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A Modified Erythropoietin Bioassay Using Protein Deprived Mice*

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A humoral factor regulating the production of erythrocytes (erythrocyte stimulating factor, ESF or erythropoietin) has been described since the turn of the century.[1] The subject has been reviewed by Gordon,[2] Gurney,[3] and recently by Krantz and Jacobson.[4]

The study of erythropoietin requires adequate assay methods. Such methods are still the subject of discussion, and a simple, fast reasonably reliable method, especially suitable for clinical use has not been described. In vivo assay methods now depend in large measure on increased Fe59 incorporation into newly formed red blood cells after administration of the test material to the assay animal.

Although the plethoric rat and especially the mouse are the assay animals generally used in highly refined work, choice of the assay animal is still a matter for debate.[5-7]

Plethora production by hypertransfusion of red blood cells or exposure to an hypoxic environment are tedious and time consuming. [8 9] Although the plethoric animal is believed to be very sensitive to exogenous erythropoietin, production of plethora is tedious and stressful to the animal. Such an animal is of limited value in clinical medicine. On exposure of animals to hypoxia, food consumption is decreased and its utilization disturbed. A diet rich in protein under these circumstances is actually harmful.[10 11] Repeated transfusions, are not without their undesirable side effects.[12] The polycythemic animal, per se, is still being studied.[13]

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Hypophysectomized and starved animals have also been employed. The objection to their use has been their sensitivity to plasma factors unrelated to the amount of erythropoietin present.[5] Adamson and his coworkers advocate a combination of fasting plus hypoxic polycythemia.[14]

In vitro assay methods have not been refined to the point where they can be routinely used to test for erythropoietin.[5]

It appears that a quick, simple and practical assay method reasonably sensitive to erythropoietin levels, is needed.

The purpose of this investigation is to explore the usefulness of protein deprived CFI adult female mice, in detecting plasma erythropoietin levels.

The first part of the study deals with the effect of various periods of protein deprivation, starvation and plethora in reducing endogenous erythropoietin production, to achieve optimum sensitization to exogenous erythropoietin. Following this, the sensitivity of these animals to various doses of erythropoietin is presented. Finally, the sensitivity of protein deprived mice to exogenous erythropoietin in the presence of non–specific plasma factors in the plasma being assayed is demonstrated by studying the effects of ESF antibodies on erythropoiesis of mice injected with ESF, or plasma.

Materials and Methods

Adult CFI female mice* weighing approximately 20-25 grams were used as assay animals.

The effectiveness of various dietary regimens and of hypoxia induced polycythemia in reducing endogenous erythropoietin production and thus increasing the sensitivity of the mice constituted the initiated study. Groups of 6 mice were placed on a protein free diet** for three, four or seven days or starved for four days following which they received their regular diet.*** Mice maintained on regular diet only served as controls (Table I). On day four and five they received daily injections of 0.5 milliliters, intraperitoneally (i.p.), of physiological saline, donor plasma or other test substances. Radioactive ferrous citrate**** (Fe59), 0.5 microcuries in 0.5 ml of saline, was injected i.p. into each mouse on the 6th day. 48 hours later, animals were briefly anesthetized

* Carworth Farm, New York City, New York.

** Nutritional Biochemical Corporation, Cleveland, Ohio.

*** Purina Lab Chow, Ralston Purina Co., Checkerboard Square, St. Louis, Missouri.

**** Ferrutope (Fe59), Mallinckroft Nuclear, St. Louis, Missouri.

TABLE I

EFFECT OF VARIOUS DIETARY REGIMENS AND HYPOXIA ON MICE

	Mean Weight - Gm.	Mean Hematocrit %	% Fe59 Incorporation Into Rbc
Regular Diet	22* ± 1.5**	36.9* ± 3**	16.8* ± 2.5**
Protein-Free Diet (3 Days)	23 ± 0.9	37.5 ± 2	7.0 ± 4.3
Protein-Free Diet (4 Days)	22 ± 1.1	36.7 ± 2	2.6 ± 2.2
Protein-Free Diet (7 Days)	16 ± 0.4	54 ± 1.4	2.0 ± 1.4
Hypoxia (3 Weeks)	17.5 ± 0.5	65 ± 1	0.5 ± 0.3
Starvation (4 Days)	22 ± 2	40.7 ± 2.4	1.5 ± 0.6

* Average of 4-6 assays
** Standard Deviation of the mean.

