JOURNAL OF NUCLEAR MEDICINE 5:595-605, 1964

Stimulation of Erythropoietin Production By Whole-Body Irradiation (Spleen Shielded)[1,2]

Abraham Gutnisky, Mary Lou Nohr, and Donald Van Dyke

Berkeley, California and Argentina

Sublethal whole body irradiation with the spleen shielded is followed within a day or so by development of increased erythropoiesis in the spleen (1). The rapid development of increased erythropoiesis in the shielded spleen occurs in the absence of any known stimulus to erythropoietin production (anemia, hypoxia, or excess cobaltous ion concentration), and has not been fully explained (2, 3, 4). This study investigated the mechanism of such stimulation in an attempt to clarify the role of erythropoietin in this response.

MATERIAL AND METHODS

Highly inbred male rats of the Buffalo strain weighing 100-200 g were used. Except where stated otherwise all rats underwent the same procedure: The spleen was surgically exteriorized, was held in a "coffin" of 3-mm lead during irradiation (or for the same time without irradiation), and then was replaced in the body. The variables were irradiation with or without shielding of the spleen, and hypertransfusion. Group I were normal controls (neither irradiated nor hypertransfused). Group II were hypertransfused (not irradiated). Group III were irradiated controls (given total body irradiation without spleen shielding and without hypertransfusion). Group IV were hypertransfused and given total body irradiation. Group V were irradiated with the spleen shielded but were not hypertransfused. Group VI were irradiated with the spleen shielded and were hypertransfused.

Rats were hypertransfused by being given 2.0 ml packed red cells per 100 g body weight (washed once with 0.9% saline solutions) intravenously on days 1 and 2. On day 3 all rats except Groups I and II were irradiated. Since hypertransfusion stops iron utilization and provides excess iron, those rats that had not been hypertransfused were given 4 mg of iron as an iron-dextran complex (Imferon) to ensure adequate iron stores in all groups.

---

[1]From the Donner Laboratory of Medical Physics, Lawrence Radiation Laboratory, University of California, Berkeley, California, and the Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentina.

[2]This work was supported in part by the United States Atomic Energy Commission.

On day 6 all rats were given 0.5 μc Fe^{59} intravenously. Five hours following Fe^{59} administration, blood was drawn from the tail vein for hematocrit and reticulocyte count, and, in some instances, for platelet and leucocyte count. The rats were anesthetized and as much blood as possible was drawn from the abdominal aorta. Through the needle in the aorta and with the jugular vein cut, the rat was perfused with 0.9% saline solution until the heart stopped beating. The completeness of perfusion was determined in a separate group of rats by giving Fe^{59} labeled red cells intravenously prior to perfusion and determining the Fe^{59} content of the spleen. The spleen was found to contain 0.16 ml of blood following perfusion and the values given for spleen uptake have been corrected for contained blood. The spleen was weighed and counted for Fe^{59} content in a well-type scintillation counter, and spleen smears were made for morphologic study. The smears were stained by LoBue's modification of Ralph's hemoglobin staining technique (5). The liver was weighed and counted for Fe^{59} content. The tibia was counted for determination of Fe^{59} uptake. The Fe^{59} content of both plasma and red blood cells was determined. The results were calculated on the basis of a blood volume of 5 per cent of body weight (6) for the nonhypertransfused and 7 per cent for hypertransfused rats.

The radiation conditions were similar to those previously used (1-4). Each rat was exposed singly under Pentomeph anesthesia. Radiation was generated at 220 kV 15 mA with a rate of 70 r per minute and a tube distance of 11 inches. Two filters were used: 1.0-mm Cu and 1.0-mm Al. The dose was calibrated by using a polyethylene phantom and a Victoreen ionization chamber. The dose to the body was 560 r in all instances. The dose inside the spleen shield was measured by inserting x-ray film and calibrating the developed film with a densitometer. The maximum dose to the shielded spleen was 0.5 r just inside the aperture for the splenic mesentery, and 0.2 r over the main mass of the spleen.

The responsiveness to erythropoietin was determined by using graded doses of hormone prepared by the collodion adsorption method (7) from the urine of a patient with aplastic anemia (8). The preparation used had been standardized against a sample of Standard A provided by the Division of Biological Standards, Medical Research Council of Great Britain, and was shown to contain 9 standard A units per mg.

