Route of ^{59}Fe Administration as a Factor Effecting the Sensitivity of an Erythropoietin Bioassay[1] (36494)

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The importance of a well-standardized and sensitive bioassay for erythropoietin (ESF) has been stressed by several investigators (1, 2). Uniformity in reporting levels of erythropoietic activity was obtained with the development of international reference preparations of ESF, Standards A and B (3). Many species of assay animals and parameters for measuring erythropoietic activity have been tested (4). The most readily accepted assay now available utilizes the incorporation of radioactive iron into erythroid cells of polycythemic mice following the injection of ESF. However, laboratories often alter the sensitivity of this assay by using a variety of mouse strains, time schedules, and techniques for producing polycythemia (5, 6).

In a previous study (7) erythropoiesis was stimulated in normal C_3H mice with daily ip injections of Step 1 ESF. An attempt was made to obtain an additional parameter for erythropoietic activity by measuring the rate of ^{59}Fe incorporation. It was obvious from the results obtained that the method used failed to reveal erythropoietic stimulation. The purpose of this study was to determine whether the route of administration of radioactive iron has an effect on the amount of iron incorporated into peripheral red blood cells.

Materials and Methods. Adult C_3H and randomly bred Swiss-Webster white mice weighing approximately 25 g were used in these studies. Mice were fed Purina Rat Chow and water *ad libitum*. Commercially produced Step 1 and Step 3 sheep plasma ESF (lot Nos. 161-2, 162-1, and 3001-35; Connaught Medical Research Laboratories, Toronto, Can.) (sp act 0.6 and 3.5 units/mg, respectively) were dissolved in 0.9% saline and used to stimulate erythropoiesis in normal and polycythemic mice. The rate of erythropoiesis was determined by injecting radioferrous citrate (^{59}Fe; Squibb) and measuring the radioactivity of the blood in a well-type scintillation counter. Experiment I was repeated twice and since the results were comparable the data was pooled.

Experiment I. One hundred and thirty-five male C_3H mice were treated daily with either 1.0, 3.0, or 8.0 units of Step 1 ESF for 9 consecutive days. Forty-five control mice received 0.2 ml of 0.9% saline. On day 9 mice were injected with 0.5 μCi of ^{59}Fe in 0.5 ml of saline. Three groups were established; mice in group A received both ESF and ^{59}Fe ip, group B mice received ESF ip and ^{59}Fe iv, and group C mice received ESF sc and ^{59}Fe ip. Twenty-four hours later mice were anesthetized with ether, 0.5 ml of blood was withdrawn via cardiac puncture, spleens were surgically removed and weighed, and the percentage of ^{59}Fe incorporated into the blood was calculated utilizing the following formula:

$$\% \text{ RBC incorporation} = \text{cpm} \times \text{total blood vol} \times 100/0.5 \text{ (vol of blood counted)}/\text{total cpm injected}$$

Total blood volumes were estimated to be 6.5% of body weight (8). Total red cell counts were made with a Coulter counter, reticulocyte counts were done on smears stained with new methylene blue, and hematocrits were determined by the microtechnique.

Experiment II. Assays for ESF were performed using Swiss-Webster female mice in which plethora was induced by transfusion.

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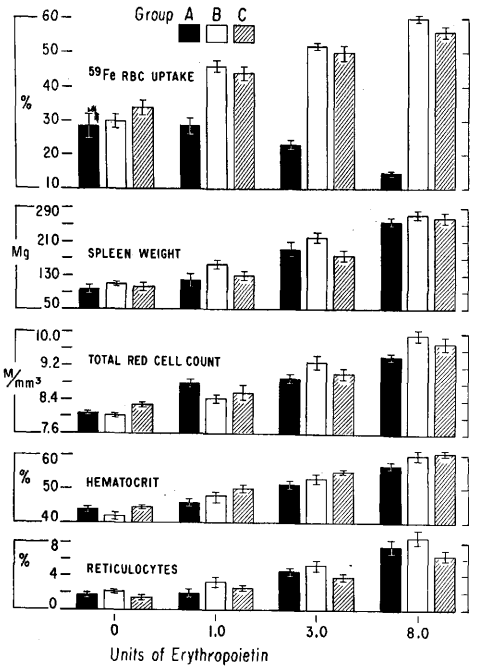

FIG. 1. Erythropoeitic response to daily injections of Step 1 ESF. Mice in group A received both ESF and ^{59}Fe ip, group B mice received ESF ip and ^{59}Fe iv, and Group C mice received ESF sc and ^{59}Fe ip. Each bar represents the data from 15 mice. The vertical lines represent the SE.

