Urinary Excretion of Erythropoietin in Normal Men and Women

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THE ROLE OF ERYTHROPOIETIN in the regulation of normal, steady-state, erythropoiesis in man has been disputed.[1-4] The relative insensitivity of currently employed technics for the detection of this hormone in normal plasma[5] and urine[6] has contributed to this problem. This report presents results derived from improvements in the polycythemic mouse bioassay method from which the daily urinary excretion of erythropoietin in normal men and women was calculated.

MATERIALS AND METHODS

Nine male and 12 female volunteers in good health were studied. Their ages ranged from 19 to 47, hematocrits from 39 to 48 volumes per cent, and reticulocyte counts from 0.4 to 2.0 per cent. The serum iron and transferrin concentrations of all subjects were within the normal range for this laboratory. Four healthy prepubertal boys with ages ranging from 6 to 11 years were also studied. Venesections had not been performed on any volunteer during the preceding 4 months.

All urine voided for 1 day was collected in polyethylene containers and frozen immediately to less than −5 C. At the time of processing, each specimen was thawed by immersion of its container in running cold tap water at 25 C., a prodecure completed within 2 hours. Sediment was removed by filtering with Whatman paper #4 at 4 C. The filtrate was concentrated by membrane dialysis at 4 C. against a polyethylene glycol compound (Carbowax, Union Carbide Company) to a volume of approximately 20 ml. At least 4 saline washes of the dialysis membrane were added to the urine concentrate. Precipitated salts in this mixture were separated by centrifugation at 500 g for 20 minutes. After transfer to a graduated cylinder, the volume of supernatant urine concentrate was measured. This material was divided in 3 equal portions and stored at −10 C.

Protein-depleted, polycythemic mice, receiving injections of urine concentrate in divided doses, were utilized for the bioassay of human urinary erythropoietin. Swiss-Webster female mice (Berkeley Pacific Laboratories, Berkeley, California), 10 to 12 weeks of age at the time of assay, were given 2.5 mg. of iron dextran (Lakeside Laboratory, Wisconsin) intramuscularly. Polycythemia was produced by exposure to 0.45 atmospheres of air in a low pressure chamber for approximately 3 weeks.[7-9] Mice were acclimatized to this degree of hypoxia by submission to approximately 0.50 atmospheres of air for the initial 48 hours. Exposure to hypoxia was continuous except for approximately 4 hours of routine care once every 48 hours. All animals received a regular laboratory diet while in the chamber and for 48 hours following their return to ambient pressure. At this time, their hematocrit was approximately 75 volumes per cent. Two days following removal from the chamber, all

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mice were divided randomly into groups of 7 or 8 and offered a USP XV protein-depletion diet (Nutritional Biochemicals Company, Cleveland, Ohio). On the next day, intraperitoneal injections of urine concentrate were initiated and repeated every 24 hours, in a constant volume for each experimental group, for a total of 3 injections. Thirty hours following the last injection of urine concentrate, approximately 2 μc. of Fe^{59} citrate (specific activity ranging from 10 to 30 μc./μg. of iron) were injected intraperitoneally. The mean weight of each group of animals was determined 3 days later. A relative weight loss of approximately 20 per cent occurred during the 7 days of protein depletion. Whole blood was collected in heparin-moistened glass pipettes from the lacerated right axillary artery of each mouse under ether anesthesia. The microhematocrit of each blood specimen was measured and mice with measurements less than 54 volumes per cent were excluded from subsequent calculations. The red blood cells of each specimen were washed once with saline and centrifuged at 200 g. The supernatant was removed and the red blood cells reconstituted with saline to a packed cell volume of approximately 44 volumes per cent.* Of this mixture, 0.5 ml. was pipetted into a counting vial. The radioactivity in each sample and in duplicate radioiron standards was measured in a gamma spectrometer. The red cell utilization of Fe^{59} was calculated as a percentage of the injected radioactivity from the formula:

$$\textit{Per cent utilization} = \frac{\textit{Total red cell radioactivity} \times 100}{\textit{Injected radioactivity}}$$

