PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 94–97 (1975)

Studies on an Inhibitor of Erythropoiesis. II. Inhibitory Effects of Serum from Uremic Rabbits on Heme Synthesis in Rabbit Bone Marrow Cultures[1] (38483)

Y. MORIYAMA, A. REGE, AND J. W. FISHER

Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Anemia is one of the important complications of chronic renal failure which cannot be corrected by dialysis therapy. Major contributing factors in this anemia are the reduced production of erythropoietin (ESF) by the diseased kidneys and an increase in the rate of hemolysis of red cells. However, the mechanisms of anemia of uremia cannot be completely explained by these factors alone because, (a) some uremic patients who have detectable ESF levels in their sera remain severely anemic (1, 2); (b) the *in vivo* response to ESF is suppressed in uremic conditions (3) even though the function of erythropoietin responsive stem cells is intact in cultures as assessed by erythroid colony formation (4); and (c) the erythroid cellularity in uremic bone marrows is often not correlated with the degree of anemia (5). These findings suggest that toxic effects of metabolic products of uremia on erythroid cells, especially the presence of inhibitor substance(s), are important in the mechanism of the anemia of uremia. A plasma factor capable of inhibiting the *in vivo* response to ESF has been described by Fisher *et al.* (6) and Moriyama *et al.* (7). In addition we have recently reported the existence of a tissue-specific inhibitor of erythropoiesis *in vitro* in sera from normal and polycythemic rabbits (8). However, the chemical nature of this inhibitor, the role of this serum inhibitor in the anemia of uremia and the relationship between the uremic inhibitor in normal and polycythemic sera are not clear.

The present studies were undertaken to determine whether a serum inhibitor of erythropoiesis is present in anephric uremic rabbits which inhibits heme synthesis in erythroid cells in bone marrow cultures and to attempt to clarify the role of such inhibitors in the mechanism of the anemia of renal insufficiency.

Materials and Methods. New Zealand albino rabbits weighing approximately 2.5 kg were bilaterally nephrectomized under pentobarbital (20 mg/kg) anesthesia. Approximately 68 hr after nephrectomy, the uremic animals (BUN 183.2 ± 12.8 mg/100 ml, Hct 32.1 ± 1.0%) were sacrificed and blood was collected by cardiac puncture and the serum separated. Sera collected from normal rabbits (BUN 16.3 ± 1.9%, Hct 41.3 ± 1.1%) served as controls. These sera were stored at −18° until used in the bone marrow cultures. The bone marrow culture technique (8) used was a modification of the method of Krantz *et al.* (9). Bone marrow cells were flushed from the normal rabbit femurs into cold NCTC-109 solution (Microbiological Associates, Inc. Bethesda, MD), dispersed through a sterile pipette and washed twice with cold NCTC-109 solution.

For cultures where ^{59}Fe incorporation into heme was determined, 2×10^6 washed normal rabbit marrow cells were plated in 35 × 10 mm tissue culture dishes (Falcon Plastics, Oxnard, CA) in 2 ml NCTC-109 solution containing varying concentrations of test sera which included normal and uremic rabbit sera and newborn calf serum (Microbiological Associates, Inc.), streptomycin (50 μg/ml), penicillin (50 units/ml) and heparin (10 units/ml). Three plates were prepared for each group. The cultures were incubated with and without the addition of human urinary erythropoietin with a specific activity of 5.29 units/mg in a vol of 20 μl NCTC-109 solution at 37° in a humidified atmosphere of 5% CO_2 in air. After 29 hr of incubation, ^{59}Fe bound to homologous transferrin was added (0.5 μCi/plate), and

[1] Supported by USPHS Contract No. AM 70-2112 from the Artificial Kidney/Chronic Uremia Program—NIAMDD.

the incubation continued for an additional 16 hr. The cells were then harvested, acidified, and the heme extracted into cyclohexanone. Radioactivity of the cyclohexanone extracts was determined with a Model 3320 Packard Scintillation Counter.

Results. The effects of normal and uremic rabbit sera on ^{59}Fe incorporation into heme in the normal bone marrow cells are shown with and without stimulation with ESF (0.02 unit) in Table I. The results indicate that the inhibitory effect of uremic sera on the erythropoietic response to added ESF in cultures was significantly ($P < 0.05$) greater than that of the normal rabbit sera. However, there was no significant difference between ^{59}Fe incorporation into heme in cultures with normal and uremic sera in the absence of ESF.

As illustrated in Fig. 1, total heme synthesis per plate decreased exponentially with increasing concentrations of both normal and uremic sera in cultures. The inhibitory activity of uremic sera at a concentration of 20% in cultures was significantly ($P < 0.01$) greater than that of normal sera.

