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Effects of Plasma Erythropoiesis Stimulating Factor (E.S.F.) at Different Time Intervals After Single Injection. (26469)

G. HODGSON* (Introduced by H. Borsook)
Biology Division, California Institute of Technology, Pasadena

It has been suggested(1,2) that erythropoiesis stimulating factor (E.S.F) stimulates differentiation of stem cells into red cell precursors. Results of histological study of the spleen of polycythemic mice treated with E.S.F. support this view(3). More detailed analysis of several effects of E.S.F. might provide evidence for or against this theory. Therefore, amino acid and iron incorporation in hemoglobin, and reticulocyte levels were measured at various time intervals after a single intravenous injection of E.S.F.

Methods. Normal Webster Swiss female mice 24-30 g obtained from Diablo, Berkeley, Cal. were used. E.S.F. extract, No. LD11, was prepared from plasma of rabbits made severely anemic with phenylhydrazine by methods already described(4). Its activity was about 0.7 CS 1 unit/mg or 7 Co units/mg. This extract is of the same type as Extract CS 1 which was assayed in several laboratories(4).

Several criteria were employed to measure the effects of the extract. a) Measurement of C^{14} amino acid uptake in globin as proposed by Dovey *et al.*(5). b) Measurement of Fe^{59} in plasma and spleen at 2 hours and in red cells at 48 hours after tracer injection. c) Reticulocyte counts. d) Measurement of reticulocytes by determining *in vitro* uptake of Fe^{59} and C^{14} leucine into washed red cells from 5 mice, by methods described by Borsook(6).

The experimental design was the following: on day 0 the mice received 0.25 ml of .9% NaCl solution containing 2 mg LD11 intravenously (tail vein). Controls received .9% NaCl solution. For the C^{14} amino acid experiments groups of mice—number of animals shown in Table I—were injected intravenously at 12, 24, 48, 72, and 96 hours after the extract with 1 μc C^{14} uniformly labelled amino acids obtained by acid hydrolysis of C^{14} labelled chlorella protein (CFB6 Radiochemical center, Amersham, England) with a specific activity of 1 mc/5.6 mg. One week later, a time when C^{14} activity globin had levelled off, mice were exsanguinated by heart puncture under ether anesthesia. In initial experiments pooled red cells from 6 mice were washed thrice with 30 vol. of ice cold .9% NaCl; 0.10 ml of the red cell suspension was dried on planchettes using the technic described by Lowy *et al.*(7), and counted in Nuclear Chicago automatic gas flow counter. The rest of the red cells were lysed with 20 vol. of distilled water, insoluble material was centrifuged down, the clear super-

* Research Fellow supported by I.C.A. under the visiting scientists program administered by Nat. Acad. Sci. Permanent address: Inst. de Fisica y Matematicas U de Chile, Santiago.

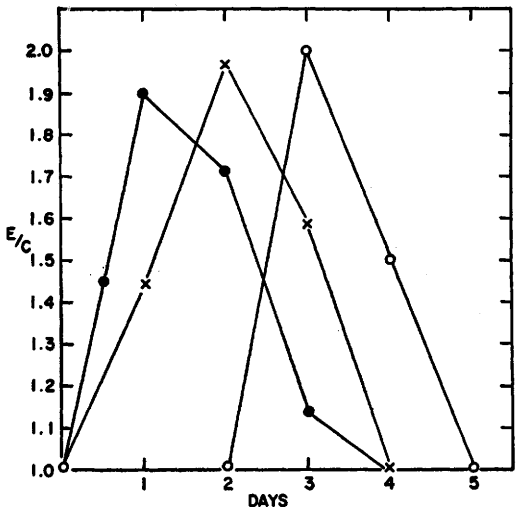

FIG. 1. Effect of E.S.F. as a function of time after intrav. inj. Ordinate: E/C = ratio of experimental to control values for ●—● C^{14} in Hb. ×—× fraction of plasma iron going to erythroid tissue per hour. ○—○ reticulocytes. Abscissa: Days after E.S.F. inj.

natant was made 5% with respect to TCA, the precipitated proteins were washed with TCA, acid acetone, alcohol-ether, ether, dried, plated and counted. Results of both methods of counting were correlated. For 6 pools with activities ranging from 60-375 cpm, the value of r was 0.985 and the slope 1. In view of this finding, C^{14} uptake into globin was measured in the experiments to be described by counting plated washed red cells. For the Fe^{59} experiments, other groups of mice were injected with Fe^{59} at 24, 48, 72, and 96 hours after injection of the extract. Two hours later, radioactivity in plasma and spleen was determined in some of the mice. The remaining mice were bled 48 hours after tracer injection and erythrocyte Fe^{59} was determined. Counting was done as in the experiments of Lowy *et al.*(7). For calculation of Fe^{59} uptake the blood volume was taken as 6.7 ml/100 g. This value was obtained by measuring the volume of distribution of Fe^{59} labelled erythrocytes.

