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Inhibition of Bone Marrow Erythroid Colony-Forming Cells (CFU-E) by Serum from Chronic Anemic Uremic Rabbits[1] (39874)

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Introduction. Anemia is a severe complication associated with chronic renal failure. Although the mechanism of this anemia is still not completely understood, several factors are known to be involved. The three primary factors postulated to play an important role in the mechanism of the anemia of renal insufficiency are: (i) a relative decrease in erythropoietin (Ep) production (1); (ii) inhibitor(s) of heme synthesis (1, 2) and/or erythroid colony-forming units (CFU-E) (3); and (iii) shortened red cell life span (4).

Serum from acutely uremic rabbits and serum from patients with anemia and uremia associated with chronic renal disease have been demonstrated to inhibit *in vitro* heme synthesis (1, 2) and *in vitro* erythroid colony formation in bone marrow (3). Chanutin and Ferris (5) have described a procedure to produce a model for chronic renal insufficiency using 5/6th nephrectomy in rats. These animals remain uremic for several months and are useful for studying the biological effects of chronic uremia.

Stephenson *et al.* (6) have reported a plasma clot method for the production of erythroid colonies in cultures which reflects the number of erythropoietin-responsive stem cells using the fetal liver. The development of erythroid colonies requires the presence of Ep in this culture system (6, 7).

The purpose of the present study was to determine the role of the inhibitor(s) of the erythroid colony-forming unit in the mechanism of the anemia of chronic renal insufficiency.

Materials and methods. Female New Zealand albino rabbits (2.5–3.0 kg) were made uremic by the two-stage surgical procedure described by Chanutin and Ferris (5) under pentobarbital (30 mg/kg) anesthesia. Sham-operated controls were prepared at the same time and studied in parallel. Thirty-five days after the 5/6th nephrectomy, blood was collected via cardiac puncture and the sera were separated by centrifugation and stored at −20° until used in the bone marrow culture system. Hematocrits, serum creatinine levels, and Ep activity were determined. Serum Ep activity was determined in our exhypoxic polycythemic mouse assay which has been described previously (8, 9).

A modification of the method of McLeod *et al.* (7) was used as an *in vitro* assay for erythroid colony formation. Rabbits were sacrificed by cervical dislocation and the bone marrows were flushed from the femurs into 2% fetal calf serum (FCS) in minimal essential medium (MEM), dispersed with a pipet several times, and washed twice with cold 2% FCS in MEM. One-tenth milliliter of this culture medium contained 5×10^4 nucleated bone marrow cells, beef embryo extract, L-asparagine, NCTC-109 medium, FCS, human urinary erythropoietin (0.02 U), and citrated bovine plasma. To determine the inhibitor(s) of CFU-E, 10% uremic or sham-operated rabbit serum was added to the culture medium. All sera used were heat inactivated at 56° for 30 min. One-tenth milliliter of this suspension was placed into wells in microtiter plates. After clotting, the cultures were incubated at 37° in a humidified atmosphere at 5% CO_2 in air. Four days later the cultures were removed from the wells and fixed with 5% glutaraldehyde in phosphate buffer at pH 7.4. After drying the cultures were stained with 1% benzidine and counterstained with

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Giemsa solution. Colonies containing eight or more cells which stained benzidine positive for hemoglobin were counted using 100× magnification. The number of erythroid colonies in each of three wells was counted and the mean plus or minus the standard error of the mean was calculated.

Results. Hematocrits and serum creatinine levels in the experimental animals are depicted in Table I. The hematocrits of partially nephrectomized rabbits decreased from 40.3 ± 0.3% (mean ± SE) on the first day to 30.3 ± 1.4% after 35 days. The mean hematocrit of the 35-day uremic rabbits was significantly ($P < 0.01$) less than that of sham-operated rabbits (41.0 ± 0.6). The serum creatinine levels increased from 1.42 ± 0.12 mg% on the first day to 3.60 ± 0.46 mg% after 35 days post-5/6th nephrectomy, and were significantly ($P < 0.01$) higher than those of the sham-operated controls. There was no significant change in the serum creatinine levels in the sham-operated controls over the 35-day period studied. Erythropoietin levels in serum (1.0 ml) from partially nephrectomized rabbits were undetectable when assayed in exhypoxic polycythemic mice (8, 9).

To determine the number of CFU-E in the bone marrows from 35-day anemic uremic and sham-operated control rabbits, an *in vitro* assay for CFU-E was performed with and without Ep. As shown in Table II, the marrows from anemic uremic and sham-operated rabbits contained 25.7 ± 2.0 and 58.0 ± 3.8 CFU-E/5 × 10^4 nucleated bone marrow cells, respectively. As seen in Table II, the number of CFU-E in chronically uremic anemic rabbit bone marrows (25.7 ± 2.0) was significantly ($P < 0.01$) less than that of the sham-operated controls (58.0 ± 3.8).

