Studies on Erythropoietin. I. Demonstration of Erythropoietin in Normal Plasma

By MORRIS REICHLIN* AND WILLIAM J. HARRINGTON

IT IS NOW generally accepted that erythropoiesis is in part regulated by humoral mechanisms.1-4 The term erythropoietin has been given to the proposed humoral factor; its chemical nature, physiologic effects and site of formation have been subjects of numerous investigations. Yet no convincing demonstration of its presence in normal plasma has been described. Accordingly, inferences regarding circulating levels of erythropoietin in disease can be made only when increases above normal values are obtained.

The present study was undertaken with the objectives of modifying assay conditions to permit better quantitation and, if possible, to allow measurement of decreases as well as increases in plasma erythropoietin relative to the normal. Both objectives were achieved, and substantial concentrations of the hormone were demonstrated in normal rat plasma.

The technic employed was a modification of that devised by Jacobson and his co-workers.5 Use was made of the well established principle that increased sensitivity to an administered hormone can be induced by suppression of formation of its endogenous counterpart. Rats rendered polycythemic by a single intravenous transfusion of homologous red blood cells were given three successive daily injections of the plasma to be assayed. The degree of erythropoietic response was expressed in terms of per cent incorporation into circulating hemoglobin of a tracer dose of radioactive iron. It was assumed that the transfusion of erythrocytes temporarily abolished the stimulus for red blood cell production in the recipient animal and therefore specifically depressed its elaboration of endogenous erythropoietin. If the premise was correct, then the extent to which the test plasma restored radioiron utilization should have paralleled its erythropoietin content. The data supported the validity of this premise.

MATERIALS AND METHODS

Female Sprague-Dawley rats† were used in all studies. Donors 300 Gm. in weight were bled by cardiac puncture into siliconized syringes which contained 0.2 mg. of heparin. A volume equal to 2.5 per cent of the body weight was withdrawn. The packed cell volume of pooled samples was adjusted to 70 per cent by removal of supernatant plasma following centrifugation in the cold for 10 minutes at 300 X g. Four ml. of the concentrated erythrocyte preparation were transfused with a 25 gage needle into tail veins of recipients weighing 200 Gm.

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Table 1.—Red Cell Mass and Blood Volume Determinations

<table>
<thead>
<tr>
<th>Red cell mass (mL)</th>
<th>Body wt. (Gm.)</th>
<th>Red cell mass/ml. 100 Gm. rat</th>
<th>Blood volume (% body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.33</td>
<td>204</td>
<td>2.12</td>
<td>4.71</td>
</tr>
<tr>
<td>4.33</td>
<td>205</td>
<td>2.11</td>
<td>4.69</td>
</tr>
<tr>
<td>5.43</td>
<td>241</td>
<td>2.26</td>
<td>5.01</td>
</tr>
<tr>
<td>5.75</td>
<td>257</td>
<td>2.24</td>
<td>4.76</td>
</tr>
<tr>
<td>5.66</td>
<td>258</td>
<td>2.19</td>
<td>4.88</td>
</tr>
<tr>
<td>5.40</td>
<td>228</td>
<td>2.37</td>
<td>5.26</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.21</td>
<td>4.89</td>
</tr>
</tbody>
</table>

Measurements of red cell mass and blood volume in normal rats yielded data in agreement with those of other investigators.

Table 2.—Red Cell Mass of Rats at Time of Assay

<table>
<thead>
<tr>
<th>Calculated mL/100 Gm.</th>
<th>Observed mL/100 Gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.91</td>
<td>3.56</td>
</tr>
<tr>
<td>3.84</td>
<td>3.61</td>
</tr>
<tr>
<td>3.54</td>
<td>3.55</td>
</tr>
<tr>
<td>3.33</td>
<td>3.55</td>
</tr>
</tbody>
</table>

Good agreement was found between calculated and observed values for expansion of red cell mass, 4 days after transfusion of homologous erythrocytes.

