Regulation of Erythropoiesis. I. Erythropoietin Assay as a Clinical Tool

By Ward D. Noyes, Bill M. Domm and Loretta C. Willis

The rate of red cell production is regulated by a plasma factor(s) termed erythropoietin. While injection of exogenous plasma rich in this substance (or substances) has been shown to induce an increase in the total circulating red cell mass by stimulation of erythropoiesis, quantitation of erythropoietin has been hampered by lack of specific chemical methods. Numerous biological assays have been developed, each capable of demonstrating the presence of potent erythropoietin, but varying in sensitivity and reliability. With the increasing availability of these assays in clinical diagnosis comes the necessity of interpreting the results in the light of the patient's disease. The purpose of this paper is to examine the meaning of an erythropoietin assay using radioiron utilization in polycythemic rats, and to interpret the results obtained from patients with refractory anemia and with polycythemia.

Methods

Human plasma for erythropoietin assay was collected as whole blood in heparinized syringes. Erythropoietin-rich rabbit plasma was also harvested from adult male rabbits four days after the intraperitoneal injection of 40 mg./Kg. of phenylhydrazine hydrochloride. At the time of phlebotomy, these animals' hematocrits were in the range of 12 to 15 per cent. The blood was centrifuged and the plasma stored in sterile containers at -16 C. until tested.

The assay procedure used the method described by Reichlin and Harrington with some modification. Assay animals were Sprague-Dawley male rats weighing 150-200 Gm. at the start of the assay. They had received 5 mg. of iron intramuscularly as iron-dextran 10 days previously, to preclude preexisting iron deficiency. On the first day each animal received 4 ml. of packed red cells intravenously from a normal rat donor. On days three, four and five, 2 ml. of test plasma were injected. No attempt was made to concentrate the plasma, since published data suggest considerable variation in the proportion of native erythropoietin recovered with concentration procedures. Four hours after the last injection, approximately 0.25 uc. of radioiron containing less than 0.05 jg. of elemental iron was injected intravenously as ferric citrate bound to normal rat plasma, or as ferric chloride. The mean value for plasma iron determinations from a group of 13 rats on day six was 209 jg. per cent and for unsaturated iron-binding capacity 264 jg. per cent, giving a mean saturation of 44 per cent. This is interpreted as indicating that all injected radioiron could be easily bound to transferrin, producing valid results for radioiron utilization. Twenty hours after radioiron injection, cardiac blood was obtained under light ether anesthesia for determination of radioactivity and hematocrit. Animals whose hematocrits on day six were less than 55 volumes per cent were discarded, as were animals who failed to gain weight during the period of assay. Radioiron utilization at 20 hours was calculated as follows:

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Per cent utilization = \( \frac{\text{Weight (Gm.)} \times 0.044 \times \text{cpm/ml. blood}}{\text{Hematocrit} \times \text{cpm injected}} \times 100 \)

The estimated red cell mass of 0.044 ml./Gm. total body weight was obtained by measuring the red cell mass in 10 rats of comparable weight, six days after hypertransfusion, using red cells from a normal donor labelled with Fe\(^{59}\).

Radioactivity was measured in a well-type scintillation detector with sufficient accumulated counts to bring the counting error below 5 per cent. Patient studies included measurements of red cell mass by radiochromium,\(^7\) plasma iron turnover,\(^8\) arterial oxygen content using the Van Slyke-Neill method, reticulocytes stained with new methylene blue, and hematocrit by centrifugation at 3000 g for 30 minutes.

**RESULTS**

**A. Characterization of the Method**

Confidence in the assay procedure and objective interpretation of results required evaluation of the method. Anemic rabbit plasma was used as a readily available source of potent erythropoietin. Observations were made on the variability of test results using a standard procedure and modifying the route of administration, length of storage at \(-16\ C.\) and the number of injections (table 1). Analysis of the variance components from the data in table 1 revealed a within-group standard deviation of \(\pm 4.6\) as an indication of the variation from test animal to test animal. It was concluded that three injections of plasma subcutaneously given after storage for not longer than three months produced reliable observations bearing on the amount of erythropoietin in the plasma.