The relationship between the hematocrit of the test animals and the effects of their sera on heme synthesis in cultures are shown in Fig. 2. Test sera were from anemic, normal and polycythemic rabbits (8). The responses to these sera were along the same regression line, indicating that the concentration of the serum inhibitor is inversely proportional to the increase seen in the hematocrit. On the other hand, the response to uremic sera was below the regression line indicating a higher level of this inhibitor of heme in uremia.

In order to determine whether such an inhibition of heme synthesis by uremic sera

TABLE I. INHIBITORY EFFECT OF UREMIC SERUM ON HEME SYNTHESIS IN NORMAL RABBIT BONE MARROW CULTURES WITH AND WITHOUT ERYTHROPOIETIN.

Treatment of cultures	No. of experiments	% ^{59}Fe incorporation[a] into heme ± SEM
10% normal rabbit serum	(10)	7.7 ± 0.4
10% normal rabbit serum + ESF (0.02 unit)	(10)	17.8 ± 0.6
10% uremic rabbit serum	(9)	7.8 ± 1.0
10% uremic rabbit serum + ESF (0.02 unit)	(9)	15.2 ± 1.1[b]

[a] Three culture plates/experiment.

[b] Mean value significantly ($P < 0.05$) less than that of normal rabbit serum.

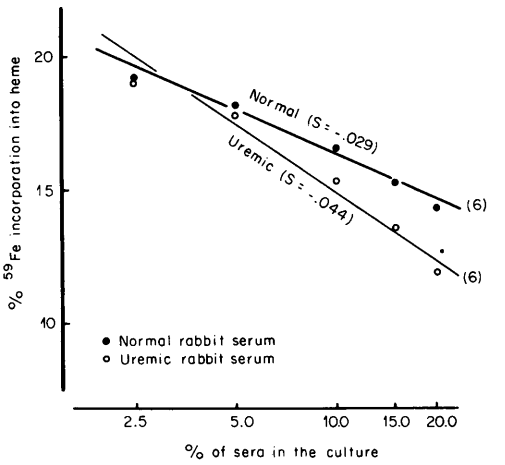

FIG. 1. Effects of various concentrations of normal and uremic rabbit sera on heme synthesis in bone marrow cultures with ESF (0.02U). * significantly ($P < 0.01$) different from that of normal rabbit sera.

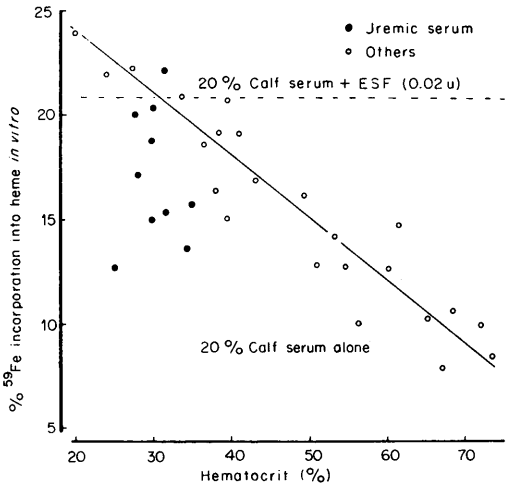

FIG. 2. Relationship between heme synthesis and hematocrit with various rabbit sera in bone marrow cultures in the presence of ESF (0.02U). Each point indicates heme synthesis with 10% test sera + 10% calf serum in the cultures. Correlation coefficient of regression line (without uremic sera) is −0.895 with $P < 0.01$.

TABLE II. SERUM IRON LEVELS IN NORMAL AND UREMIC RABBITS.

Treatment	No. of experiments	Iron level mg/100 ml ± SEM
Normal rabbit serum	(5)	134.6 ± 42.3
Uremic rabbit serum	(5)	111.3 ± 34.5

in cultures is due to the amount of cold iron in the test sera added to the cultures, serum iron levels in normal and uremic rabbits were measured according to the technique of Young and Hicks (14) and the results are shown in Table II. Serum iron levels in the uremic sera were slightly decreased but were not significantly different from that of the normal controls.