The red cell mass of protein-depleted, polycythemic mice in this laboratory was approximately 6.5 per cent of body weight using an isotope-dilution procedure with Fe^{59}-tagged red cells. This figure was used to calculate the relationship between the measured radioactivity in each sample of 0.22 ml. red blood cells (product of 0.5 ml. and 0.44) and the total red cell radioactivity. The median per cent utilization of Fe^{59} in each group of experimental mice was derived by subtracting the median per cent utilization in a group of control mice previously injected with 5 per cent human albumin. Simultaneous assays were performed, employing an identical program of fractionation, with measured amounts of erythropoietin standard B (Medical Research Council, London, England). The median per cent utilization of Fe^{59} in each urine assay was converted to equivalent units of erythropoietin by comparison with a dose-response curve for erythropoietin standard B. From the product of this figure (erythropoietin units) and the factor relating the available volume of urine concentrate to the volume administered to each animal, the daily urinary excretion of erythropoietin was calculated.

Results

Erythropoietin Yield following Urine Concentration

The ineffectiveness of normal, unconcentrated urine in stimulating erythropoiesis in polycythemic mice required the preparation of urine concentrates. A convenient and efficient method for achieving this goal was provided by membrane dialysis against "Carbowax." Erythropoietin yield following this procedure was evaluated by comparing the concentration of erythropoietin in a specific quantity of urine from an anemic donor before and after concentration and reconstitution with saline to the original volume. As indicated in Table 1, urine concentration to approximately 1 per cent of the original volume was associated with high erythropoietin yield.

*Reconstitution to a standardized hematocrit was achieved by adding a volume of saline in a constant proportion to the measured volume of packed red cells in narrow 9.5 × 55 mm. glass vials. The mean hematocrit of more than 50 washed red cell samples prepared in this manner in different experiments was 44 ± 2 volumes per cent.

Table 1.—*Erythropoietin Yield Following Concentration of Urine from an Anemic Donor*

	Number of Mice	Red Cell Utilization Fe^{59} (%)		
		Median	Mean	Range
Untreated urine	7	3.7	3.7	(2.5–6.9)
Treated urine, concentrated × 100 and rediluted	7	5.6	4.5	(2.2–6.2)

Response following Different Schedules of Erythropoietin Administration

The magnitude of response was evaluated following different durations of fractionation of a constant amount of erythropoietin. Erythropoietin standard B and urine with high erythropoietin concentration were given in separate experiments to polycythemic mice either as a single injection or in equally divided fractions every 12 or 24 hours for 1 to 6 days. All groups received Fe^{59} on the day following the last erythropoietin injection, except for mice receiving a single injection which received the isotope 52 hours later. Per cent utilization was expressed relative to the radioiron uptake in animals injected with erythropoietin every 24 hours for 4 days, considered as 100 per cent. A curve was calculated from all measurements using the formula, $y = a + bx + cx^2$, and the method of least squares. As indicated in Figure 1, fractionation of a constant dose of erythropoietin for 3, 4 or 5 days produced the greatest utilization of radioiron. Less stimulation was apparent following longer or shorter durations of erythropoietin injection.

In order to evaluate the magnitude of response following more frequent stimulation, graded amounts of erythropoietin standard B, in doses ranging from 0.04 to 1.0 unit, were given to groups of polycythemic mice either (a) in a single injection, (b) in equally divided daily fractions for 3 days, or (c) in equally divided fractions every 8 hours for 3 days. Radioiron was administered in a manner identical to that described in the preceding experiment. Curves were fitted to the data using the polynomial function, $y = a + b \log x$, and the method of least squares. Each of these injection regimens detected 0.05 units of erythropoietin, but failed to discriminate less than this amount from the effect following serum albumin. Despite this similarity, the nature of the dose/response curves differed with each injection program (Fig. 2). While the response to graded doses of erythropoietin injected every 8 hours for 3 days was almost linear, the relative increment in response was least following single injections of erythropoietin, and intermediate following daily fractionation for 3 days. The latter schedule was used for all subsequent assays of human urine concentrate reported here.