The effect of erythropoietin antibodies on spleen-shielded rats was tested by use of serum from rabbits immunized with erythropoietin (9). The anti-erythropoietin activity of the rabbit serum had been determined by its ability to inhibit erythropoiesis in normal mice (10). Serum from normal rabbits or saline solution was given to the controls.

The studies on the rats were made on the third day postirradiation in order to be well in advance of the development of anemia. Five hours after Fe^{59} administration was chosen for autopsy, as that has been shown to be the time of maximal iron incorporation in the spleen of normal rats (11).

The rats used in this study were demonstrated to be free of Bartonella when splenectomized rats of the same strain failed to develop anemia after splenectomy.

TABLE I

EFFECT OF SPLEEN SHIELDING OR HYPERTRANSFUSION OR BOTH ON RED CELL PRODUCTION OF RATS AFTER 560 R WHOLE BODY X-IRRADIATION

Group	*Number of rats*	*Fe^{59} uptake (%)*		*Normoblasts in spleen (%)*	*Reticulocytes (%)*	*Hematocrit (%)*
		Spleen	*RBC*			
I. Normal controls (not irradiated, not hypertransfused)	4	5.8 ± 1.6*	15.8 ± 0.2	7.0 ± 2.6	7.5 ± 1.4	36.2 ± 0.7
II. Hypertransfused controls (not irradiated)	4	1.5 ± 0.1	7.0 ± 3.4	—	0.5 ± 0.4	70.0 ± 1.0
III. Irradiated controls (total body irradiation but no hypertransfusion)	4	0.1 ± 0.1	0.1 ± 0.1	0.1**	0	36.8 ± 1.0
IV. Total body irradiation plus hypertransfusion	4	0.1 ± 0.04	0.3 ± 0.3	0.2 ± 0.1	0.02 ± .01	67.9 ± 2.0
V. Irradiation with spleen shielded (not hypertransfused)	5	15.3 ± 2.1	6.0 ± 0.7	27.0**	0.3 ± 0.1	40.7 ± 1.5
VI. Irradiation with spleen shielded plus hypertransfusion	6	1.3 ± 0.8	0	0.4 ± 0.1†	0	68.3 ± 0.3

*Standard error of the mean.
**Average from 2 rats only.
†Average from 5 rats.

RESULTS

The results for the six primary groups are presented in Table I. As can be seen from the table, irradiation of the body with the spleen shielded (Group V) resulted in the well-known increase in erythrocyte precursors (normoblasts) in the spleen and in splenic radioiron incorporation (1-4). Splenic iron incorporation in this group was twice that in the nonirradiated controls (Group I). Combining hypertransfusion with irradiation and spleen shielding (Group VI) not only abolished the increase in splenic erythropoiesis that characterically follows irradiation, but even reduced the iron uptake to the level of the nonirradiated hypertransfused controls (Group II).

Administering rabbit serum (2 ml subcutaneously) containing erythropoietin antibodies immediately after irradiation and on each of the two following days (Table II) again abolished the increase in splenic Fe^{59} incorporation expected in the shielded spleen, and reduced the incorporation nearly to the level found after total body irradiation (Table I, Group III) or in hypertransfused spleen-shielded rats (Table I, Group VI). Platelet and leucocyte counts and leucocyte differential counts done on the rats in this experiment showed no significant difference between the antibody-treated and the control groups. These results indicate that erythropoiesis in the shielded spleen is controlled by erythropoietin.

The sensitivity of normal rats and irradiated spleen-shielded rats to various doses of erythropoietin was compared. Spleen-shielded rats were given erythropoietin subcutaneously 1 hour after irradiation and on the two days following; normal rats were given the same doses of erythropoietin at the same times; all were autopsied by the same schedule for comparison. The results are summarized in Table III and Figure 1. As can be seen from Figure 1, the injection of two units of erythropoietin, which had little effect on splenic iron incorporation in normal rats, had a maximal effect on Fe^{59} incorporation into the spleen of irradiated spleen-shielded rats. The dose-response curve rises rapidly for the shielded spleen. These data demonstrate that normal rats would require a very large dose of erythropoietin to induce splenic Fe^{59} incorporation comparable to that found in irradiated spleen-shielded rats.