Packed cell volumes of normal rats ranged from 47 to 49 per cent; those in recipient animals were consistently 59 ± 2 per cent one day after transfusion and 57 ± 3 per cent four days later. Red cell mass was also determined in a group of rats before and at intervals following transfusion. Dilution of red blood cells labeled with Fe59 was employed. The observed value in normal animals of 2.21 mL/100 Gm. was similar to those reported by other investigators (table 1); furthermore, good agreement was found between predicted and observed values following transfusion (table 2). These data were required in order to calculate the per cent of the tracer dose of Fe59 incorporated into the circulating erythrocyte mass during the actual assay for erythropoietin and in addition were desired in order to demonstrate conclusively that the injected cells used to render the recipients polycythemic did recirculate in a predictable pattern throughout the time period of the study.

For the determination of radioiron incorporation, 0.5 μc. of Fe59* was injected intravenously, and blood samples were obtained thereafter as described below. Their radioactivities were assayed in a well-type gamma scintillation detector and the per cent of the dose of Fe59 present in circulating erythrocytes was calculated as follows:

\[
\% \text{RI} = \left( \frac{\text{cpm mL WB.}}{\% \, \text{PCV}} \right) \left( \frac{\text{B. Wt.}}{100} \times 2.21 \right) + 2.80
\]

where 2.21 is the mean red cell mass in mL/100 Gm. of normal rat (table 1), 2.80 is the volume in ml. of transfused erythrocytes (4.0 ml. × 70 per cent, RI = radioiron incorporation, cpm = counts per minute, WB. = whole blood, and B. Wt. = body weight in Gm.

At the outset studies were designed to define the degree and rate of suppression of iron incorporation consequent to the transfusion of erythrocytes. Rats were given an intra-

*Ferrous sulfate: S.A. 3-7 mc. mg., Abbott Laboratories, North Chicago, Ill.
†Nancy Wood Counterlab, Chicago, Ill.
Table 3.—Effects of Test Materials on Per Cent of Dose of Fe$^{59}$ Incorporated

<table>
<thead>
<tr>
<th>Isotonic saline*</th>
<th>Normal plasma</th>
<th>4 day post-transfusion plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.35</td>
<td>9.80</td>
<td>7.95</td>
</tr>
<tr>
<td>4.50</td>
<td>10.10</td>
<td>4.61</td>
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<tr>
<td>5.00</td>
<td>7.92</td>
<td>4.78</td>
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<td>5.13</td>
<td>9.60</td>
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<td>5.37</td>
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<td>8.32</td>
<td>5.07</td>
</tr>
<tr>
<td>3.45</td>
<td>8.46</td>
<td></td>
</tr>
<tr>
<td>3.60</td>
<td>8.40</td>
<td></td>
</tr>
<tr>
<td>3.72</td>
<td>13.02</td>
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</tr>
<tr>
<td>5.75</td>
<td>12.00</td>
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<td>4.75</td>
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<td></td>
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<tr>
<td>5.45</td>
<td>10.20</td>
<td></td>
</tr>
<tr>
<td>4.35</td>
<td>10.30</td>
<td></td>
</tr>
<tr>
<td>7.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{x}$ = 4.56</td>
<td>10.03</td>
<td>5.06</td>
</tr>
<tr>
<td>$s$ = 1.27</td>
<td>1.50</td>
<td>1.26</td>
</tr>
</tbody>
</table>

$*0.03$ mgm. heparin/ml.

All materials given i.v. in volumes of 4 ml./day for 3 successive days. Analysis of data revealed that normal plasma induced significantly greater radioiron incorporation into circulating erythrocytes than did saline or plasma obtained from rats hypertransfused 4 days previously. No significant difference was noted between the effects of isotonic saline and plasmas from rats hypertransfused 4 days previously. At least 4 different lots of test material were assayed in each group.

Venous injection of 0.5 $\mu$C. of Fe$^{59}$ either before or at various times after transfusion. Depending on whether single or multiple determinations were to be made, either 5 ml. of blood were withdrawn from the heart or 0.02 ml. from the tail. The radioactivity was determined in 2 ml. of the larger sample or in the entire volume of the smaller sample (added to 2.0 ml. of distilled water). In addition, packed cell volumes were measured by a micro-hematocrit technic. The results of these studies are given in figures 1 and 2.