Yield of Testosterone Following Urine Concentration

The yield of 17-ketosteroids in unconcentrated and concentrated 24-hour urine collections was compared in separate studies in 2 normal male subjects. In addition, a measured quantity of C^{14}-testosterone was added to each un-

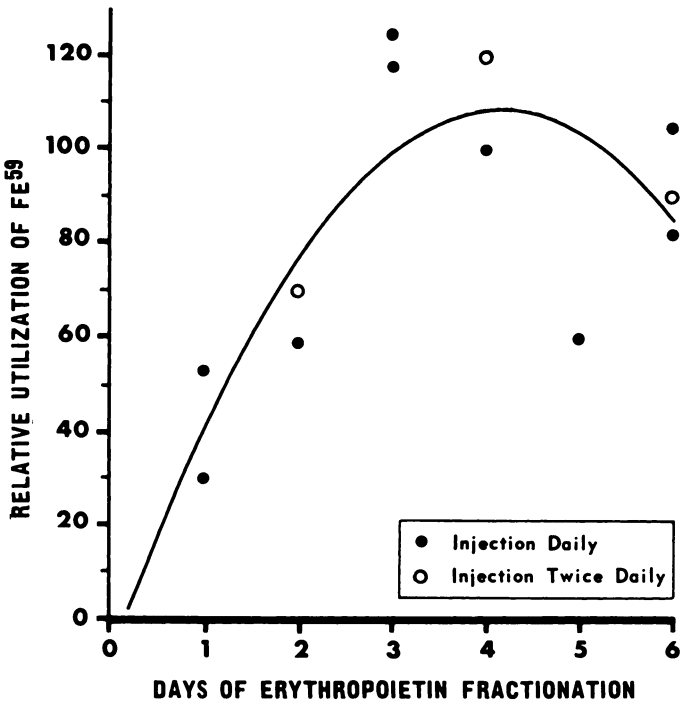

Fig. 1.—Relative utilization of Fe^{59} in polycythemic mice following different durations of erythropoietin injection. Daily injections for 4 days = 100 per cent. Each point represents the median value in a group of at least 6 animals.

treated urine specimen and the total remaining radioactivity following concentration was calculated from measurements of aliquots in a liquid scintillation counter (Packard Instrument Company, LaGrange, Illinois). As indicated in Table 2, less than 1 per cent of the original quantity of 17-ketosteroids and C^{14}-testosterone remained in the urine concentrates.

Daily Urinary Excretion of Erythropoietin in Normal Men and Women

The median red cell uptake of radioiron in all groups of mice receiving human urine concentrate exceeded that measured in albumin-injected controls. While the median per cent utilization of 10 control groups ranged from 0.01 to 0.09 per cent (mean, 0.03 per cent), the median per cent utilization of all groups receiving concentrates of normal urine ranged from 0.4 to 5.2 per cent.

As indicated in Table 3, the mean daily erythropoietin excretion calculated for 9 men was 2.8 units, with a range from 1.5 to 5.2 units. The mean daily erythropoietin excretion for 12 women was 0.9 unit with a range from 0.5 to 1.8 units, as shown in Table 4. Using the "t-test" this difference between men and women was statistically significant ($p < .01$). The mean excretion in 4 prepubertal boys was 1.0 unit, with a range from 0.6 to 1.2 units, as demonstrated in Table 5.