The response of the assay animal to the concentration of erythropoietin in the injected material was evaluated by diluting a single erythropoietin-rich rabbit plasma with various amounts of normal rabbit plasma and administering a constant volume of 2 ml. on three successive days. The results as shown

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>TEST MATERIAL</th>
<th>NUMBER OF ANIMALS</th>
<th>% RADIOIRON</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Animals</td>
<td>Plasma A</td>
<td>10</td>
<td>41.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Plasma B</td>
<td>8</td>
<td>32.7</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>13</td>
<td>7.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Route of Administration</td>
<td>Plasma A, 1-V</td>
<td>10</td>
<td>41.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Plasma B, 1-V</td>
<td>8</td>
<td>32.7</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Plasma B, 1-P</td>
<td>3</td>
<td>41.5</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>Plasma A, Sub-Cut</td>
<td>4</td>
<td>48.4</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Plasma B, Sub-Cut</td>
<td>5</td>
<td>45.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Length of Storage</td>
<td>Plasma B, Fresh</td>
<td>8</td>
<td>32.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma C, Fresh</td>
<td>5</td>
<td>35.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma C, 80 days</td>
<td>4</td>
<td>37.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma B, 90 days</td>
<td>4</td>
<td>37.1</td>
<td></td>
</tr>
<tr>
<td>Number of Injections</td>
<td>Plasma B, 1 inj.</td>
<td>5</td>
<td>11.1</td>
<td>4.6</td>
</tr>
<tr>
<td>(Subcutaneous)</td>
<td>Plasma B, 2 inj.</td>
<td>4</td>
<td>26.2</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>Plasma B, 3 inj.</td>
<td>5</td>
<td>45.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Table 2.—Response to Varying Concentrations of Erythropoietin

<table>
<thead>
<tr>
<th>CONCENTRATION OF ERYTHROPOIETIN-RICH PLASMA</th>
<th>NUMBER OF ANIMALS</th>
<th>% RADIOIRON UTILIZATION</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>4</td>
<td>7.2</td>
<td>2.5</td>
</tr>
<tr>
<td>10%</td>
<td>4</td>
<td>7.7</td>
<td>3.0</td>
</tr>
<tr>
<td>25%</td>
<td>4</td>
<td>21.1</td>
<td>3.9</td>
</tr>
<tr>
<td>50%</td>
<td>4</td>
<td>26.8</td>
<td>4.8</td>
</tr>
<tr>
<td>100%</td>
<td>5</td>
<td>38.2</td>
<td>5.6</td>
</tr>
</tbody>
</table>

The gross radioiron utilization in assay animals is corrected for response observed in saline-injected animals.

The data in table 2 are plotted in figure 1 using the analysis suggested by Stetten for dose-response relationships. The raw data were modified by subtracting from each response the utilization observed in saline-injected animals. The dose is plotted as the concentration of erythropoietin-rich plasma given versus the ratio of dose/corrected response.

B. Clinical Results

To delineate the clinical usefulness of the assay, plasma was tested from selected patients in whom the level of erythropoietin might be anticipated to be of crucial importance. The first group of patients was that with refractory anemia in which a failure of effective erythropoiesis could be demonstrated. Patients in this group were selected on the basis of a persistent normochromic anemia accompanied by a low reticulocyte count without evidence of infection, renal disease, endocrine disorder or other primary disease. Specific etiology was unknown except in case N. L., in whom the marrow changes were felt to be related to anticonvulsant therapy. Patients D. F. and T. F. were siblings who developed refractory anemia within six months after birth, which failed to respond to corticosteroids. Patients were separated into those with a hypoplastic erythroid marrow and those with erythroid hyperplasia, on the basis of examination of the marrow aspirate, and—where possible—the plasma iron turnover. The results of the erythropoietin assays are shown in table 3 where cases are ranked in order of increasing peripheral hematocrit. The results are also shown in figure 2 where the response observed in excess of that in saline-injected animals is plotted against the patient’s hematocrit. Consistently elevated levels were found in all but two patients. Analysis of the variance components gave a within-patient assay standard deviation of ± 4.1.