Blood for transfusion was obtained from donor mice, using acid-citrate–dextrose as an anticoagulant, centrifuged, and the supernatant was drawn off. The cells were washed twice with 0.9% saline, centrifuged, and the blood was reconstituted to a hematocrit of approximately 80%. Each assay mouse was transfused ip for 2 consecutive days with 1.0 ml of this concentrated blood. On days 5 and 6 after transfusion, 3 groups of mice (27–41 mice/groups) received 0.1 ml of the following test materials ip: (a) 4.0 units of Step 1 ESF, (b) 4.0 units of Step 3 ESF, or (c) 0.9% saline. The following day each group was divided into 3 subgroups and 0.5 μCi of ^{59}Fe in 0.5 ml of saline was injected either iv, ip, or sc. Forty-eight hours later 0.5 ml of blood was withdrawn via cardiac punture, its radioactivity was determined in a well-type scintillation counter, and the RBC utilization was calculated on the basis of radioactivity recovered related to the amount given. The formula presented above was used, however, in the polycythemic mouse; total blood volumes were estimated to be 7.3% of body weight. In this and the following experiment hematocrits were obtained on each animal at the time of autopsy; however, none were excluded for all had values above 60%. The schedule utilized for assaying ESF is summarized:

						0.1 ml Test material		0.5 μCi ^{59}Fe		
Transfused	–	–	–	–					–	Autopsy
Days	0	1	2	3	4	5	6	7	8	9

Experiment III. Four units of Step 1 ESF in 0.2 ml of saline were incubated with 0.2 mg of ferric citrate for 1 hr at 37°. This material was injected ip on days 5 and 6 and assayed in 14 polycythemic Swiss-Webster female mice. The next day 0.5 μCi of ^{59}Fe was also injected ip. Two control groups (10 and 15 mice/group) received either 0.2 ml of saline or 0.2 mg of ferric citrate dissolved in saline.

Two additional groups of mice (12 and 15/group) also received a total of 4.0 units of Step 1 ESF during days 5 and 6 after transfusion. However, on the following day, an attempt was made to block the reticuloendothelial system by injecting 0.2 ml of colloidal carbon (lot No. C11/1431a; Gunthar-Wagner, Hanover, Germany) iv into one group of mice while 0.5 μCi of ^{59}Fe was injected ip into both groups. Mice treated with saline and receiving colloidal carbon ip or iv prior to ^{59}Fe administration served as controls.

Results. Experiment I. The peripheral blood values and spleen weights for all groups of mice (Fig. 1) indicate increased erythropoietic activity in response to treatment with high dosages of ESF. However, group A mice (both ESF and ^{59}Fe ip) did not reflect this increased activity in the percentage of ^{59}Fe incorporated into the blood.

Experiment II. Polycythemic mice treated with 4.0 units of either Step 1 or Step 3 ESF incorporated similar quantities of ^{59}Fe (30.4–33.8% of the injected dose) into their peripheral blood when ^{59}Fe was administered either iv or sc (Fig. 2). However, ^{59}Fe uptake was approximately halved ($p < .01$) when both Step 1 ESF and ^{59}Fe were injected ip,

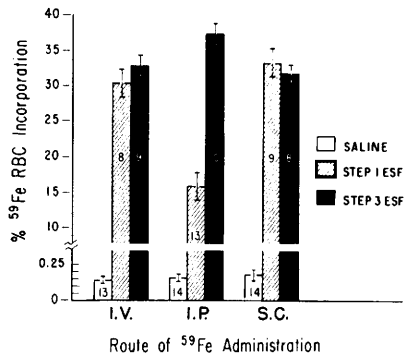

FIG. 2. Effect of the route of administration of ^{59}Fe on RBC uptake in polycythemic mice. Four units of ESF were injected ip. The numbers in each bar indicate the number of mice per group and the vertical lines represent the SE.

a phenomenon not observed following ip administration of both Step 3 ESF and ^{59}Fe. All saline-treated control mice incorporated approximately 0.15% of the injected dose of ^{59}Fe into their peripheral blood.

