

Effects of Erythropoietin on Erythroid Colony Formation in Uremic Rabbit Bone Marrow Cultures

By Y. Moriyama and James W. Fisher

Erythropoietin-responsive stem cell (ERC) kinetics in anephric uremic rabbits were studied *in vitro* using the growth of erythroid colonies in a methyl cellulose system in cultures with and without the addition of erythropoietin (ESF). Approximately 68 hr after bilateral nephrectomy, an increase in BUN and decreases in hematocrit and marrow erythroid cellularity were seen. However, the numbers of erythroid colonies formed in response to ESF on plates inoculated with 2×10^5 cells were greater in anephric rabbit marrows than in normal controls. In addition, the numbers of erythroid colonies produced by

the uremic and normal marrows in the presence of ESF were increased in proportion to the number of precursors plated. These findings suggest that, in uremia, the concentration of ERC is increased and that the ERC are capable of responding normally to ESF. The increase in the number of erythroid colonies in uremia may be due to the undisturbed flow of uncommitted hematopoietic stem cells into the ERC compartment in the presence of a delay of differentiation of ERC into heme-synthesizing nucleated erythroid cells due to a lack of ESF.

ANEMIA IS A FREQUENT complication of chronic renal failure which may be partially relieved, but not completely corrected, by dialysis therapy. The primary mechanisms involved in the anemia of renal insufficiency are postulated to be (1) reduced production of erythropoietin (ESF) by the diseased kidneys, (2) hemolysis and shortened red cell life span, (3) changes in the response of the bone marrow to ESF, and (4) plasma inhibitors of erythropoiesis.¹ However, the role of pluripotential hematopoietic stem cell (CFC) and erythropoietin-responsive stem cell (ERC) proliferation in the anemia associated with renal insufficiency is not clear and heretofore has not been investigated. In addition, in considering therapy (such as the administration of ESF and/or androgens) of the anemia of renal failure, it is important to know whether the erythroid stem cell compartment is intact. We showed in our previous studies^{1,2} that the relative response of uremic marrows to ESF *in vitro* was significantly greater than that of normal bone marrows, although total baseline heme synthesis in the uremic bone marrow cultures was significantly less than that of normal controls. This suggests that, in uremia, the ERC compartment is either more sensitive to ESF *in vitro*, or else that it has an increase in concentration of ERC when compared with that of normal controls.

In this study, we attempt to assess the role of the ERC compartment in the

From the Department of Pharmacology, Tulane University School of Medicine, New Orleans, La. 70112.

Submitted June 20, 1974; accepted November 12, 1974.

Supported by USPHS Contract AM-70-2112 from the Artificial Kidney/Chronic Uremia Program—NIAMDD.

Address for reprint requests: James W. Fisher, Ph.D., Department of Pharmacology, Tulane University School of Medicine, 1430 Tulane Ave., New Orleans, La. 70112.

© 1975 by Grune & Stratton, Inc.

mechanism of the anemia of uremia using an *in vitro* assay for erythroid cell colony formation in bone marrows from anephric uremic rabbits. There was a significant increase in the numbers of erythroid colonies in cultures, both with and without ESF, in uremic rabbit marrows compared with those of normal rabbit bone marrow cultures.

MATERIALS AND METHODS

Young New Zealand albino rabbits weighing approximately 2.5 kg were made uremic by the removal of both kidneys under pentobarbital (20 mg/kg) anesthesia. Normal intact rabbits were used as controls. Approximately 68 hr after bilateral nephrectomy, the rabbits were sacrificed by exsanguination via cardiac puncture. Reticulocyte counts, hematocrits, and serum BUN were determined for each rabbit. Femoral bone marrow smears were prepared, and the erythroid cellularity was determined as per cent of total nucleated marrow cells using May-Grünwald and Giemsa stains.

A modification of the method of Iscove et al.³ was used in the *in vitro* assay for erythroid colony formation as follows: bone marrow cells were flushed from the femurs into alpha-medium (Flow Laboratories, Inc., Inglewood, Calif.), dispersed through a sterile pipette, and washed twice with cold alpha-medium containing 10% fetal calf serum (Grand Island Biological Company, Grand Island, N.Y.). In order to remove as many granulocytes from the marrow cells as possible, a portion of the washed marrow cells (5×10^7 nucleated cells) in 10 ml alpha-medium containing 25% fetal calf serum was placed in a plastic 100-mm tissue culture dish and incubated at 37°C in 95% air and 5% CO₂ for 1 hr. The suspended nonadherent cells were then pipetted into the culture dishes.