Demonstrates the effect of various dietary regimes and hypoxic plethora on erythropoietic activity of CFI adult female mice.

with ether and sacrificed by exanguination. The radioactivity of 0.5 ml of whole blood was then determined in a well type scintillation counter. Erythropoietic activity (therefore the level of erythropoietin) was expressed as per cent incorporation of injected Fe59 (standard solution) into circulating red blood cells of the mice in 48 hours using the following equation:

$$\% \text{ Fe59 incorporation} = \frac{\text{Total radioactivity in circulating blood} \times 100}{\text{Radioactivity of injected Fe59}}$$

Total circulating blood of the mouse on 4 days protein deprivation diet was calculated to be 5.5% of the body weight, based on determinations made by exanguination.

Hypoxic polycythemia was produced by placing animals in semi-permeable silicone rubber membrane enclosures* for three weeks as described elsewhere.[15-17] They were then placed in room air. On day four and five following return to room air they received i.p. injections of assay materials. The assay then followed the routine procedure. These animals were maintained on regular diet throughout.

Preparation of the mice by placing them on protein free diet for 4 days described in detail above was accepted as the best method (See Discussion) and was used routinely for the remainder of the studies. Dilutions of all materials were made in physiological saline. Groups of 5-6 animals were used for each assay and averages taken. Saline controls were kept throughout.

The sensitivity of the assay animals to various doses of exogenous erythropoietin was tested by injecting 0.01 to 4 units of Step III erythropoietin obtained from phenylhydrazine treated sheep** (Figure 1).

In order to ascertain the specificity of the assay method in detecting erythropoietin, and not simply non-specific plasma factors rabbit anti-human erythropoietin*** was utilized in various doses together with human plasmas, Step III sheep erythropoietin and saline (Table II).

Results

The effect of various dietary regimens and polycythemia, induced by hypoxia, on red cell production of the mouse, as measured by per

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* General Electric Corporation, Schenectady, New York.

** Step III sheep erythropoietin was obtained from Connought Medical Research Laboratories, Toronto, Canada.

*** Rabbit anti-human erythropoietin was obtained through the courtesy of Drs. J. Schooley and J. Garcia at the University of California, Berkeley, Calif.

TABLE II

SUPPRESSION OF ERYTROPOIETIC EFFECT OF PLASMA AND ESF BY ANTI-ESF
% Fe59 Incorporation into RBC of Mice

Plasma (Donors)	Hb (Gm %)	Without Anti-EsF	With Anti-ESF	Amount of Anti-ESF (Units)***
1	14	27.40* ± 1.98**	18.00 ± 2.26	1.0
2	15.5	23.17 ± 4.26	15.45 ± 3.92	1.0
3	16.8	27.75 ± 5.26	14.96 ± 1.69	0.2
			11.16 ± 1.24	0.4
4	12.8	16.80 ± 5.45	8.72 ± 4.34	0.1
ESF (Step III) 0.1 U.	–	20.92 ± 3.77	10.50 ± 0.31	1.0
Saline	–	2.35 ± 0.46	0.39 ± 0.30	0.4
		2.88 ± 1.02	1.37 ± 0.65	0.1

* Average of 5-6 Assays
** Standard Deviation
*** Units capable of neutralizing an equal number of IRP units
(International Reference Preparation units of erythropoietin)

Erythropoietic effect of human plasma and sheep erythropoietin is suppressed by simultaneous administration of the rabbit -anti-human erythropoietin.