A protocol was thereupon developed for assays of erythropoietin activity in test plasmas. Sterile normal plasma was obtained from rats bled a volume equal to 2.5 per cent of their body weights; the blood was centrifuged at 800 $\times$ g. for 20 minutes at 5 C. and the plasma separated and frozen. Twenty-four hours later plasma was harvested from the same animals, now profoundly anemic, and stored at $-10$ C. On the first thawing of the plasmas a precipitate of altered fibrinogen was repeatedly noted; it was removed by low speed centrifugation at 5 C. In addition, neutral fat which accumulated on the surface was discarded. Thereafter freezing and thawing could be carried out without further loss and the plasmas were used directly for assay.

The intravenous route of administration of all materials was found to be the most satisfactory. A tail vein was employed, and particular attention was given to the prevention of blood loss. For optimal results the test plasmas had to be given daily starting one day after the erythrocyte transfusion. If administration of the test plasma was delayed for four days, whereupon iron incorporation was minimal (figs. 1 and 2), the impaired utilization of the isotope was only moderately affected. Plasma given in a single dose immediately after transfusion also produced only a moderate hematopoietic response. But if the total volume was apportioned into three equal doses given on three successive
DEMONSTRATION OF ERYTHROPOIETIN IN NORMAL PLASMA

Accordingly, for assay of test materials the total volume was divided into three equal doses; and these fractional amounts were given at 24, 48 and 72 hours following transfusion. One day after the final infusion of plasma 0.5 μc. of Fe²⁹ was injected intravenously. On the next day, five days after the transfusion of red blood cells and 24 hours after administration of the tracer dose of radioiron, exactly 5 ml. of blood were withdrawn by cardiac puncture into a syringe containing 0.2 mg. of heparin. The radioactivity in 2.0 ml. of whole blood was assayed, a packed cell volume was determined on the remainder of the sample and the per cent incorporation of the isotope into the circulating red cell mass was calculated. An occasional sample was found to have a packed cell volume less than 53 per cent and data on it were discarded.

RESULTS

In the preliminary studies it was demonstrated that the transfused cells survived normally in the hypertransfused animal, at least for the duration of the experimental period of five days. The value for red cell mass in normal control rats is given in table 1: 2.21 ml./100 Gm. is similar to results reported by other investigators; and good agreement is evident in table 2 between observed and expected values on the fourth day after transfusion, the day when the radioiron was injected in the assay of test plasmas.

In the development of the assay, observations were made on the pattern of radioiron incorporation into circulating blood in normal animals and in rats given the isotope at intervals after the transfusion. In all groups there was an initial interval of rapidly decreasing radioactivity as the tracer dose of Fe²⁹ was cleared from the plasma. Shortly thereafter radioiron again appeared in the blood, but now it was in the red cells. The slope of increment in isotope concentration in the circulating cell mass was found to be approximately linear for 30 hours and thereafter to taper and approach a plateau at 72 hours. At 24 hours normal animals incorporated 19 ± 3 per cent of the radioiron into their circulating erythrocytes and after three days a value of 40 ± 5 per cent was obtained (fig. 1).

In the groups of hypertransfused rats iron utilization declined as illustrated in figure 1. Little if any difference was noted between the pattern obtained with normal rats and that observed in rats transfused 24 hours previously. Thereafter, however, the rate of uptake declined until the fourth day after transfusion whereupon a "basal" level was reached which persisted for at least 48 hours. The actual incorporation of radioiron during this "basal" period approximated 4 per cent of the administered dose of the isotope; similar values have been obtained by others when either hypertransfused or starved animals were tested.