The second group of patients in whom erythropoietin assay would seem to be of significance is that of polycythemia. In these cases, the rate of red cell production is in excess of that in comparable normal individuals and the mechanism of its regulation is of interest. Patients were classified into those with hypoxic polycythemia and those with polycythemia vera, on the basis of arterial oxygen saturation, radiochromium red cell mass, peripheral blood studies and clinical findings. No evidence of significant infection or inflamma-
tion was present. Patients with relative polycythemia were not included in this group. Results of erythropoietin assay are shown in table 4. Increased levels were found in hypoxic polycythemia only in those patients treated with phlebotomy within the past six weeks. No elevation was demonstrated in patients with polycythemia vera, even when nearly normal hematocrits had been achieved by venesection. Analysis of variance components gave a within-patient assay standard deviation of ±3.7.

**DISCUSSION**

The confidence with which one employs a bio-assay partially depends upon the knowledge of variables affecting the results and the deviations of the observed results from the experimental mean. An attempt has been made to limit the known variables resulting in a within-group standard deviation of ±4.6. The values reported here are not directly comparable to those reported by others and emphasize the necessity for each laboratory to establish its own range of expected values and variability.

Of considerable interest is the relation between the dose of erythropoietin given to the test animal and the response elicited. Previous reports have employed a log dose versus response analysis to produce a linear relation. A more pertinent analysis of hormone-response data was suggested by Stetten using the Langmuir sorptive isotherm and more recently emphasized by Strickler et al. to which the response of graded doses obtained in this study is well fitted (fig. 1). The validity of this approach is further substantiated by similarly plotting data from other sources using various measurements of response.
Table 3.—Erythropoietin Assay in Refractory Anemia

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P. M.</td>
<td>48</td>
<td>L</td>
<td>15</td>
<td>†</td>
<td>0.2</td>
<td>0.68</td>
<td>57.3</td>
<td>5.8</td>
</tr>
<tr>
<td>K. B.</td>
<td>28</td>
<td>L</td>
<td>17</td>
<td>†</td>
<td>0.0</td>
<td>0.44</td>
<td>50.5</td>
<td>6.5</td>
</tr>
<tr>
<td>S. C.</td>
<td>72</td>
<td>N</td>
<td>19</td>
<td>†</td>
<td>0.0</td>
<td>0.0</td>
<td>55.9</td>
<td>3.2</td>
</tr>
<tr>
<td>D. F.</td>
<td>1</td>
<td>N</td>
<td>20</td>
<td>†</td>
<td>0.0</td>
<td>0.1</td>
<td>0.58</td>
<td>10.9</td>
</tr>
<tr>
<td>T. M.</td>
<td>2</td>
<td>N</td>
<td>23</td>
<td>†</td>
<td>0.1</td>
<td>0.5</td>
<td>0.58</td>
<td>52.4</td>
</tr>
<tr>
<td>E. C.</td>
<td>9</td>
<td>L</td>
<td>25</td>
<td>†</td>
<td>0.0</td>
<td>0.0</td>
<td>61.0</td>
<td>5.3</td>
</tr>
<tr>
<td>E. L.</td>
<td>68</td>
<td>L</td>
<td>29</td>
<td>†</td>
<td>0.5</td>
<td>0.32</td>
<td>33.9</td>
<td>1.4</td>
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<tr>
<td>M. B.</td>
<td>53</td>
<td>L</td>
<td>30</td>
<td>†</td>
<td>0.4</td>
<td>0.32</td>
<td>18.4</td>
<td>5.8</td>
</tr>
<tr>
<td>F. D.</td>
<td>66</td>
<td>N</td>
<td>30</td>
<td>†</td>
<td>0.0</td>
<td>0.39</td>
<td>68.7</td>
<td>2.8</td>
</tr>
<tr>
<td>T. F.</td>
<td>1/2</td>
<td>N</td>
<td>31</td>
<td>†</td>
<td>0.4</td>
<td>0.39</td>
<td>17.0</td>
<td>0.8</td>
</tr>
<tr>
<td>N. L.</td>
<td>7</td>
<td>L</td>
<td>38</td>
<td>†</td>
<td>0.0</td>
<td>0.6</td>
<td>21.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

(P.B.) 76 N 23 † 0.2 2.6 5.9 2.3
S. A. 68 N 24 † 0.3 3.1 25.0 2.3
R. Mc. 68 N 25 † 1.0 2.0 26.9 3.9
R. R. 78 N 29 † 0.5 2.0 24.8 2.3
C. W. 77 N 29 † 1.0 2.0 9.6 0.7

Patients are arranged in order of increasing hematocrit. The gross response in the assay animal is corrected for that seen in saline-injected controls.