TABLE II

SUPPRESSION OF SPLENIC ERYTHROPOIESIS BY ANTI-ERYTHROPOIETIN AFTER WHOLE BODY X-IRRADIATION WITH SPLEEN SHIELDED

Treatment	*Number of rats*	*Fe^{59} uptake (%)*		*Normoblasts in spleen (%)*	*Reticulocytes (%)*
		Spleen	*RBC*		
Anti-erythropoietin	6	0.5 ± 0.1*	0	0.6 ± 0.4	0.02 ± 0.02
Normal rabbit serum	4	33.6 ± 2.4	9.5 ± 3.3	21.6 ± 3.9	0.7 ± 0.4
Saline	3	21.6 ± 0.7	7.3 ± 2.9	36.6 ± 19.9	0.6 ± 0.3

*Standard error of the mean.

Comparison of the starting points of the curves in Figure 1 suggests that the irradiated rat has either a very high concentration of erythropoietin or a marked increase in sensitivity to normal concentrations of erythropoietin, or a combination. Twelve attempts were made to measure the titer of erythropoietin in the serum of irradiated, spleen-shielded rats, using the hypertransfused mouse assay (12). On five occasions, there was a suggestion of an elevated titer in serum; on seven occasions the results were clearly negative.

To demonstrate further the effect of irradiation of the rest of the body on the slope of the erythropoietin dose-response curve in the spleen, rats were hypertransfused to inhibit endogenous erythropoietin production; half the group was irradiated with the spleen shielded and the other half was not irradiated (sham-operated). Each group was then given various doses of erythropoietin and the effect on splenic Fe^{59} incorporation was compared. Table IV and Figure 2. Although the minimum effective dose for the irradiated and nonirradiated rats was the same, the slope of the curve was increased by irradiation.

Comparing the control values from Figures 1 and 2 indicates that endogenous erythropoietin in the nonirradiated rat is equivalent to the injection of 1.5 units of erythropoietin, whereas endogenous erythropoietin in the irradiated rat is equivalent to the injection of approximately 3 units; this suggests that irradiation

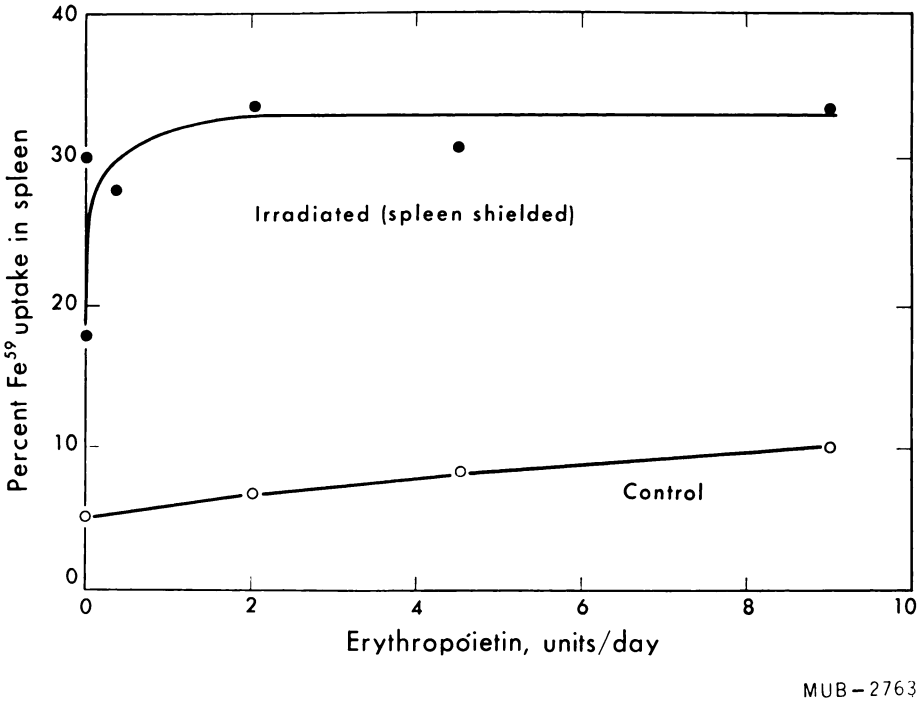

Fig. 1. Effect of various doses of erythropoietin on splenic iron incorporation in irradiated (spleen shielded) and normal (sham-operated) rats. These data demonstrate that normal rats would requre a very large dose of erythropoietin to induce splenic Fe^{59} incorporation comparable to that found in irradiated spleen-shielded rats.