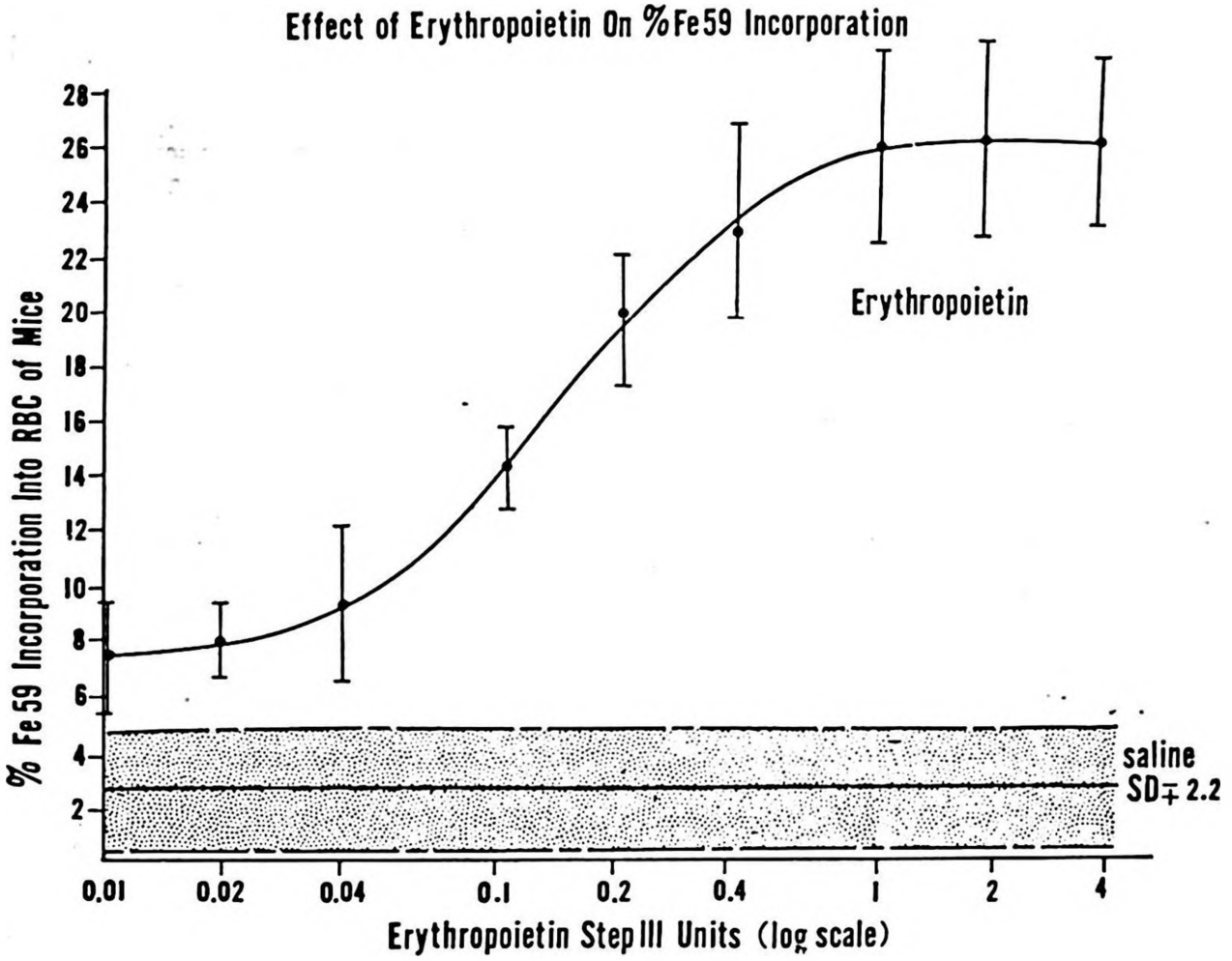

Figure 1

The degree of erythropoietic stimulation in protein deprived CFI mice is proportional to the amount of injected Step III sheep erythropoietin. A plateau is reached with doses over 1 unit. Vertical bars indicate one standard deviation.

cent Fe59 incorporation into circulating red blood cells in 48 hours is presented in Table I. Erythropoiesis was suppressed to the greatest degree in animals subjected to hypoxia for 3 weeks and then placed in room air, as compared to the normal high rate of erythropoiesis observed in those maintained on a regular diet (0.5 % ± 0.3 and 16.8 % ± 2.5).

Incorporation of injected Fe59 in mice on protein free diets for 3,4 and days was 7 % ± 4.2, 2.6 % ± 2.2 and 2 % ± 1.4 respectively. Starvation for 4 days reduced Fe59 incorporation to 1.5 % ± 0.6.

Animals on a protein free diet for 7 days or on a starvation diet, as well as those sujected to hypoxia, became emaciated and a number of them died.

Using 4 days protein deprivation to reduce erythropoiesis of the mouse (thus endogenous erythropoietin production), the stimulatory

effect of exogenous erythropoietin injections on red cell production was measured. Increases in per cent Fe59 incorporation were proportional to the amount of Step III erythropoietin injected (Figure 1). The effect of 0.02 or 0.1 units of erythropoietin could be detected using one or two standard deviations of the mean respectively. The dose response curve a reached a plateau after 1 unit of erythropoietin.

The specificity of the assay in detecting erythopoietin was investigated further by using antibodies to erythropoietin (anti-ESF) together with Step III erythropoietin or donor plasmas (Table II). Normal plasma injections resulted in an Fe59 red cell incorporation of 16.80 % to 27.40 %. Each plasma was assayed 5-6 times. Table II shows that as little as 0.1 unit of anti-ESF (capable of neutrilizing an equal number of International Reference Preparation units of erythropoietin) significantaly depressed the erythropoietic effect of donor plasmas. Anti-ESF also suppressed the effect of injected Step III sheep erythropoietin as well as that of endogenous erythropoietin of the mouse.