It would seem unlikely that only a single absorptive phenomenon with reversible binding to a target acceptor site has produced the dose-response relationship here observed. The value of this analysis, however, lies in provoking new experiments, such as to observe whether competitive or noncompetitive inhibition may be demonstrated with substances like uremic plasmas or to determine whether the maximum response is related to the quantity of stem cells acting as binding sites in the recipient.

The erythropoietin assays in patients with refractory anemia are clearly elevated in 14 of 16 patients, and to this extent agree with findings recently reported by Lange, McCarthy and Gallagher. The exceptions remain an enigma. They showed no evidence of renal, endocrine, or inflammatory disorder, failed to respond to vitamin B12 or folic acid, and had ingested no known toxic agent. The morphology of their erythroid marrow was so bizarre as to suggest di Guglielmo's disease. However, transfusion to a hematocrit of 40 in P. B. was accompanied by return of his marrow smear toward normal, and some decrease in his plasma iron turnover, but he continued to have severe cytopenia and his hematocrit gradually fell to below 30 per cent. In C. W., the marrow remained bizarre after transfusion to normal levels and he died three months later from complications associated with progressive leukopenia and thrombocytopenia. These two patients may represent a different clinical entity. The large preponderance of patients with refractory anemia acquire a
remarkably elevated level of erythropoietin in response to their anemia. This increase in many patients may be calculated to be 10-50 times the normal level, indicating that in those with aplastic marrow the erythron is incapable of reacting with normal proliferation to a substance which does elicit a response in the assay animal.

Attempts have been made to correlate the quantity of available erythropoietin to the degree of anemia and to the cellularity of the erythroid marrow. Van Dyke et al. have suggested that in those anemias exclusive of iron deficiency, a demonstrable titer of plasma erythropoietin is shown only at hemoglobin levels below 8 Gm. per cent. The data shown here (fig. 2) suggest a general relationship between hematocrit and plasma erythropoietin with elevated levels present even at near-normal hematocrit values. Perhaps a closer relation with the oxygen carrying capacity of the blood should not be expected since the tissue availability of oxygen depends not only on the blood content, but also on the tissue blood flow. The latter is influenced by independent factors such as cardiac output and variations in the local vascular bed. In addition, if there is a relation between erythropoietin levels and marrow cellularity as a reflection of erythropoietin consumption by proliferating erythroid tissue as suggested by Stohlman, it is not apparent from our studies. This relationship requires further examination by measurement of plasma erythropoietin turnover before definitive conclusions can be reached.
Patients with polycythemia provide a second crucial category in which to examine the usefulness of erythropoietin assay. In hypoxic polycythemia one might anticipate that tissue oxygen deficits had led to increased release of erythropoietin with subsequent elevation of the oxygen-carrying capacity of the blood. The degree of this alteration might be modified by compensatory changes in cardiac output or local vascular beds. The end result of these compensatory mechanisms in many patients may be normal tissue oxygen concentration so that the change in erythropoietin levels may be only that which is required to maintain the elevated red cell mass. Indeed, of five patients with hypoxic polycythemia, only two demonstrated elevations of erythropoietin and in each the degree of polycythemia had been modified within the past six weeks by phlebotomy. Increase of plasma erythropoietin in hypoxic polycythemia may be detected only if the compensatory equilibrium has been modified as by phlebotomy or if the summation of these adaptive mechanisms has failed to restore normal tissue oxygen.