Reticulocytes were stained using new methylene blue and the fraction of these cells was determined by counting a total of 100 reticulocytes in incubation experiments, and 1000 red cells in other experiments. Pooled red cells from blood drawn 72 hours after injection of the extract were used for incubation experiments.

Results. Fig. 1 and Table I show that C^{14} amino acid uptake into globin was at a maximum when the tracer was injected 24 hours after the extract. Uptake was also above normal when tracer was injected 12 and 48 hours after extract, but at 72 hours it was equal to controls. Plasma iron turnover rate and uptake of iron by the spleen were slightly above control values at 24 hours, maximum at 48, slightly elevated at 72 hours, and equal to the controls at 96 hours. Fe^{59} uptake in red cells was high from 24 to 72 hours after E.S.F. administration. The product of plasma iron turnover rate and percentage of Fe^{59} appearing in the red cells at 48 hours was taken as an estimate of the fraction of plasma iron going to erythroid tissue (F.I.Er.) Values of spleen Fe^{59} and F.I.Er are in close agreement (Table I). Fig. 1 represents the variation of F.I.Er and reticulocyte count as a function of time after E.S.F. injection. The increased reticulocyte count manifested itself by increases in uptake of C^{14} leucine and Fe^{59} into the incubated red cells of blood drawn 72 hours after injection of the extract (Table II). Analysis of the dose response study showed that incorporation of C^{14} leucine and Fe^{59} was a function of the log dose of extract injected. The increase in C^{14} leucine and Fe^{59} uptake is proportionately greater than the increase in reticulocyte percentage. This probably reflects the fact that Hb synthesis is not only a function of reticulocyte number but also of degree of maturity. The more immature reticulocytes synthesize more Hb(8).

Discussion. The first observed effect of E.S.F. in an increased uptake of C^{14} amino acids into globin, as early as 12 hours after injection, with a maximum at 24 hours. Maximum iron uptake into Hb is at 48 hours. Reticulocytosis appears at 72 hours.

The increased uptake of C^{14} leucine and Fe^{59} by incubated erythrocytes from blood drawn 72 hours after injection of extracts represents an objective method of measuring reticulocytes. It is conceivably more precise than reticulocyte counts, which have a high

TABLE I. Effects of E.S.F. as a Function of Time after Intravenous Injection of Extract. First column indicates time interval between injection of E.S.F. and of tracer. Number in parentheses indicates number of mice in the group in which measurement was carried out.

	1	2	3	4	5	6	7
Time, hr	Retic., %	C^{14}Hb, cpm	Fe^{59}, % ID plasma (2 hr)	PITR, %/hr	% Fe^{59} erythrocytes (at 48 hr)	F.I.Er, %/hr	% Fe^{59} spleen (2 hr)
0	2.05 ±.15(10)	790 ± 41.5 (14)	24.5 ±1.24 (13)	70	46.8 ± 4.08(10)	32.9	3.8 ±.60(10)
12	—	1160 ± 60.0 (7)	—	—	—	—	—
24	—	1500 ± 71.0 (14)	20.5 ± .63 (6)	79.5	59.6 ± 2.45 (6)	47.4	4.8 ±.69 (5)
48	2.00 ±.46 (5)	1360 ± 71.0 (14)	13.5 ± .69 (10)	100	64.8 ± 1.96 (6)	64.8	7.6 ±.95 (5)
72	4.00 ±.33 (5)	850 ±102.0 (6)	20.3 ± .62 (9)	80	65.5 ± 2.95 (6)	52.4	5.9 ±.91 (5)
96	3.05 ±.42 (6)	810 ± 77.0 (5)	24.3 ±1.43 (5)	70	—	—	3.6 ±.52 (5)
120	1.93 ±.15 (6)	—	—	—	—	—	—

PITR = plasma iron turnover rate, fraction of plasma iron turned over per hr (%/hr) = $\frac{1}{2} \ln \frac{1}{\text{plasma } Fe^{59}}$ where plasma Fe^{59} = fraction of inj. Fe^{59} remaining in plasma at 2 hr. F.I.Er = fraction of plasma iron going to erythroid tissue = PITR × % Fe^{59} in erythrocytes at 48 hr.

standard deviation, especially at low levels (9).