In figure 2 are plotted data selected from figure 1. Only the one day values of individual curves were transposed because they should have fallen on the linear portion of the curve of iron incorporation, and accordingly assays made at this interval could be expected to reflect the slope and hence the rate of the process. The decline in iron incorporation proved to be exponential when the data were transposed from figure 1 on a log y = x coordinate system. It may be inferred, therefore, that iron incorporation decreased at a constant
Fig. 1.—Observations on radioiron incorporation into circulating erythrocytes. Numbers of rats employed are given in parentheses. Note that 24 hours after hypertransfusion Fe\textsuperscript{59} incorporation remained normal, but when the isotope was administered after a 48, 72, 96 or 120 hour lag, there was a well defined suppression of uptake into the peripheral blood.

percentage of the rate present at any time $t$, once the stimulus to erythropoiesis was removed. The cell believed to be most subject to stimulation by erythropoietin is the pronormoblast,\textsuperscript{7} and it may be that these cells were most affected. The fact that depression of iron uptake in circulating red cells was delayed 24 hours after transfusion may have a corollary in other recent observations,\textsuperscript{7} wherein it was noted that reticulocytes did not appear in the peripheral circulation for 24 hours following the onset of a protracted anoxic stress. It would seem that removal or application of the anoxic stimulus to the bone marrow may not be manifest in the peripheral blood cells for 24 hours, since erythropoietin most probably acts at the precursor level.

Assays on Plasmas from Normal, Anemic and Hypertransfused Rats

With “anemic” plasmas, it was possible to obtain a five-fold stimulation of iron uptake over that observed in saline-treated animals. The maximal response was obtained if 2 ml./per day was administered at 24, 48 and 72 hours, a total of 6 ml./200 Gm. of body weight. The response could not be increased by doubling the dose of the “anemic” plasma; it appeared probable, therefore, that there was a definable limitation on the potential for red cell production in these animals.
Fig. 2.—Data from figure 1, plotted as log per cent uptake at 24 hours, of the values found when radioiron was given at daily intervals after hypertransfusion. It is suggested that radioiron incorporation disappears exponentially as the stimulus for erythropoietin formation is removed.

The relationship between per cent of dose of Fe$^{59}$ incorporated and the dose of plasma administered appeared to be linear (fig. 3), thereby permitting an appreciable degree of quantitation. The technic proved to be capable of detecting variations of 1.5 ml. in total dosage of "anemic" plasma. The difference in response of a hypertransfused animal to 0, 1½, 3, 4½ and 6 ml. of "anemic" plasma administered over a 3 day period could be ascertained with a high degree of confidence; an increase in iron uptake of approximately 4 per cent accompanied each 1.5 ml. increment in volume of plasma tested.

Since quantitation of erythropoietin in "anemic" plasma was possible, within certain limits, it was hoped that even small amounts of circulating erythropoietin in normal animals might be detected if enough plasma were given. The results are summarized in table 3. Sixteen animals were given 4 ml. of saline daily for 3 days; an average iron incorporation of 4.56 per cent was observed, with a standard deviation of 1.27 per cent. However, 15 recipients given 4 ml. of normal plasma daily for 3 days had an average Fe$^{59}$ uptake of 10.03 per cent, with a standard deviation of 1.50 per cent. The difference between the two groups is highly significant, the $t$ value being equal to 10.94, and $p$ less than 0.01.

A simple calculation revealed that normal plasma contained approximately
DOSE RESPONSE ANALYSIS

Fig. 3.—A dose-response analysis on the effects of plasma from anemic rats revealed that for the particular preparation and plasma employed, 2 ml./day for 3 successive days (a total of 6 ml.), produced a maximal response. Smaller volumes induced an uptake of isotope intermediate between that of saline and 6 ml. of “anemic” plasma, whereas larger volumes failed to augment the response to 6 ml. of “anemic” plasma.

25 per cent of the activity noted in “anemic” plasma, in that a dose of 12 ml. of normal plasma induced an increase in radioiron incorporation equivalent to that obtained with 3 ml. of “anemic” plasma (fig. 3).

In order to prove that the response to normal plasma was specific, plasma was obtained from hypertransfused donors. On theoretical grounds this plasma should contain less erythropoietin than is present in normal plasma. The results are summarized in table 3. Plasma obtained four days after transfusion was inactive. It would seem, therefore, that the highly significant response to normal plasma was due specifically to its content of erythropoietin and that plasma obtained four days after transfusion contained virtually no erythropoietin.