results in a doubling of endogenous erythropoietin. Thus, the increase in splenic erythropoiesis that follows irradiation is apparently the result of a doubling of concentration of erythropoietin as well as an increased responsiveness of the spleen to the hormone. That increased responsiveness alone does not account for the accelerated erythropoiesis in the shielded spleen is further demonstrated by the experiment summarized in Table V. This experiment was designed to demonstrate that following suppression of endogenous erythropoietin by hypertransfusion, a dose of 1.5 units of erythropoietin was sufficient to increase splenic Fe^{59} incorporation to the level of the nonhypertransfused, nonirradiated control rats, but was not sufficient to raise splenic Fe^{59} incorporation to the level of the irradiated control. By comparing these results with the dose-response curves in Figure 1, one again sees that the response obtained in the shielded spleen (Group V, Table I) would require twice the dose needed to obtain the Fe^{59} uptake of the nonirradiated rat (Group I, Table I).

Liver uptake of Fe^{59} in all cases was the reverse of the splenic uptake; uptake in tibiae was suppressed by irradiation and did not contribute to interpretation of the results.

DISCUSSION

It has been demonstrated that extramedullary erythropoiesis in the lead-shielded spleen following total body irradition can be enhanced by administration

TABLE III

RESPONSE OF NORMAL OR IRRADIATED (SPLEEN-SHIELDED) RATS TO ERYTHROPOIETIN

Dose (Std. A units)	*Number of rats*	*Fe^{59} uptake (%)*		*Normoblasts in spleen (%)*	*Reticulocytes (%)*
		Spleen	*RBC*		
Irradiated					
9	5	33.2 ± 2.2*	18.0 ± 3.6	25.4 ± 4.9	1.8 ± 0.6
4.5	9	30.7 ± 0.6	20.3 ± 1.8	15.6 ± 1.9	2.7 ± 0.3
2	5	33.5 ± 2.0	9.0 ± 0.8	24.9 ± 6.2	1.5 ± 0.2
0.7	4	27.5 ± 1.1	4.6 ± 2.2	16.5 ± 1.4	0.7 ± 0.2
0.07	5	29.9 ± 3.4	5.5 ± 2.7	18.0 ± 3.2	0.7 ± 0.2
(Saline)	16	17.5 ± 2.0	8.0 ± 1.7	17.6 ± 2.2**	0.9 ± 0.3
Normal					
9	5	9.8 ± 0.7	29.2 ± 2.4	9.9 ± 4.8	7.7 ± 1.2
4.5	5	8.0 ± 1.0	14.8 ± 1.8	——	9.4 ± 0.8
2	5	6.5 ± 0.7	17.6 ± 3.6	6.6 ± 1.3	9.4 ± 0.4
(Saline)	14	5.1 ± 0.7	10.6 ± 1.4	4.8 ± 1.3***	5.7 ± 0.6

*Standard error of the mean.
**Average from 12 rats.
***Average from 9 rats.

of exogenous erythropoietin, and depressed by prior hypertransfusion or by the administration of erythropoietin antibodies. This is considered to be evidence that splenic erythropoiesis is, like normal medullary erythropoiesis, under erythropoietin control. Thus, the increase in splenic erythropoiesis that occurs following irradiation of the rest of the body is due to an effective increase in erythropoietin concentration in the absence of any known stimulant to erythropoietin production (anemia, hypoxia, or cobalt).