The experimental findings can be explained by assuming that initially, under the influence of E.S.F., a greater number of pro- and basophylic erythroblasts appear in the marrow(3). The presence of more cells of this type manifests itself early (12 hours) by an increased uptake of amino acids into globin, at a rate greater than the uptake of iron into heme. Hammarsten *et al.*(10) have shown that these early cells synthesize globin faster than heme, and autoradiographic studies(11) indicate that they take up 4 times more amino acids than polychromatic normoblasts and 60 times more than orthochromic normoblasts. Some time later (48 hours) when the newly formed cells have matured to the polychromatic stage, heme synthesis is at a maximum as indicated by histochemical studies (12) and by autoradiographic Fe^{59} determinations in rat marrow(13). At this period, 48 hours after E.S.F. injection when plasma iron turnover and Fe^{59} incorporation into Hb is at its maximum, C^{14} amino acid uptake is still high, for although individual polychromatic normoblasts take up less amino acid than the earlier forms(11), more cells of this type are present. Later, 72 hours after E.S.F. injection, the cells have matured to the reticulocyte stage in which, as the incubation studies show, terminal globin and heme synthesis go on at approximately the same rates(6).

TABLE II. Leucine C^{14} and Fe^{59} Uptake by Incubated Erythrocytes from Blood Drawn 72 Hr after E.S.F. Injection in Female Webster Swiss Mice.

E.S.F. inj.	Retic., %	C^{14}, cpm/.1 ml	Fe^{59}, cpm/.1 ml
Control	1.65	61	740
.25 mg	3.35	169	2483
.50 "	4.4	219	3031
1.5 "	4.4	312	3766

In conclusion, these data can be tentatively correlated with those of Filmanowicz and Gurney(3) in the polycythemic mouse spleen, by interpreting the early increase in C^{14} amino acid uptake into globin as a reflection of an increase in the early forms of erythroid cells 24 hours after E.S.F. injection and the increase in Fe^{59} uptake at 48 hours as reflecting maturation of these cells to normoblasts. The reticulocyte peak at 72 hours seen in normal mice coincides with the maximum reticulocytosis observed in polycythemic mice. Taken together, these data indicate that the sequence and timing of the events are similar in normal and physiologically depressed erythropoietic tissue.

Summary. Several effects of E.S.F. in normal mice were studied as a function of time after injection. The first change was a marked increase of C^{14} amino acid uptake by globin at 24 hours. This was followed at 48 hours by increase in iron consumption by erythroid tissue, and at 72 hours by an increase of reticulocyte count in peripheral blood as well as by increase in Fe^{59} and leucine C^{14} uptake

by incubated red cells.

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Rate of Localization of Anti-Rat Lung Antibody.*† (26470)

ITSURO TAMANOI, YASUO YAGI AND DAVID PRESSMAN
Department of Biochemistry Research, Roswell Park Memorial Institute, Buffalo, N. Y.

Antibody prepared in rabbits against rat lung tissue has been shown to be fixed in the lung and other organs when injected intravenously into rats(1). A similar situation exists with antibody against rat kidney in that when it is injected intravenously it localizes in the kidney with some cross-localization elsewhere(2,3). Rate of localization in kidney has been determined for the latter case(4) and it was found that for the most part the antibody is removed from the circulation as it passes through that organ. There is a second component which appears to be fixed at a somewhat slower rate. In the experiments reported here, rate of localization of anti-lung antibody in the lung was determined.

Preliminary results with the globulin fraction of anti-lung serum indicated that the lung localizing activity was too low to permit very accurate measurement in view of the relatively high background localization in the lung. Therefore, the study was made primarily with antibodies in which the lung localizing activity had been concentrated and purified by adsorption and elution from lung tissue. The major part of the kidney localizing activity was removed from the purified preparation by treatment with kidney sediment.

Materials and methods. Sprague-Dawley rats were used throughout the studies. *Sera.* Anti-rat lung sera were obtained by injecting perfused rat lung homogenate intraperitoneally into 8 rabbits 3 times a week for 4 weeks and bleeding one week after last injection. The dose given was 0.5 g wet tissue per injection. The γ-globulin fraction of each antiserum (γ G Anti-R Lu) and of normal rabbit serum (γ GNS) (Pentex, Inc.) was prepared by ethanol fractionation(5). The γ G anti-R Lu from a single rabbit showing the highest lung localizing activity relative to localization elsewhere was selected for this study. The proteins were radioiodinated as described previously(6,7). *Preparation of sediments*: Frozen rat lungs previously perfused with saline were minced with scissors and homogenized with 4.5 vol. physiological saline

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