The erythropoietin excretion for each individual was plotted against his

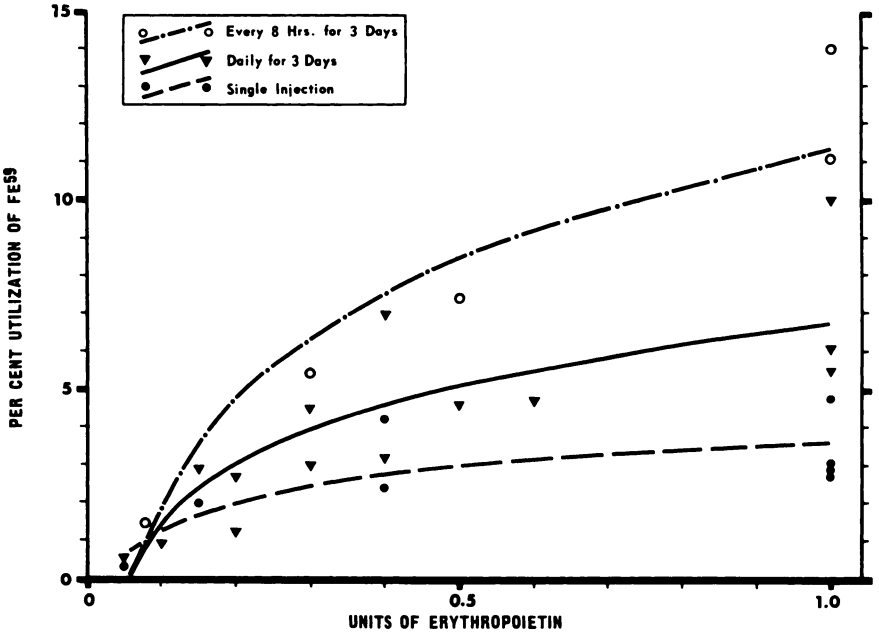

Fig. 2.—Per cent utilization of Fe^{59} in protein-depleted, polycythemic mice following different schedules of erythropoietin injection. Each point represents the median value in a group of at least 6 animals.

height, weight, body surface area, hematocrit, reticulocyte concentration, serum concentration of iron, per cent saturation of transferrin, and the assumed red cell mass calculated from the formula of Nadler.[10] There was a tendency for individuals with a greater height, weight, surface area, and red cell mass to have a higher level of erythropoietin excretion. When these factors were correlated separately for males and females, no relationship was apparent.

Discussion

In the absence of specific biochemical or immunologic procedures, the polycythemic mouse bioassay provides the most sensitive available method for the demonstration of small quantities of erythropoietin.[11,12] These animals are useful as a result of the increased sensitivity to exogenous erythropoietin derived from the marked suppression of endogenous erythropoiesis produced by

Table 2.—*Urine Androgens before and after Concentration*

	Unconcentrated Urine			Concentrated Urine		
Subject	24-hour volume (ml.)	24-hour 17-Ketosteroids* (mg.)	C^{14}-Testosterone added (cpm)	Volume urine concentrate (ml.)	17-Ketosteroids* in concentrate (mg.)	C^{14}-Testosterone in concentrate (cpm)
R. A.	850	3.69	763,000	25	<0.04	1,100
W. C.	2200	4.00	1,162,000	40	<0.04	500

*Androsterone, epiandrosterone, dehydroepiandrosterone.

Table 3.—*Urinary Excretion of Erythropoietin in 9 Normal Men*

Subject	Hct.	Retic. Conc. (%)	Fe (μg. %)	Iron-binding Capacity (μg. %)	Volume 24-Hour Urine Conc. (ml.)	Volume Conc. Injected Per Mouse (ml.)	Median Fe^{59} Uptake (%)	Equivalent Erythropoietin Per Mouse (St. B units)	24-Hour Excretion Erythropoietin (St. B units)
F. H.	46	1.1	111	353	28	3.0	2.5	0.16	1.5
A. I.	44	0.6	84	257	41	4.2	2.4	0.16	1.6
J. F.	42	1.0	120	273	34	3.6	2.7	0.18	1.7
O. W.	44	1.0	126	314	34	4.2	3.2	0.22	1.8
R. A.	41	1.8	114	337	25	3.0	3.7	0.28	2.3
J. Si.	45	1.0	124	320	53	5.4	4.2	0.34	3.4
J. Sh.	46	2.0	78	216	43	4.2	4.1	0.33	3.4
K. G.	48	0.4	84	310	60	6.0	4.6	0.40	4.0
S. H.	46	1.1	97	361	42	4.2	5.2	0.52	5.2
								Mean	2.8
								±1 S.D.	±1.3