DISCUSSION

Several bioassays have been devised for the measurement of erythropoietin. The objective sought with most existing assay animals is to depress their erythropoiesis and thereafter to restore it toward normal by injections of plasma presumed to contain an excess of this erythropoietic factor. However, too often the test animals have been subjected not only to depressed erythropoiesis but also to alterations along other broad physiologic parameters.

A bioassay animal that is unaltered physiologically is the normal rabbit.
However this animal responds only to “anemic” plasma and is insensitive to the small amounts of erythropoietin in normal plasma.

Three common ways of preparing rats are hypophysectomy, starvation and sublethal irradiation. All of these animals are subjected to severe physiologic handicaps. The first two methods are believed to induce depression of erythropoiesis because of a discrepancy between circulating red cell mass and tissue requirements for oxygen. Hence, there exists a relative polycythemia and red cell production ceases until a new equilibrium is achieved between circulating red cell mass and tissue oxygen requirements. In addition, though, to hematopoietic responses to erythropoietin, the hypophysectomized or starved animal may respond to other plasma constituents which tend to replace the deficiency created by the hypophysectomy or starvation. Unknown degrees of nonspecificity are thereby inherent in the response. That nonspecificity does not confer sensitivity is attested to by the fact that normal homologous unconcentrated plasmas do not contain erythropoietin as measured in either of these animals. The marrow depression in sublethal irradiation is probably due to direct damage to dividing red cell precursors; the basis of response in these animals to anemic plasma is not clear. In each instance insensitivity is a major shortcoming. Nevertheless, in all three assay animals red cell production is depressed and reticulocytes and iron incorporation fall to low basal levels from which erythropoiesis can be restored to normal or near normal levels by the injection of erythropoietically active plasmas from anemic donors.

The technic employed herein is a modification of a method previously described wherein polycythemic mice and rats were found to be sensitive recipients for erythropoietin. The earlier assays, however, involved repeated intraperitoneal injections of washed red cells, until a packed cell volume of 70 per cent was achieved in the recipient animals. The procedure was more time consuming and may have been less sensitive than the method presented in this communication. Nevertheless, its advantages were clear; it possessed all of the desirable characteristics claimed for other technics coupled with a probable high degree of specificity.

Does the assay described measure the apparent specific effect of the anoxic stimulus, the differentiation of stem cells to pronormoblasts, or does it also measure other effects on more mature red cell precursors? Although a categorical answer is not possible, the fact that plasma from normal donors affects iron incorporation and that from hypertransfused animals does not would suggest that the effect is not due to any other hormonal or nutritional factor but is in fact a reflection of erythropoietin activity.

Summary

1. A modification of a technic wherein hypertransfused rats are used for the bioassay of erythropoietin is described, and some kinetics of iron incorporation following transfusion are presented.
2. It was possible to detect activity in normal plasma and demonstrate that plasma of animals whose erythropoiesis is depressed below normal by hypertransfusion possesses less erythropoietin than normal plasma.
3. The variation of response under standardized conditions is sufficiently
small and sufficiently predictable so that within certain limits, quantitation is possible. A dose-response analysis is presented.

4. Plasma from normal rats possesses approximately 25 per cent of the activity found in rats rendered acutely anemic by a standardized blood loss.

**SUMMARY IN INTERLINGUA**

1. Es describite un modificate technica pro le bioessayage de erythropoietina, utilisante rattos in stato de hypertransfusion. Certe aspectos del cinetica del incorporation de ferro post le transfusion es presentate.

2. Esseva possibile deteger activitate in plasma normal e demonstrar que le plasma de animales in que le erythropoiese es deprimite per hypertransfusion usque a nivellos infranormal possede minus erythropoietina que plasma normal.

3. Le variation del responsas, sub conditiones standardisate, es sufficientlye micre e sufficientemente predicibile pro render quantitation possible, al minus intra certe limites. Un analyse del relation inter dose e responsa es presentate.

4. Le plasma de rattos normal possede approximativemente 25 pro cento del activitate trovate in rattos que es acutemente anemic como effecto de un standardisate perdita de peso.

**REFERENCES**


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