By contrast, patients with polycythemia vera failed to demonstrate abnormally increased levels of erythropoietin even in those patients who had received therapeutic phlebotomy. This is in agreement with Penington but is in
contradistinction to results of others reported previously. However, the lack of elevated erythropoietin is more consistent with the concept of polycythemia vera as a myeloproliferative disorder in which cellular multiplication is no longer under normal regulation. This is further supported by the evidence that administration of oxygen to patients with polycythemia vera fails to depress the plasma iron turnover in contrast to patients with hypoxic polycythemia.

Sufficient experience with erythropoietin assay has accumulated from many laboratories to now consider its potentialities as a clinical tool. In anemia uncomplicated by decreased metabolic activity, the normal response is an increased release of erythropoietin. The appearance of morphologically normal erythroid hyperplasia in the marrow is sound evidence of increased erythropoietin and may be considered as an in vivo assay. Since the normal response to anemia is elevated erythropoietin, we need not be concerned about measuring decreased levels in this situation, as the absence of an increased titer is abnormal. On the other hand, erythroid hypoplasia in the presence of anemia may be due either to lack of erythropoietin increases or to inability of the marrow to respond. In patients with refractory anemia, our observations and those from other laboratories indicate that erythropoietin stimulation is usually present. The few exceptions are of great theoretical interest, but useful clinical interpretation must await further information. In patients with endocrine abnormalities it seems probable the metabolic alterations or changes in tissue blood flow influence the normal erythropoietin response. Patients with polycythemia in a steady state are unassociated with detectable changes in erythropoietin titer from normal. These studies suggest that erythropoietin assay following phlebotomy may be useful in separating hypoxic polycythemia from polycythemia vera. From these observations it seems likely that far more definitive knowledge will be obtained not by more “sensitive” bioassay systems, but by manipulation of the patients’ hemoglobin level so as to bring out more clearly the functional abnormality.
REGULATION OF ERYTHROPOIESIS I

SUMMARY

An erythropoietin assay using hypertransfused rats and radioiron utilization has been evaluated in regard to modifying factors and its variability. It is proposed that the dose-response relationship may be regarded as an adsorptive process which suggests new investigative approaches in terms of hormone binding sites and erythropoietin inhibition.

Elevated levels of erythropoietin were found in 14 of 16 patients with refractory anemia indicating a basic defect in the response of the erythroid marrow. The two exceptions are of great theoretical interest, but insufficient information limits further conclusions. Patients with hypoxic polycythemia demonstrated increased levels only when their compensatory equilibrium had been modified by phlebotomy. Seven patients with polycythemia vera had erythropoietin levels within the normal range whether or not therapy had modified their red cell mass. This is further evidence that polycythemia vera is a proliferative disorder usurping the normal regulation. The clinical use of erythropoietin assay may provide the most information when correlated with alterations of oxygen-carrying capacity as a functional test of the patient's erythropoietic regulatory mechanism.

SUMMARIO IN INTERLINGUA

Un methodo pro le essayage de erythropoietina. basate in le utilisation de radioferro per hypertransfusionate rattos, esseva evalutate con respecto al factores capace a modificare su resultatos e con respecto al variabilitate de illo. Es proponite que le relation dose-responsa pote esser considerate como un processo de adsorption, lo que suggere nove tacticas de investigation concernite con le sitos de ligation hormonal e le inhibition de erythropoietina.

Nivellos elevate de erythropoietina esseva trovate in 14 de 16 patientes con anemia refractori, lo que indica un defecto basic in le responsa del medulla erythroide. Le duo exceptiones es de grande interesse theorico, sed insufficientia de informationes restringe le possibilitate de formular conclusiones plus definitive. Patientes con polycythemia hypoxic habeva nivellos elevate de erythropoietina solmente quando lor equilibrio compensatori habeva essite modificate per phlebotomia. Septe patientes con polycythemia ver habeva nivellos de erythropoietina intra le limites normal sin reguardo a si o non le therapia habeva modificate le massa de lor erythrocytos. Isto constitue evidentia additional que polycythemia ver es un disordine proliferative que usurpa le functiones del regulation normal. In uso clinic le essayage de erythropoietina pote esser le plus informative quando illo es correlatoate con alterationes del capacitate de portar oxygeno, como un test functional del mechanismo responsabile pro le regulation del erythropoiese del patiente.

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