The response of the shielded spleen to increasing doses of erythropoietin has demonstrated an increase in the slope of the response curve over that in nonirradiated controls. To achieve the type of response seen in the shielded spleen requires increasing (doubling) the amount of erythropoietin introduced into the circulation. A decrease in rate of removal of erythropoietin from the circulation (13) or an increase in number of responsive cells (14) could account for the increased slope of the dose-response curve, an effect that would be equally operative for endogenous and exogenous erythropoietin. In order to duplicate the response obtained with endogenous erythropoietin, twice as much erythropoietin must be introduced into the irradiated spleen-shielded rat (in which endogenous

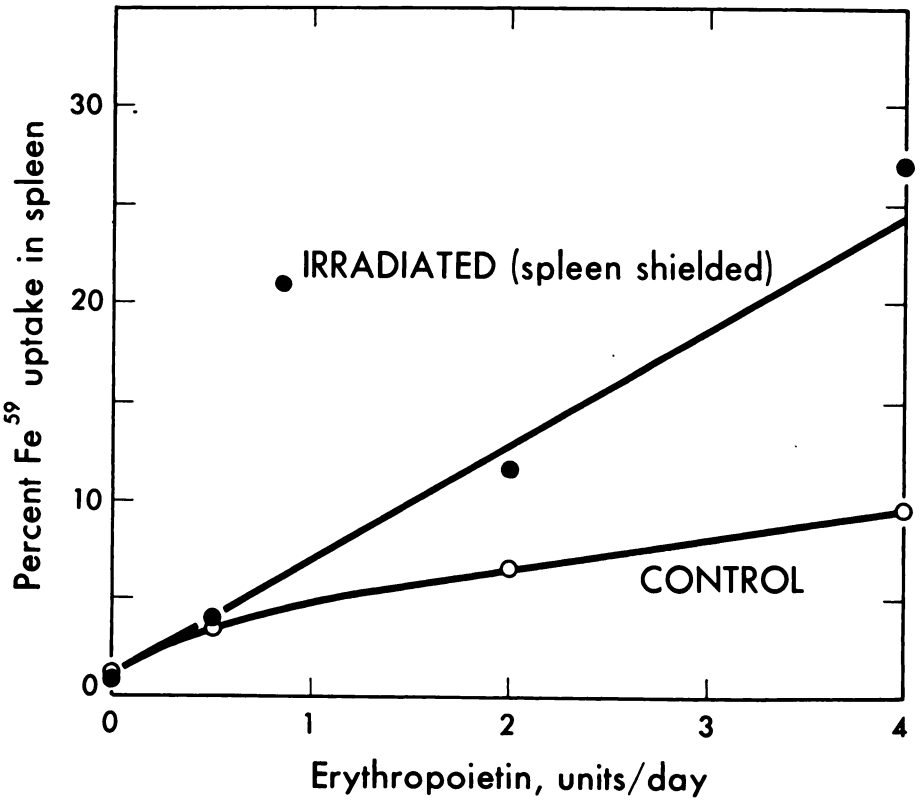

Fig. 2. Effect of various doses of erythropoietin on splenic radioiron incorporation in irradiated (spleen shelded) and normal rats in which endogenous erythropoietin production had been abolished by hypertransfusion.

erythropoietin production has been suppressed by hypertransfusion) as into the nonirradiated control; this indicates that whatever other changes occur, endogenous erythropoietin production doubles following irradiation. The concentration of erythropoietin may be considerably more than doubled if decreased rate of removal of the hormone accompanies a doubling of production.

Multiple attempts to measure the increased erythropoietin level in serum of irradiated spleen-shielded rats were inconclusive. The titer following irradiation was not sufficiently elevated to be demonstrable by the relatively insensitive assay methods currently available.

Although Eskuche and Hodgson (15) have concluded that total body irradiation does not further elevate the erythropoietin titer resulting from a given degree of anemia in rats, Stohlman and Brecher (13) found higher erythropoietin levels in irradiated than in nonirradiated animals exposed to a similar degree of hypoxia. Pesic *et al.* (16) have recently presented evidence suggesting that following total body irradiation in normal dogs the titer of erythropoietin may rise to measure levels. The present results confirm the suggestion by Stohlman and Brecher (17) that immediately following irradiation an animal is more sensitive to erythropoietic stimuli.

