The Measurement of Erythropoietin

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ABSTRACT

The bioassay of erythropoietin in animals is reviewed. The techniques employed are those of hypertransfusion and hypoxia using polycythemic mice.

Introduction

Erythropoietin can be measured by in vivo or in vitro methods. In vivo assays are performed in erythropoietically suppressed animals in which the effects of injected erythropoietin can be followed by measuring a variety of parameters, e.g. the increase in reticulocytes, in hemoglobin or hematocrit, in marrow erythroblasts, in iron turnover rate or in red blood cell ⁵⁹Fe incorporation.²,¹³ The most widely used methods today depend on ⁵⁹Fe incorporation and the mouse [or the rat] is employed as the test animal.

In vitro assays are more diversified; they include such techniques as marrow tissue culture, inhibition of hemagglutination, immunoochemical double diffusion, and radioimmune assays.² The results are about parallel to those of in vivo assays but in general the techniques are less developed and still less accurate than the in vivo bioassays.⁵ Tissue culture techniques depend upon the measurement of the rate of ¹⁴C glucosamine or ⁵⁹Fe incorporation into newly formed erythroblasts in bone marrow cell culture.⁴,⁸,¹²,¹⁸ Tissue culture techniques may be as sensitive as in vivo assays but are not well adapted for the assay of plasmas with low concentrations of erythropoietin because of the large sample volumes required which introduce non-specific effects owing to protein contamination and are better suited for the measurement of purified erythropoietin. Immunoochemical assays are extremely sensitive; however, they have been difficult to standardize and compare with in vivo assays. The success of these techniques ultimately is dependent upon the availability of a purified antibody to erythropoietin.¹⁴ Purified erythropoietin antigen with a specific activity close to 10,000 units per mg has been prepared¹¹ in extremely small amounts and is not generally available for the preparation of antisera. The radioimmune assay has an additional problem, that of iodinating erythropoietin without causing a loss of biologic identity by denaturation. Nevertheless, it is the most promising technique and it undoubtedly, when first perfected, will be preferred to other immunochemical types of assay.¹⁰

The procedures described are bioassays for erythropoietin in the polycythemic mouse in which erythropoiesis is depressed
by hypertransfusion with mouse erythrocytes or by hypoxia. The hypertransfusion technique with its extremely low baseline is the preferred one for exact measurement of erythropoietin, while the hypoxia technique is preferred for routine screening tests.

**Principle**

The measurement of erythropoietin is based on the increase in the rate of red cell production produced by the injected hormone in erythropoietically depressed mice. The red cell production is estimated from the amount of injected $^{59}$Fe which becomes incorporated into red cells and this amount is proportional to the dose of injected erythropoietin. The endogenous erythropoietin production in the mouse almost ceases when the demand for tissue oxygen is satisfied by polycythemia induced by hypertransfusion or hypoxia. At that point the injection of small doses of erythropoietin will cause an easily measurable increase in the rate of red cell production. If $^{59}$Fe is injected one to two days after erythropoietin at a time when normoblasts reach their peak concentration in bone marrow, it will be incorporated into hemoglobin and the radioiron so utilized will appear in the fully developed red cells subsequently released from the bone marrow into the circulation. Maximum $^{59}$Fe incorporation takes place in a few days after the injection of radioiron and the increased utilization or incorporation is calculated from the measurement of radioactivity in the circulating red blood cells. A standard curve relating units to radioactivity is essential for estimating the activity of the test sample. Different laboratories have adopted various minor modifications of the controllable parameters of the assay. The bioassays used in our laboratory are modifications of that of Rosse and Waldman and of Coates.

**Animals**

Virgin female mice of the ICR (International Cancer Research) or Swiss-Webster strain, weighing between 22 and 25 grams are maintained on Tecklad mouse/rat diet. Water is provided ad libitum. It is necessary to acclimatize the mice for a period of at least one week in order to stabilize the hematocrit. CF-1, B6D2F and other strains of mice have been used in different laboratories.

**Reagents**

**Reference Standard**

The Reference Standard is a human urinary erythropoietin, Standard B. The earliest standard unit was the Cobalt Unit which was the erythropoietic response to 5$\mu$ M $\text{CoCl}_2\cdot6\text{H}_2\text{O}$ in the fasted rat assay. The first International Standard (A) was a step 4 anemic sheep plasma erythropoietin (23 units per mg protein) which was equal to the erythropoietic response of one Cobalt Unit. The current erythropoietin standard (a human urinary preparation) was set up as International Reference Standard B of which 1.48 mg is equivalent to 1 unit of Standard A.