Table 4.—*Urinary Excretion of Erythropoietin in 12 Normal Women*

Subject	Hct.	Retic. Conc. (%)	Fe (μg. %)	Iron-binding Capacity (μg. %)	Volume 24-Hour Urine Conc. (ml.)	Volume Conc. Injected Per Mouse (ml.)	Median Fe^{59} Uptake (%)	Equivalent Erythropoietin Per Mouse (St. B units)	24-Hour Excretion Erythropoietin (St. B units)
S. S.	39	0.6	85	312	17	2.4	0.6	0.07	0.5
C. H.	42	1.0	141	299	34	3.6	0.4	0.06	0.6
C. W.	42	0.8	93	278	32	3.6	0.6	0.07	0.6
L. M.	39	0.4	119	311	29	3.6	1.0	0.08	0.6
V. P.	41	1.6	153	342	42	4.8	0.8	0.08	0.7
P. C.	44	0.6	114	429	20	2.4	1.0	0.08	0.7
M. M.	43	0.9	160	380	24	3.0	1.3	0.10	0.8
L. A.	42	0.6	77	279	31	2.4	0.6	0.07	0.9
L. R.	41	0.8	114	291	39	4.8	2.0	0.13	1.1
M. B.	39	2.0	67	278	22	2.1	1.6	0.11	1.2
B. Y.	42	2.0	60	324	31	3.6	2.4	0.16	1.4
B. B.	44	1.3	189	374	37	4.2	2.9	0.20	1.8
								Mean	0.9
								±1 S.D.	±0.4

experimental polycythemia. As in rats,[13,14] protein depletion decreases the per cent utilization of radioiron in mice to approximately 15 per cent of normal.[6] Although this degree of inhibition of erythropoiesis is less than in polycythemic[8,12] or in starved[15] mice, a further decrease results from combining protein depletion with polycythemia.[6,16] Despite this maximal suppression of erythropoiesis, the response of these animals to graded doses of erythropoietin is less than in polycythemic mice receiving a normal diet.[8,12] In contrast to protein-depleted rats,[14] this finding indicates that an impaired bone marrow response to erythropoietin may contribute to the suppression of erythropoiesis in protein-depleted or starved mice. The use of a divided dose injection schedule enhances the response but does not improve bioassay sensitivity in terms of detecting less than 0.05 units of erythropoietin. Nevertheless, the more pro-

Table 5.—*Urinary Excretion of Erythropoietin in 4 Normal Boys*

Subject	Age	Volume 24-Hour Urine Conc. (ml.)	Volume Conc. Injected Per Mouse (ml.)	Median Fe^{59} Uptake (%)	Equivalent Erythropoietin Per Mouse (St. B units)	24-Hour Excretion Erythropoietin (St. B units)
M. T.	11	24	3.0	0.5	0.07	0.6
W. G.	11	27	3.3	1.8	0.12	1.0
R. R.	11	12	1.8	2.7	0.17	1.1
P. A.	6	21	2.4	2.1	0.14	1.2
					Mean	1.0

nounced suppression of endogenous erythropoiesis in polycythemic mice receiving a protein-depletion diet may provide more precise quantitation of low levels of erythropoietin in the 0.05 to 0.20 unit range.

The differentiation of primordial bone marrow cells into the erythron is a major function of erythropoietin.[17,18] The level of red cell production is related to the amount of erythropoietin available to the bone marrow,[8,12] as well as to the number and/or responsiveness of primitive cells sensitive to erythropoietin.[19,20] The increased erythropoiesis in polycythemic mice following progressive fractionation of erythropoietin may result from greater recruitment of primordial marrow cells in a state receptive to the action of this hormone, as suggested by Gurney[3] and Schooley.[21] A maximum effect occurred when polycythemic mice were stimulated by divided injections of erythropoietin for 3, 4 and 5 days. The relatively lower response after longer periods of fractionation was attributed to the unavailability for Fe^{59} labeling of a proportion of the red cell population differentiated by the initial erythropoietin injections. The advantage of a divided injection schedule in the bioassay of erythropoietin resides in the improved accuracy of a more linear dose/response relationship. In addition, the degree of urine concentration required for the injection of a complete 24-hour urine concentrate is less with multiple injections than with a single injection.