If one accepts the evidence as indicating an increased rate of entry of erythropoietin into the circulation, what is the mechanism? The known stimuli to increased erythropoietin production are hypoxia, anemia, and an excess of cobaltous ion, none of which appears to be a factor in this experiment. Is it possible that what appears to be increased production of erythropoietin is simply

TABLE IV

EFFECT OF VARIOUS DOSES OF ERYTHROPOIETIN ON RETICULOCYTES AND Fe^{59} UPTAKE IN SPLEEN AND RED CELLS OF HYPERTRANSFUSED RATS, IRRADIATED (SPLEEN SHIELDED) AND NOT IRRADIATED

Dose (Std. A units)	*Number of rats*	*Reticulocytes (%)*	*Fe^{59} uptake (%)*	
			Spleen	*RBC*
Irradiated, hypertransfused				
4	5	0.07 ± .02*	26.8 ± 1.6	4.7 ± 1.3
2	5	0.04 ± .02	11.3 ± 1.7	1.3 ± 0.3
0.5	5	0.02 ± .02	3.6 ± 1.5	0.4 ± 0.2
(Saline)	3	0	0.6 ± 0.3	0.5 ± 0.5
Hypertransfused control				
4	5	2.4 ± 0.3	9.1 ± 0.7	18.1 ± 1.6
2	5	1.6 ± 0.1	6.2 ± 0.8	14.7 ± 0.6
0.5	5	1.1 ± 0.1	3.1 ± 0.5	6.9 ± 0.6
(Saline)	4	0.4 ± 0.1	0.8 ± 0.1	1.5 ± 0.9

*Standard error of the mean.

TABLE V

EFFECT OF 1.5 STANDARD A UNITS OF ERYTHROPOIETIN ON SPLEEN UPTAKE OF HYPERTRANSFUSED AND HYPERTRANSFUSED IRRADIATED (SPLEEN SHIELDED) RATS

Group	*Number of rats*	*Fe^{59} uptake (%)*		*Reticulocytes (%)*	*Hematocrit (%)*
		Spleen	*RBC*		
Hypertransfused irradiated (spleen shielded), 1.5 units per day for 3 days	7	6.1 ± 0.8*	0.5 ± 0.2	0.08 ± 0.04	68.7 ± 1.4
Hypertransfused, sham-operated, 1.5 units per day for 3 days	7	4.1 ± 0.3	14.3 ± 0.8	1.5 ± 0.2	61.1 ± 0.7
Irradiated (spleen shielded), 1 ml saline per day for 3 days. (4 mg Imferon at time of irradiation)	6	13.5 ± 3.2	9.5 ± 2.9	2.0 ± 0.5	39.4 ± 1.2
Sham-operated, 1 ml saline per day for 3 days. (4 mg Imferon at time of operation)	6	4.2 ± 0.5	15.7 ± 1.5	6.2 ± 0.6	39.3 ± 0.8

*Standard error of the mean.

release of stored hormone into the circulation through damaged cell membranes? This does not appear to be the case, as hypertransfusion—which would not be expected to alter cell permeability significantly—completely abolishes the effect. The dose of radiation used may cause sufficient alteration in hemodynamics to result in areas of local hypoxia, producing an erythropoietin release. These studies do not answer the questions that might be raised, and such speculations are presented only because one hesitates to propose radiation as an entirely new and different stimulus to erythropoietin production.

SUMMARY

Increase in erythropoiesis in the spleen as a result of irradiating the remainder of the body has again been demonstrated. That the erythropoiesis in the shielded spleen can be abolished by hypertransfusion or administration of erythropoietin antibody and enhanced by administration of exogenous erythropoietin indicates that erythropoiesis in the shielded spleen is, like normal marrow, under erythropoietin control.

Since erythropoietin production is apparently increased in the absence of any known stimulant (anemia, hypoxia, or increased cobaltous ion concentration), it is suggested that irradiation may be a stimulus to the release of increased amounts of erythropoietin into the circulation via a mechanism not yet understood.

The results of this study indicate that increased erythropoiesis in the shielded spleen following irradiation results from a combination of doubling the erythropoietin production and increasing the sensitivity to erythropoietin.

ACKNOWLEDGMENT

The authors are greatly indebted to Dr. John H. Lawrence for his support of this project.