Discussion

One of the difficulties encountered in erythropoietin bio-assay is the rapid rate of red cell production of the assay animals; the other is the small quantity of the hormone normally present in human plasma. This necessitates either the injection of large quantities of plasma or concentrated urine (with possibilty of loss of potency or sensitizing animals by stopping or reducing endogenous erythropoietin production. Polycythemia induced by hypoxia or transfusions, starvation and protein deprivation are the methods generally utilized to sensitize assay animals. The polycythemic mouse assay (CF1) is considered superior to the staved or protein deprived rat assays.[7] However the relative mertis of the starved or protein deprived CFI female mouse assay has not been sufficiently investigated .The proportion of injected test plasma (1 ml in general) to the total blood volume of the animal could influence the sensitivity of the assay as well as species of the animel per se.

Hypoxic polycythemia, starvation and protein deprivation were all effective in suppressing erythropoiesis of CF1 adult female mice as measured by per cent Fe59 incorporation into circulating red cells. Although the suppression was greatest in the polycythemic animals, this was time consuming and requiring special equipment to maintain hypoxic condition. A number of animals died either during preparation or shortly after being exposed to atmospheric air. Hypoxia was stressful to tne animal ,disturbing their metabolism and producing significant weight losses.[11]

Starvation also resulted in the loss of animals by cannibalism thus fluctuating the level of endogenous erythropoietin suppression. Animals on the 7 days protein deprivation diet lost considerable weight and became rather irresponsive to exogenous erythropoietic stimulation. Erythropoiesis in mice on 4 days protein deprivation diet was considerably depressed. This group of animals appeared to be in good health and responsive to subsequent erythropoietic stimulations, thus most suitable for the erythropoietin assay.

CFl adult female mice on 4 days protein deprivation diet were quite sensitive in detecting exogenous erythropoietin. As little as 0.02 units of Step III sheep erythropoietin could be detected using one standard deviation or 0.1 units using two standard deviations from the mean (Figure 1). This compares favorably with the levels detectable with the polycythemic mouse.[7] [18]

Erythropoietic stimulation of normal human plasma could also be measured with this assay as demonstrated in Table II. These results are similar to those obtained in a previous study.[19] Since non-specific stimulation of erythropoiesis by non-specific plasma factors can occur in protein deprived animals, specificity of the assay was further studied by using antibodies against erythropoietin. Injecitons of various doses of anti-erythropoietin only partially suppressed the stimulatory effect of human plasma or sheep erythropoietin, indicating that some non-specific stimulation of Fe59 incorporation was present (Table II). However, the fact that there was significant suppression indicates the sensitivity of the method to ESF in plasma. Since rabbit anti-human erythropoietin used here suppressed the effect of human erythropoietin (in plasma) as well as the effect of sheep erythropoietin (Step III) and endogenous erythropoietin of the mouse (saline controls), the lack of species specificity of the erythropoietin and its antibody is indicated.

The non-specific stimulation of the erythropoiesis was not due to B complex vitamins absent in the diet nor to degraded protein products since the injections of B complex vitamins and blood hydrolysates failed to increase Fe59 incorporations.[20] The non-specific stimulation of the erythropoiesis could not be explained by the presence of various hormones (e.g. androgens, pitressin) in the injected plasma, since the hormones exert their erythropoietic effect through the stikulation of erythropoietin production which would have been neutralised when anti-erythropoietin was used simultaneously.[21]

Although the assay is somewhat influenced by non-specific plasma factors, it is quite sensitive to plasma erythropoietin levels. It may be

of value as a routine screening procedure in clinical medicine, to measure total erythropoietic effect of the plasma, because of ease in performance, reasonable sensitivity and reproducibility. Furhermore, by subtracting non-specific stimulation of Fe59 incorporation obtained with simultaneous use of adequate amount of anti-erythropoietin (e.g. 0.5-1 unit) from the total stimulation of Fe59 uptake, erythropoietic activity of the plasma due to erythropoietin alone may be calculated.

Summary

A modified erythropoietin bio-assay using protein deprived CFI adult female mice is described. After 4 days of protein deprivation, the assay is capable of detecting 0.02 units of Step III sheep erythropoietin as measured by per cent increases in Fe59 incorporation into circulating RBC of mice in 48 hours.

The specificity of the method is demonstrated by suppression of the effect of erythropoietin or plasma by simultaneous injetions of anti-erythropoietin.

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