To study further the role of inhibitor(s) of CFU-E in the mechanism of chronic renal failure, the effects of 35-day uremic sera on CFU-E in plasma clot cultures were compared with sham-operated rabbit sera in normal rabbit bone marrows. When 10% sera from 35-day uremic or sham-operated rabbits were added to the culture system, the number of CFU-E was 37.1 ± 6.4 and 68.7 ± 6.7/5 × 10^4 nucleated bone marrow cells, respectively (Table III). Thus, the uremic sera significantly ($P < 0.01$) in-

TABLE II. ERYTHROID COLONIES IN UREMIC AND SHAM-OPERATED RABBIT BONE MARROW CULTURES.

Treatment	No. of experimental rabbits	No. of erythroid colonies/5 × 10^4 nucleated bone marrow cells
Sham	5	58.0 ± 3.8[a]
35-Day uremic	5	25.7 ± 2.0*

[a] Mean ± SE.
* Significantly different ($P < 0.01$) from sham-operated bone marrow controls.

TABLE III. EFFECTS OF UREMIC AND SHAM-OPERATED RABBIT SERA ON ERYTHROID COLONIES IN NORMAL RABBIT BONE MARROW CULTURES.

Source of serum	No. of experimental rabbits	No. of erythroid colonies/5 × 10^4 nucleated normal bone marrow cells
Sham	6	68.7 ± 6.7[a]
35-Day uremic (5/6th nephx)	6	37.1 ± 6.4*

[a] Mean ± SE.
* Significantly different ($P < 0.01$) from sham-operated rabbit serum.

TABLE I. HEMATOCRITS AND SERUM CREATININE VALUES IN 5/6TH NEPHRECTOMIZED AND SHAM-OPERATED RABBITS ($n = 5$).

	Treatment	Day of 2nd operation[a]	35 Days after 2nd operation
Hematocrit (%)	Sham	40.5 ± 0.7[b]	41.0 ± 0.6
	5/6th Nephx	40.3 ± 0.3	30.3 ± 1.4*
Serum creatinine (mg%)	Sham	1.30 ± 0.05[b]	1.42 ± 0.08
	5/6th Nephx	1.42 ± 0.12	3.60 ± 0.46*

[a] Mean ± SE.
[b] Determination was made immediately after the 2nd operation and was designated as Day 0 of the study.
* Significantly different ($P < 0.01$) from sham-operated control.

hibited erythroid colony formation in normal rabbit bone marrows when compared with that of the normal sham-operated controls.

Discussion. The present studies demonstrated a significant increase in serum creatinine levels and a significant decrease in hematocrits in 5/6th nephrectomized rabbits after 35 days. Erythropoietin was not detected in sera from chronically uremic anemic rabbits 35 days post 5/6th nephrectomy using the exhypoxic polycythemic mouse bioassay. Anagnostou *et al.* (10) recently reported a significant decrease in the percentage of injected ^{59}Fe incorporated into the RBC of 5/6th nephrectomized uremic anemic rats when compared with that of sham-operated rats. Lack of an appropriate rise in serum levels of Ep in uremic anemic animals is probably a critical factor in the reduction of the heme-synthesizing cell compartment in this type of anemia (1, 10). Only a few reports have demonstrated changes in the stem cell compartment, especially CFU-E, in uremia (3, 11). Moriyama and Fisher (11) have reported previously that the number of erythroid colonies is higher in the bone marrows of acutely uremic anemic rabbits than in normal controls. On the other hand, the number of CFU-E in 35-day chronically uremic anemic rabbit marrows was found to be significantly less than that in sham-operated controls. We also found in the present studies that the chronically uremic anemic rabbit sera from 5/6th nephrectomized rabbits decreased the number of CFU-E in normal rabbit bone marrow cell cultures. These data suggest that there is an inhibitor(s) of CFU-E in the sera of 35-day chronically uremic anemic rabbits and that this inhibitor(s) probably decreases the number of erythroid-committed stem cells in the CFU-E compartment *in vivo,* resulting in a decrease in the differentiated nucleated erythroid cells. Inhibitor(s) in uremic plasma or serum from acutely uremic rabbits which inhibits heme synthesis has also been reported using an *in vitro* bone marrow culture (1, 3). This inhibitor(s) has been reported to be in a low-molecular-weight range (1, 3) and is effective in inhibiting heme synthesis but not CFU-E (12). We do not know whether our inhibitor(s) of CFU-E is the same as that which inhibits heme synthesis *in vitro.*

The findings in the present study suggest that a decrease in the number of erythroid colony-forming units in 35-day uremic anemic rabbits not only is dependent upon the failure of erythropoietin production but also is due to the presence of inhibitor(s) of the erythroid colony-forming unit.

Summary. The 5/6th nephrectomized uremic rabbit was demonstrated to develop a marked anemia. Erythropoietic activity in the sera of these chronically uremic anemic rabbits was not detectable. A decrease in the number of erythroid colony-forming cells (CFU-E) was found in chronically anemic uremic rabbit bone marrows and a significant inhibitory effect of the uremic serum on the erythroid colony-forming unit in normal rabbit bone marrow cultures was also seen in the 5/6th nephrectomized rabbit after 35 days. These findings support the hypothesis that an inhibitor of the primary target cell for erythropoietin, the CFU-E, plays an important role in the anemia of chronic renal failure.

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