BIBLIOGRAPHY

1. JACOBSON, L. O., MARKS, E. K., GASTON, E., ROBSON, M., and ZIRKLE, R. E.: The Role of the Spleen in Radiation Injury. *Proc. Soc. Exper. Biol. & Med.* **70**:740, 1949.
2. JACOBSON, L. O., MARKS, E. K., ROBSON, M., GASTON, E., and ZIRKLE, R. E.: The Effect of Spleen Protection on Mortality Following X-radiation. *J. Lab. Clin. Med.* **34**:1538, 1949.
3. JACOBSON, L. O., SIMMONS, E. L., MARKS, E. K., ROBSON, M. S., BETHARD, W. F., and GASTON, E. O.: The Role of the Spleen in Radiation Injury and Recovery. *J. Lab. Clin. Med.* **35**:746, 1950.
4. HUFF, R. L., BETHARD, W. F., GARCIA, J. F., ROBERTS, B. M., JACOBSON, L. O., and LAWRENCE, J. H.: Tracer Iron Distribution Studies in Irradiated Rats with Lead-Shielded Spleens. *J. Lab. Clin. Med.* **36**:40, 1950.
5. LOBUE, J., DORNFEST, B. S., GORDON, A. S., HURST, J., QUASTLER, H.: Marrow Distribution in Rat Femurs Determined by Cell Enumeration and Fe^{59} Labeling. *Proc. Soc. Exper. Biol. and Med.* **112**:1058, 1963.
6. GARCIA, J. F.: Changes in Blood, Plasma and Red Cell Volume in the Male Rat, as a Function of Age. *Am. J. Physiol.* **190**:19, 1957.
7. VAN DYKE, D. C.: Sources and Properties of Human Urinary Erythropoietin. *Haemopoiesis*, ed. Wolstenholme, G.E.W. and O'Connor, M. London. J & A Churchill, Ltd., 1960.

8. VAN DYKE, D. C., LAYRISSE, M., LAWRENCE, J. H., GARCIA, J. F., and POLLYCOVE, M.: Relation Between Severity of Anemia and Erythropoietin Titer in Human Beings, *Blood* **18**:187, 1961.
9. SCHOOLEY, J. C. and GARCIA, J. F.: Immunochemical Studies of Human Urinary Erythropoietin. *Proc. Soc. Exper. Biol. & Med.* **109**:325, 1962.
10. SCHOOLEY, J. C. and GARCIA, J. F.: Immunologic Studies on the Mechanism of Action of Erythropoietin. *Proc. Soc. Exper. Biol. & Med.* **110**:636, 1962.
11. GARCIA, J. F.: Radioiron Time-Distribution Studies at Various Ages in the Normal Male Rat. *Am. J. Physiol.* **190**:31, 1957.
12. DEGOWIN, R. L., HOFSTRA, D., and GURNEY, C. W.: A Comparison of Erythropoietin Bioassays. *Proc. Soc. Exper. Biol. & Med.* **110**:48, 1962.
13. STOHLMAN, F., JR. and BRECHER, G.: Humoral Regulation of Erythropoiesis. V. Relationship of Plasma Erythropoietine Level to Bone Marrow Activity. *Proc. Soc. Exper. Biol. & Med.* **100**:40, 1959.
14. NOYES, W. D., FINCH, C. A., WASSERMAN, H., and GLICKMAN, K.: Partial Marrow Shielding and Total-Body Irradiation. *J. Appl. Physiol.*, **18**:629, 1963.
15. ESKUCHE, I. and HODGSON, G.: Sustained High Levels of Erythropoiesis Stimulating Factor(s) in Plasma of Irradiated Phenylhydrazine-Treated Rats. *Acta Physiol. Latino Am.* **12**:228, 1962.
16. PESIC, N., RADOTIC, M., and HAJDUKOVIC, S.: Erythropoietin Production Following Gamma Irradiation and Hemorrhage in Dogs. *Science* **143**:49, 1964.
17. STOHLMAN, F., JR. and BRECHER, G.: Stimulation of Erythropoiesis in Sublethally Irradiated Rats by a Plasma Factor. *Proc. Soc. Exper. Biol. & Med.* **91**:1, 1956.