Urine was employed as the bioassay material after it was found that large volumes could be concentrated without significant loss of erythropoietin. The capacity of assay animals to tolerate without significant mortality the intraperitoneal injection of more than 90 per cent of the available urine concentrate supported the feasibility of this procedure. Despite the difficulty in detecting erythropoietin in normal human plasma,[5] significant erythropoietin-like activity has been demonstrated in urine concentrates from normal individuals.[22] Neutralization of this activity by rabbit antihuman erythropoietin serum suggests strongly that this activity is identical with human erythropoietin.[16,23] The parallel dose/response relationship following injections of urine concentrate and erythropoietin standard B supports this conclusion,[16] and justifies the calculation of urinary erythropoietin unitage in terms of the erythropoietin standard.

The detection of erythropoietin in the urine of normal human beings suggests that normal, steady-state erythropoiesis in man is probably dependent upon the continuous activity of this hormone. This conclusion is consistent

with observations in animals indicating that the differentiation of erythroid cells is prevented by hypertransfusion,[11] hyperoxygenation,[24] and antierythropoietin antibody,[25] and may be restimulated by exogenous[18] or endogenous[26] erythropoietin. The higher level of urinary erythropoietin excretion in men may reflect greater erythropoietin production in this sex, providing an explanation for the higher red cell mass in adult males in comparison with adult females.[27]

The virtual elimination of testosterone from urine concentrates of male donors indicates that an indirect stimulation of erythropoiesis in polycythemic mice by this hormone could not account for the greater activity resulting from these concentrates. Less than 0.5 μg. of testosterone was injected in the assay animals, an amount far below the minimum level of 500 μg. necessary for the stimulation of erythropoiesis in polycythemic mice.[28]

Conclusion

The bioassay of erythropoietin in polycythemic mice was modified to include a protein-depletion diet and a divided erythropoietin injection schedule. Although less than 0.05 unit of standard erythropoietin was not detected, a more linear dose/response relationship resulted from increasing doses of erythropoietin in the 0.1 to 1.0 unit range. The amount of erythropoietin in concentrated specimens prepared from the 24-hour urinary excretion of 25 normal subjects was measured in comparison with known quantities of standard erythropoietin. A mean daily erythropoietin excretion in men of 2.8 $\pm$ 1.3 units, in women of 0.9 $\pm$ 0.4 unit and in prepubertal boys of 1.0 unit was calculated. The higher erythropoietin excretion in adult males may be secondary to a greater production of erythropoietin in this sex.

Summario in Interlingua

Le bioessayage pro erythropoietina in muses polycythemic esseva modificate de maniera a includer un dieta a depletion de proteina e un dividite horario de injectiones de erythropoietina. Ben que minus que 0,05 unitates de erythropoietina standard non esseva detegite un relation plus linear inter dose e responsa resultava ab crescente doses de erythropoietina in le region inter 0,1 e 1,0 unitates. Le quantitate de erythropoietina in specimens concentrate preparate ab le excretion urinari in 24 horas esseva mesurate in 25 subjectos normal in comparation con cognoscite quantitates de erythropoietina standard. Un valor medie del excretion diurne de erythropoietina in homines de 2,8 $\pm$ 1,3 unitates esseva calculate. In feminas, le correspondente valor esseva 0,9 $\pm$ 0,4 unitates. In pueros de etate prepubertal, illo esseva 1,0 unitates. Le plus elevate excretion de erythropoietina in masculos adulte es possibilemente secundari a un plus grande production de erythropoietina in iste sexo.

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