythropoiesis could be approached by other methods than TIR, such as the marrow erythroid cellularity, retic index, and ETU.

The present model only deals with the pathophysiology of anemia and has no pretension to replace a marrow examination to identify specific clinical disorders or to recognize iron deficiency along with a serum ferritin level. A marrow sample can also be used to evaluate overall erythroid cellularity, but can only describe it in a semiquantitative fashion as normal, hypocellular, or hypercellular. The erythropoietic response to anemia cannot be measured quantitatively from the marrow alone, and a hypoproliferative component cannot be identified in patients with normocellular or hypercellular erythroid marrow. Furthermore, there are many instances in which a representative sample cannot be obtained because of nonhomogeneous marrow distribution, or in which an abnormally expanded white blood cell population precludes a meaningful interpretation of the erythroid/myeloid ratio and the erythroid mass.

As illustrated in Table 1, the retic percentage does not provide a quantitative evaluation of total erythropoiesis, as the majority of the groups of patients were indistinguishable from normal individuals and several groups of patients characterized by hypoproliferative anemia as defined here had elevated counts. The retic count showed no correlation with the Hct, TIR, or ETU. After corrections were applied, a retic index greater than 3 was rarely encountered outside the AIHA and spherocytosis groups, although many of the patients had increased erythropoietic activity. Patients with a hypoproliferative component, ineffective erythropoiesis, or both remained indistinguishable. Therefore, with the exception of patients with a purely hemolytic anemia, the retic count and index alone do not provide a quantitative evaluation of erythropoiesis. However, when used in conjunction with quantitative measurements, they provide information complementary to TIR, particularly when one desires to separate hemolysis from ineffective erythropoiesis.

When transferrin saturation decreases below 16% or the plasma radioiron t½ is less than 30 minutes, ferrokinetic measurements are no longer valid. On the other hand, when plasma iron is elevated, the presence of nontransferrin-bound iron whose turnover is not related to the erythron but to the hepatocyte can overestimate erythropoiesis. This problem was probably encountered in our BMT patients (Fig 3). Because its calculation is based on three separate measurements (plasma iron, radioiron t½, and Hct), the ETU is more vulnerable to experimental error than TIR levels and, indeed, in the present study its coefficient of variation was higher in normal subjects as well as in patients, its correlation with Hct weaker, and its 95% confidence limits wider. Although the overall correlation between ETU and TIR was excellent, the greater precision and simpler methodology of the TIR determination should make it the method of choice for estimating erythroid activity.

ACKNOWLEDGMENT

The authors thank Georges Weber (Department of Experimental Nuclear Physics, University of Liège) for preparing the figures.

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Quantitative assessment of erythropoiesis and functional classification of anemia based on measurements of serum transferrin receptor and erythropoietin

Y Beguin, GK Clemons, P Pootrakul and G Fillet

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