For cultures of erythroid colonies, 2×10^5 nucleated marrow cells, from which granulocytes had been removed as described above, were plated on 35 × 10-mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.) in 1 ml alpha-medium containing 0.8% methyl cellulose, 30% fetal calf serum, 1% deionized bovine serum albumin,⁴ penicillin (50 U), and streptomycin (20 μg).

Human urinary erythropoietin* with a specific activity of 5.29 U/mg was dissolved at several concentrations in alpha-medium, sterilized by passage through Millipore filters (0.45 μ), and added in 5-μl volumes to each culture plate. Controls with and without ESF were incubated with each experiment. Three plates were prepared for each group.

Cultures were incubated for 4 days at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Erythroid colonies containing ten or more cells were scored on one quarter of the total plate area using an inverted microscope at 75 × magnification. Erythroid colonies were identified after staining with benzidine.⁵ For each experiment, the numbers of erythroid colonies on each of three replicate plates were averaged and the mean and standard error determined.

RESULTS

Table 1 shows the hematologic changes in the normal and bilaterally nephrectomized rabbits. In this study, 68 hr after bilateral nephrectomy, anephric rabbits had a decrease (−22.3%) in hematocrit, an increase (+11.3 ×) in BUN, and a decrease in reticulocyte counts when compared with that of normal rabbits. The marrow erythroid cellularity in uremia was also significantly decreased (−39.5%), indicating a marked suppression in erythropoiesis in the uremic rabbits following bilateral nephrectomy.

In order to determine whether or not the ERC compartment is still intact in this type of uremia, an *in vitro* assay for erythroid colony formation was per-

*The erythropoietin was collected and concentrated by the Department of Physiology, University of The Northeast, Corrientes, Argentina, further processed and assayed by the Hematology Research Laboratories, Childrens Hospital of Los Angeles, Los Angeles, Calif. under Research Grant HE 10880 from the National Heart and Lung Institute.

Table 1. Comparison of Hematologic Values in Normal and Anephric Uremic Rabbits*

Treatment of Rabbits	No. Animals	Hematocrit (%)	BUN (mg/100 ml)	Reticulocytes (%)	Marrow Erythroid Cellularity (%)†
Normal rabbits	10	41.3 ± 1.1	16.3 ± 1.9	2.8 ± 0.4	35.2 ± 1.0
Anephric rabbits	7	32.1 ± 1.0‡	183.2 ± 12.8‡	1.3 ± 0.2‡	21.3 ± 2.1‡

All values represent mean ± SEM.

*Sixty-five to seventy-two hours after bilateral nephrectomy.

†Represents the mean of only five animals.

‡Significantly different ($p < 0.05$) from normal rabbits.

formed with and without the addition of ESF. These cultures contained 2×10^5 nucleated marrow cells obtained from normal and anephric rabbits. As illustrated in Fig. 1, the numbers of erythroid cell colonies in both normal and anephric uremic rabbit bone marrow cultures were increased in proportion to the increasing concentrations of ESF, reaching a plateau at 0.1 U of ESF. However, the numbers of erythroid colonies in the uremic marrows, with and without ESF, were significantly ($p < 0.05$) greater than those of normal controls. The marrow erythroid cellularity was markedly decreased in uremia.

The results of the addition of a single concentration of ESF (0.02 U) to varying concentrations of marrow cells in cultures are shown in Fig. 2. In these experiments the regression line for the numbers of erythroid colonies versus the number of erythroid precursors in the anephric rabbit marrows was shifted to the left but was still parallel to that of normal controls. There were no significant differences between the response to ESF in vitro of marrows obtained from anephric uremic and normal rabbits.

DISCUSSION

In the present studies, bilateral nephrectomy in rabbits induced a marked uremia and anemia indicated by the significant increase in BUN and decreases in hematocrit and marrow erythroid cellularity. Erythroid colony formation,

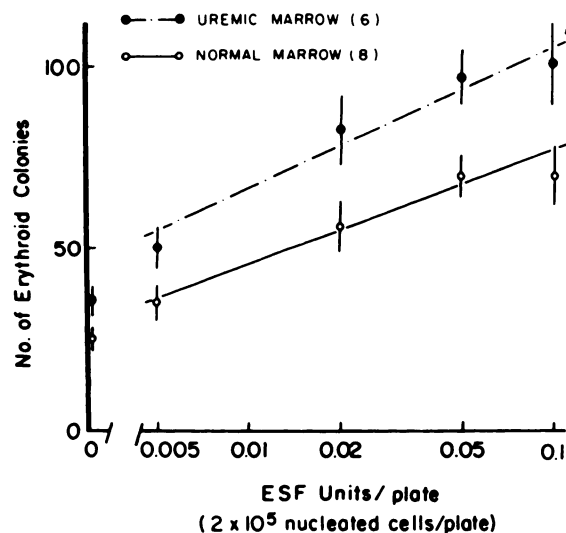


Fig. 1. Dose-response regression lines for erythroid colonies in normal and uremic rabbit bone marrow cultures treated with erythropoietin. * indicates significant ($p < 0.05$) difference from normal marrow at all doses of ESF.