**Working Standard**

This can be prepared from human urinary erythropoietin supplied by the National Heart and Lung Institute Committee on Erythropoietin. This erythropoietin was procured from the Department of Physiology, University of the Northeast, Corrientes, Argentina, and it is processed by the Hematology Research Laboratory, Children's Hospital of Los Angeles, Los Angeles, CA for distribution by the NHLI Committee on Erythropoietin under research grant HE 10880.
standards also can be prepared from an active specimen of anemic plasma or from anemic urine, preferably in a partially purified form. Anemic plasma can be processed through Step III of Goldwasser.\textsuperscript{20} Urine can be processed through steps 1 or 2 of Lowy and Borsook.\textsuperscript{15}

**Chemicals**

\[^{59}\text{FeCl}_3\] is obtained\textsuperscript{¶} and a solution is prepared containing 0.5 $\mu$Ci in 0.9 percent sterile sodium chloride solution. Carbowax 6000 is needed.\textsuperscript{||}

**Packed Red Blood Cells For Rendering Mice Polycythemic**

These are obtained by bleeding large normal donor female mice. The mice are lightly anesthetized with ether in a jar and their carotid arteries are severed with a scalpel. The blood is collected in a beaker containing anticoagulant and antibiotics (125 ml blood plus 5 ml saline containing 1000 units heparin, 20,000 units penicillin and 20 mg streptomycin). The blood is centrifuged\textsuperscript{*} at 2900 rpm for 30 minutes and the packed cells are washed twice with saline. The hematocrit of the washed cell suspension is adjusted to 85 percent and cells are stored at 4° for approximately 24 hours.

**Methods**

**Hematocrit Determinations**

These are obtained in duplicate by a microhematocrit method. The hematocrit is determined when the mice are sacrificed; all results from mice with a hematocrit of less than 55 percent are eliminated.

\textsuperscript{¶}Abbott Laboratories, Oakridge, TN.

\textsuperscript{||}It may be obtained from the J. T. Baker Co., Phillipsburg, NJ 08865 or from Phillips and Jacobs, Inc., Philadelphia, PA 19123. It is manufactured by Union Carbide Corp., New York, NY 10017.

\textsuperscript{*}PR-1 Refrigerated International Centrifuge.

**Radioactivity**

This is determined with a gamma scintillation counter\textsuperscript{†} and expressed as counts per minute (CPM) per ml of blood.

**Preparation of Sample**

Plasma or serum is assayed as such. Urine, however, must be concentrated by dialysis-concentration with Carbowax. Three hundred ml of urine in a cellophane dialysis bag is placed in a beaker, and covered with an excess of Carbowax-6000. The beaker is held at refrigerator temperature until the urine volume is reduced 50-fold. Carbowax may contaminate the sample; however, it is not necessary to remove it by dialysis, as it does not interfere with the test.

**Special Apparatus**

**Hypoxic Chamber**

A steel enclosed cylindrical decompression chamber with a capacity of 18 cubic feet is modified to provide a hypobaric enclosure. A Welch Duo-Seal Vacuum Pump\textsuperscript{‡} provides partial vacuum, and a self-regulating intake valve which opens to normal atmospheric pressure allows an air-flow of 48 liters per minute at 0.4 atmospheric pressure. A safety pressure relief valve, two manually operated regulating valves and an electric light (40 watts) with a switch complete the assembly. A thermometer is enclosed to read temperature. The chamber is maintained at room temperature (76°F) in an air conditioned room. The chamber will accommodate approximately 60 mice under healthful conditions, and it should not be overloaded with a greater number of mice.

**Procedure for Use with Hypertransfusion Polycythemia**

Day 0 Mice (minimum of four) are injected intraperitoneally (i.p.) with 0.9 ml packed cells.

\textsuperscript{†}Nuclear, Chicago, IL.

\textsuperscript{‡}Model 1405, Sargent-Welch Scientific Co.
Day 1  A second injection of 0.9 ml packed cells is made.

Day 6  A test sample of 0.5 to 1.0 ml is injected subcutaneously (s.c.) into the test animals and a similar volume of sterile saline solution into the control animals. A zig-zag injection technique is used to prevent seepage loss.

Day 7  The s.c. injection of 0.5 to 1.0 ml of test sample is repeated as on Day 6.

Day 8  Precisely 0.2 ml saline solution containing 0.5 $\mu$Ci $^{59}$Fe is infused i.p. The long s.c. approach is used before entering the peritoneal cavity.

Day 11 The mice are lightly anesthetized with ether and weighed. Blood samples are obtained by severing the carotid arteries with a scalpel; the blood is allowed to collect on a square of Parafilm without anticoagulant. Exactly 0.2 ml of blood is quickly transferred to 2.0 ml of saline, using a “to contain” pipet, and mixed thoroughly by drawing in and discharging the diluted blood sample several times. This mixture is used to determine $^{59}$Fe concentration as cpm per ml of blood.

A second sample of blood is drawn into a capillary tube for the determination of the microhematocrit.

Procedure for Use with Hypoxic Polycythemia

Day 0  Each mouse is injected with 1 mg of iron dextran (Imferon) intramuscularly (i.m.). The mice are placed in cages in the low pressure chamber at 0.4 atmosphere for 16 hours each day, including weekends. Feed and fresh water are provided, and the cages and chamber are cleaned each day.