Discussion. The results of the present studies indicate that uremic serum obtained from anephric rabbits 68 hr after bilateral nephrectomy produces a significant inhibition of ^{59}Fe incorporation into heme in erythroid cells in normal rabbit bone marrow cultures with ESF when compared with that of normal rabbit serum. In addition, this inhibitory activity was increased exponentially with increasing concentrations of sera in the cultures. However, uremic serum did not affect heme synthesis in the bone marrow cultures in the absence of ESF, indicating that this inhibitory effect on heme synthesis seen in the presence of ESF is unlikely to be due to cytotoxic factors, such as BUN and other toxic metabolites in uremia. Serum iron values in normal rabbit sera were not significantly different from that of uremic sera which clearly indicates that changes in the unlabeled iron pools in our cultures did not affect our results. This inhibitor of heme synthesis in uremic serum was estimated in earlier studies to be a low molecular weight substance between 2000 and 5000 using an Amicon filtration system and Sephadex G-50 fractionation (10). This suggests that it is not an antibody to ESF and also that its molecular weight is very similar to that of the inhibitor which we have reported in normal and polycythemic rabbit sera (8). It is quite possible that the inhibitor found in normal, uremic and polycythemic sera are the same, although the concentration may be different in each condition. This inhibitory activity was directly proportional to the increase seen in the hematocrit of the polycythemic rabbits. The reason for the higher concentration of the inhibitor in uremia may be related to a failure to excrete a normal inhibitor following nephrectomy.

The presence of a substance(s) in uremic plasma or serum which inhibits erythropoiesis has been suggested by several investigators using *in vivo* (3, 6, 7) and *in vitro* techniques (11, 12). The relationship between the inhibitors reported by others and our present findings as well as the role of serum inhibitors of erythropoiesis in the mechanism of the anemia of renal insufficiency are not known.

In studies of erythroid colony formation with the use of normal rabbit bone marrow in a methyl cellulose gel system, the serum inhibitors did not produce a change in the numbers of erythroid colonies formed per plate (13). Therefore, these data suggest that this inhibitor does not affect the ability of the erythroid stem cells to form erythroid colonies in response to ESF *in vitro* but probably acts more specifically to inhibit heme synthesis in the differentiated erythroid cell compartment by altering their cell cycles. Bozzini *et al.* (3) have suggested that a decrease in the responsiveness of the erythroid cell compartment to ESF *in vivo* in acutely uremic rats may also be involved in the mechanism of the anemia of uremia. Thus, it is important to emphasize that the existence of a serum inhibitor of erythropoiesis should be considered in the treatment of patients with anemia of uremia with ESF.

Summary. The effects of sera from anephric rabbits on the rate of heme synthesis in erythroid cells were studied *in vitro* in rabbit bone marrow cultures. Sera from nephrectomized rabbits significantly ($P < 0.05$) inhibited ^{59}Fe incorporation into heme in normal rabbit bone marrow cultures incubated with erythropoietin (ESF) when compared with the response in control cultures with normal serum and ESF. This inhibitory activity increased exponentially with increasing concentrations of the serum in cultures suggesting that a higher concentration of the inhibitor is present in

uremia and may be the result of the failure to excrete the inhibitor. This suggests that the retention of this inhibitor may play an important role in the mechanism of the anemia of uremia. The inhibitor of heme synthesis is a low molecular weight substance.

1. Moriyama, Y., Acta Haematol. Jap. **33,** 540 (1970).
2. Eschbach, N. W., Funk, D., Adamson, J. W., Kuhn, I., Scribner, B. H., and Finch, C. A., New Engl. J. Med. **276,** 653 (1967).
3. Bozzini, C. E., DeVoto, F. C. H., and Tomio, J. M., J. Lab. Clin. Med. **68,** 411 (1966).
4. Moriyama, Y., and Fisher, J. W., Blood (submitted) (1974).
5. Mirand, E. A., and Murphy, G. P., J. Amer. Med. Ass. **209,** 392 (1969).
6. Fisher, J. W., Hatch, F. E., Roh, B. L., Allen, R. C., and Kelley, B. J., Blood **31,** 440 (1968).
7. Moriyama, Y., Saito, H., and Kinoshita, Y., Haematologia **4,** 15 (1970).
8. Moriyama, Y., Lertora, J. J. L., and Fisher, J. W., Proc. Soc. Exp. Biol. Med. (submitted) 1974.
9. Krantz, S. B., Gallien-Lartigue, O., and Goldwasser, E., J. Biol. Chem. **238,** 4085 (1963).
10. Rege, A. B., Moriyama, Y., and Fisher, J. W., Fed. Proc. **33,** 597 (1974).
11. Markson, J. L., and Rennie, J. B., Scot. Med. J. **1,** 320 (1956).
12. Fisher, J. W., Lertora, J. J. L., Lindholm, D. D., Tornyos, K., and Moriyama, Y., Proc. Clinical Dialysis and Transplant Forum **3,** 22 (1973).
13. Moriyama, Y., Rege, A. B., and Fisher, J. W., International Symposium on Erythropoiesis. Tokyo, Japan (August 1974).
14. Young, D. R., and Hicks, J. M., J. Clin. Pathol. **18,** 98 (1965).

Received June 21, 1974. P.S.E.M.B. 1975, Vol. 148.