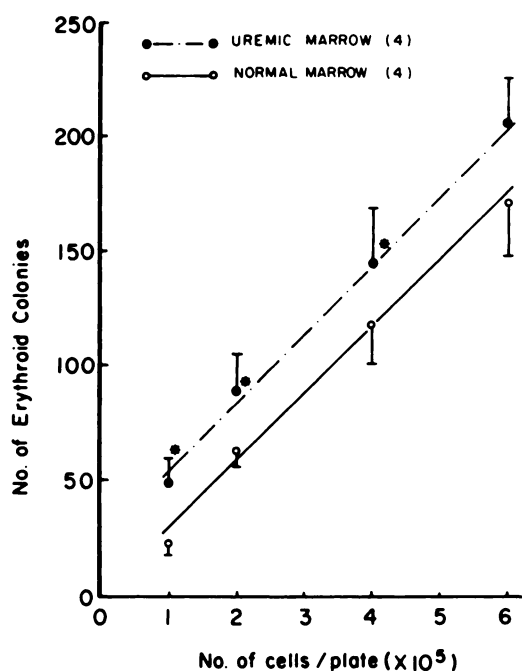


Fig. 2. Regression lines for numbers of erythroid colonies versus numbers of cells in culture from normal and uremic rabbits in the presence of ESF. * indicates significant ($p < 0.05$) difference from normal rabbit bone marrow.

assessed with the use of a methyl cellulose gel system,³ was greater in uremic marrows than in normal control marrows. Similar increases in the numbers of colonies in response to several doses of ESF were seen. In addition, the number of erythroid colonies formed by the uremic marrows was found to be proportional to the number of erythroid precursors plated, and the response to ESF paralleled the increase seen in normal controls, indicating no significant difference between the response of anemic uremic and normal marrows to ESF in vitro. The ability of ESF to stimulate erythroid colony formation using this culture method has been confirmed by other investigators.^{3,6} In the presence of ESF, the number of erythroid colonies is postulated to be directly proportional to the number of precursors inoculated.⁷ Thus, this culture system was very useful to us as a technique for evaluating the number of erythropoietin-responsive stem cells (ERC).^{6,8}

Several previous reports have appeared which have attempted to clarify the mechanism of the anemia of renal insufficiency as well as the role of inhibitor(s) of erythropoiesis in uremia. However, the role of erythroid stem cell kinetics in the anemia associated with renal failure has not been heretofore investigated. In addition, further clarification of the role of the erythroid stem cell compartment in anemia of uremia and the response of uremic marrow to ESF may provide a more rational basis for the treatment of the anemia of renal insufficiency.

In previous studies^{1,2} we demonstrated that the in vitro ESF response of marrows from anephric rabbits and patients with chronic renal failure was greater than that seen with normal controls, using the relative change in ⁵⁹Fe incorporation into heme. However, total heme synthesis per plate in uremic marrows was less than that of normal controls. These earlier findings support

our present observations of an increase in the numbers of erythroid colonies in anephric uremic marrow cultures, indicating that the ERC compartment responds to ESF stimulation, even in uremia, to cause ERC to differentiate into heme-synthesizing cells. The decrease in total heme synthesis per plate in uremic marrow cultures is probably due to the decreased numbers of heme-synthesizing marrow erythroid cells following nephrectomy as the result of ESF deficiency and/or the presence of inhibitors of erythropoiesis.