Experiment III. Treatment of assay mice with colloidal carbon prior to ip administration of ^{59}Fe and incubation of Step 1 ESF with ferric citrate were procedures which enhanced incorporation of ^{59}Fe into circulating erythrocytes (Table I).

Discussion. A review of the literature shows that a number of investigators (9–12) administered both test material and ^{59}Fe ip when assaying for ESF. The data from this study indicate that if crude preparations of ESF (Step 1) are given ip, accurate measurements of the rate of erythropoiesis cannot be obtained when ^{59}Fe is also injected ip; instead ^{59}Fe should be injected via an alternate route. However, this precaution may not be necessary when assaying more highly purified preparations of ESF (Fig. 2). In this regard it should be noted that there is a sixfold difference in the amount of contaminant present in the two preparations of ESF; Step 1 having a potency of approximately 0.6 units/mg while Step 3 has 3.5 units/mg. Gel electrophoretic studies by Goldwasser and Kung (13) have shown that a major contaminant of ESF is desialated ESF. However, there is no evidence that desialated ESF is present in the preparations used in this study. It is possible that a foreign protein which could be an inhibitor to ESF is present in this Step 1 anemic sheep plasma preparation and that this factor has been removed with further purification.

Several other possible mechanisms may explain how the increased impurity of Step 1 ESF may have caused unreliable measurements of erythropoiesis when both Step 1 ESF and ^{59}Fe were injected ip. The iron may have been bound to some protein or contaminant which was injected with the ESF and was not readily absorbed from the peritoneal cavity. Secondly, the contaminant, present in higher concentrations in the impure preparations of ESF, may have initiated an inflammatory response in the peritoneal cavity, thus interfering with passage of the iron into the portal circulation. This bound iron could then have been phagocytized by inflammatory cells, thus accounting for decreased iron uptakes in

TABLE I. Effects of Ferric Citrate and Colloidal Carbon on ^{59}Fe RBC Uptake in Polycythemic Mice.

No. of mice	Material injected ip	Treatment prior to ip injection of ^{59}Fe	^{59}Fe RBC uptake[a]
15	Saline		0.12 ± 0.02
10	Saline + ferric citrate		0.14 ± 0.03
13	ESF[b]		18.4 ± 1.74
14	ESF[b] + ferric citrate		25.6 ± 2.12
15	Saline	Colloidal carbon (iv)	0.11 ± 0.02
14	Saline	Colloidal carbon (ip)	0.09 ± 0.03
15	ESF[b]	Colloidal carbon (iv)	38.4 ± 1.90
12	ESF[b]	Colloidal carbon (ip)	38.9 ± 0.99

[a] Mean ± standard error of mean.
[b] 4.0 units of Step 1 erythropoietin.

the blood. However, this iron complex would have to be unlike normal transferrin for Finch *et al.* (14) have shown that reticuloendothelial cells are unable to take up iron directly from transferrin.

Several observations seem to support the latter theory. Brush smears of the peritoneal fluid of mice treated with large doses of ESF demonstrated increased numbers of inflammatory cells. Also, the amount of radioactivity remaining in the peritoneal cavity was much less in mice which received colloidal carbon prior to ^{59}Fe administration. It may have been the blocking of the reticuloendothelial system with colloidal carbon (15) which enhanced incorporation of ^{59}Fe into the peripheral blood. The phagocytic potential of these cells may have been saturated thus allowing the ^{59}Fe to pass from the peritoneal cavity into the vascular compartment. Similarly, ferric citrate may have bound to the contaminant and then become incorporated into the inflammatory cells, thus allowing ^{59}Fe to pass from the peritoneal cavity.

Summary. Several critical factors which interfere with the effects of ESF in iron incorporation may alter the sensitivity of a bioassay for ESF. Among these factors may be included the route of administration of ^{59}Fe. Depressed incorporation of ^{59}Fe into the blood occurs when crude preparations of ESF and ^{59}Fe are both administered ip. The results suggest that the problem is one of absorption and that this obstacle can be avoided by injecting test materials and ^{59}Fe by different routes.

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