Day 14 The animals are removed from the chamber.

Day 19 Precisely 0.5 ml of test sample is injected s.c. into the test group and 0.5 ml of 0.9 percent sterile saline into the control group. A zig-zag technique is used.

Day 20 The test sample and sterile saline are injected as on Day 19.

Day 21 Exactly 0.5 $\mu$Ci $^{59}$Fe in 0.2 ml of sterile saline solution is injected i.p. (long s.c. approach is used).

Day 24 The mice are weighed and sacrificed and the hematocrit and radioactivity are determined according to directions described for Day 11 of the hypertransfused mouse procedure.

Calculations

The mice are sacrificed on the last day of each procedure so as to obtain a 66-hour utilization of $^{59}$Fe. A blood volume of 7 percent is assumed and all samples from animals with hematocrits of less than 55 percent are discarded. The 66-hour utilization is calculated thus:

$$\%^{59}\text{Fe utilization} = \frac{\text{cpm/ml blood} \times \text{blood volume in ml}}{\text{total cpm of injected }^{59}\text{Fe}} \times 100$$

Conversion of Percent $^{59}$Fe Utilization to Erythropoietin Units

Erythropoietin units in the test sample are routinely estimated from a log-log plot of the percent $^{59}$Fe utilization of a standard reference erythropoietin in the assumed reliable range of 0.05 to 1.0 units per ml of the standard (figure 1). A large number of mice (>25 for each dose) are required for the construction of a reliable standard curve.
Reproducibility

$^{59}$Fe utilizations obtained by assays for one unit of Standard B Erythropoietin are shown in Table I.

Sources of Error

The response of mice to the hormone is the greatest source of variability and of error. A minimum of four or five mice is essential per assay but the assay may need to be repeated to obtain reproducible data.

Loss or seepage of the subcutaneously injected samples, and loss or seepage of the intraperitoneally injected $^{59}$Fe often causes erroneously low values. $^{59}$Fe injection into the intestinal cavity may result in zero results.

Mice with low hematocrits (<55 percent) must be excluded from the calculation of percent $^{59}$Fe utilization since they are not sufficiently polycythemic to respond to the hormone.

Units of erythropoietin cannot be estimated from the ends of the standard curve and the curves are not interchangeable for the two assay procedures.

Control saline solution must be tested to determine that the mice are not responding to non-specific stimuli. Controls usually give estimates of <0.1 percent $^{59}$Fe utilization.

Occasionally, for unknown reasons, a group of mice will not perform properly in the assay. To be aware of this occurrence, a solution containing one unit of standard (secondary or working) erythropoietin is included in each group of assays to ascertain that the specifications of the bioassay meet those of the reference standard assays. It is not practical to run a standard curve daily, although the occasional inclusion of a range of erythropoietin concentrations is good practice since it extends the meaningfulness of the standard curve by increasing the number of observations on which it is based.

Clinical Interpretation

With a standard curve, the concentration of erythropoietin in plasma or in a 24-hour urine collection can be estimated, provided the sample contains more than 0.05 units erythropoietin per ml. The plasma and urinary erythropoietin concentrations of normal individuals are too low to be measured by this technique or by other techniques described in the literature.

Physiologically, the body responds to anemia or hypoxic hypoxia by the production of erythropoietin, and elevated erythropoietin concentrations are found in patients with anemia or secondary polycythemias. The relationship between serum erythropoietin levels and hemoglobin concentrations in patients with anemias is de-
picted (figure 2). Unfortunately, the assay of serum erythropoietin suffers from an inability to detect the slight changes in erythropoietin concentration found in mild or moderate anemias or in mild or moderate secondary polycythemias. The relationship between urinary erythropoietin and hemoglobin concentration is depicted in figure 3 and illustrates that the assay of urinary erythropoietin is better able to detect mild or moderate anemias.

Anemia and polycythemia are not always characterized by increases in erythropoietin. Polycythemia vera does not have increased levels of erythropoietin, and the anemia of chronic renal disease and possibly the anemia of chronic disease rarely exhibit measurable levels in plasma or urine. On the other hand, inappropriate secretion of erythropoietin may occur in patients with hydronephrosis, renal cysts or tumors and in tumors in a variety of other organs.

Discussion

The bioassay of erythropoietin provides a crude estimate of the concentration of this hormone. Variability in response of the animals is large so that a large number of animals must be included in an assay to obtain a reasonably correct measurement of erythropoietic activity. Although there obviously is much room for improvement, the bioassay has practical diagnostic value in its present form. For the assay to have merit as a tool for experimental investigations, many groups of four or five animals each must be used, preferably on different days, and the assay becomes cumbersome. Hypertransfused mice have a lower baseline than ex-hypoxic mice and the presence or absence of small concentrations of erythropoietin can be demonstrated more accurately; however, the hypoxic assay is less expensive and more convenient.

References