Our finding in the present study of an increase in the numbers of erythroid colonies in uremia with a normal response to ESF suggests that, when a sufficient amount of ESF is available in the anemia of uremia, the marrow may be capable of responding to correct the erythropoietic defect. However, the presence of a substance(s) in uremic serum which inhibits erythropoiesis^{1,2,9-12} must also be considered. Perhaps continuing renal dialysis may also be necessary to remove these inhibitors to completely correct the anemia of renal insufficiency. The increase in concentration of ERC in the marrows of rabbits in renal insufficiency may occur because of the undisturbed flow of uncommitted hematopoietic stem cells (CFC) into the ERC compartment in association with a delay of ERC differentiation due to lack of ESF. It is possible that, since there is a reduced cellularity in the bone marrows from the uremic rabbits, that the increase in concentration of ERC in the marrows is a reflection of the reduced numbers of differentiated cells. However, this is difficult to assess because no techniques are available to measure the absolute numbers of ERC in the total body marrow compartment. In previous studies,^{1,2} sera from anephric rabbits and plasma from human subjects with chronic renal failure were found to significantly inhibit ⁵⁹Fe incorporation into heme in bone marrow cultures. The inhibitory activity in uremic serum was increased exponentially with increasing concentrations of sera in cultures. The inhibitor was estimated to be a low-molecular-weight substance, with a molecular weight between 2000 and 5000 as measured by an Amicon filtration system and Sephadex G-100 fractionation.¹⁴ This inhibitor of heme did not modify the number of erythroid colonies formed per plate in our *in vitro* system.¹⁵ Therefore, these data suggest that this inhibitor in uremic serum does not affect the ability of the erythroid stem cells to form erythroid colonies in response to ESF *in vitro*, but that it probably acts more specifically to inhibit heme synthesis in the differentiated nucleated erythroid cell compartment. Bozzini et al.⁹ have suggested that a decrease in the responsiveness of the erythroid cell compartment to ESF in acutely uremic animals may also be involved in the mechanism of the anemia of uremia. The level of inhibitors of heme synthesis in the plasma of these uremic rats should be known before it is concluded that the marrow *per se* is less responsive to ESF. Thus, it is important to emphasize that the existence of a serum inhibitor of erythropoiesis should always be considered in the treatment of patients with anemia of uremia with ESF.

REFERENCES

1. Fisher JW, Lertora JLL, Lindholm DD, Tornoyos K, Moriyama Y: Erythropoietin production and inhibitors in serum in the anemia of uremia. Proceedings of the Third Annual Clinical Dialysis and Transplant Forum vol. 3. Washington, D.C., 1973, p 22-32
2. Moriyama Y, Lertora JLL, Fisher JW: Response of uremic rabbit bone marrow cultures

in vitro to erythropoietin and testosterone. *Clin Res* 22:67, 1974

3. Iscove NN, Sieber F, Winterhalter KH: Erythroid colony formation in cultures of mouse and human bone marrow: Analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A¹. *J Cell Physiol* 83:309, 1974

4. Worton RG, McCulloch EA, Till JE: Physical separation of hemopoietic stem cells from cells forming colonies in culture. *J Cell Physiol* 74:171, 1969

5. Borsook H, Ratner K, Tattie B: Studies on erythropoiesis. II. A method of segregating immature from mature adult rabbit erythroblasts. *Blood* 34:32, 1969

6. Stephenson JR, Axelrad AA, McLeod DL, Shreeve MM: Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. *Proc Natl Acad Sci USA* 68:1542, 1971

7. Cooper MC, Levy J, Cantor LN, Marks PA, Rifkind RA: Effect of erythropoietin (ESF) on colonial growth of erythroid precursor cells in vitro. Sixteenth Annual Meeting, American Society of Hematology, Chicago, 1973

8. Moriyama Y, Fisher JW: Effects of testosterone and erythropoietin on erythroid colony formation in rabbit bone marrow cultures. *Life Sci* 15:1181-1188, 1974

9. Bozzini CF, DeVoto FCH, Tomio JM: Decreased responsiveness of hematopoietic tissue to erythropoietin in acutely uremic rats. *J Lab Clin Med* 68:411, 1966

10. Eschbach JW, Funk D, Adamson JW, Kuhn I, Scribner BH, Finch CA: Erythropoiesis in patients with renal failure undergoing chronic dialysis. *N Engl J Med* 276:653, 1967

11. Fisher JW, Hatch FE, Roh BL, Allen RC, Kelley B: Erythropoietin inhibitor in kidney extracts and plasma from anemic uremic human subjects. *Blood* 31:440, 1968

12. Moriyama Y, Saito H, Kinoshita Y: Erythropoietin inhibitor in plasma from patients with chronic renal failure. *Haematologia (Budap)* 4:15, 1970

13. Moriyama Y, Lertora JLL, Rege AB, Fisher JW: Inhibitors of erythropoiesis in uremia. *Fed Proc* 33:597, 1974

14. Rege AB, Moriyama Y, Fisher JW: Characterization of a serum inhibitor of erythropoiesis. *Fed Proc* 33:597, 1974

15. Moriyama Y, Rege AB, Fisher JW: Effects of normal and uremic sera on heme synthesis and on erythroid colonies in vitro in rabbit bone marrow cultures. *International Symposium on Erythropoiesis, Tokyo (in press)*