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Selective Inhibition of Erythropoiesis by Sera from Patients with Chronic Renal Failure[1] (42582)

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Abstract. The pathogenesis of anemia in patients with chronic renal failure was studied by analyzing the effect of uremic sera on the *in vitro* colony growth of erythroid (CFU-E) and granulocyte–macrophage (CFU-GM) progenitor cells. Uremic sera from 20 of 30 patients inhibited erythroid colony growth below 70% of control even when cultured with normal human bone marrow of the same blood type. On the other hand, only one of the sera inhibited colony growth of CFU-GM as compared with normal sera. On Sephadex G-15 gel filtration, the CFU-E-inhibiting activity appeared in two different fractions: the void volume peak and the delayed eluant before the second peak. The inhibiting activity in the former fraction was noted only in uremic sera. The results of this study suggest the existence of a serum inhibitor(s) of erythropoiesis with a relative molecular mass of more than 1500 Da which are virtually impossible to dialyze by conventional membranes.

Anemia is a common complication of chronic renal failure and an important limiting factor influencing patients' quality of life. In addition to hemolysis, blood loss from various sources, and hypoproduction of erythropoietin, metabolic inhibitors of erythropoiesis accumulating in plasma and body fluids are also considered to be causes of anemia associated with chronic renal failure (1, 2). During the past decade, the existence of erythropoiesis-inhibiting activities in sera of uremic patients using semisolid bone marrow cultures has been well documented (3–8). However, the reported frequency and degree of erythropoiesis inhibition by uremic sera vary, probably because of the differences in culture condition, especially the source of bone marrow as target cells.

In this study we have examined the effects of sera from patients with chronic renal failure undergoing maintenance hemodialysis on the colony growth of erythroid and granulocyte–macrophage progenitors (CFU-E and CFU-GM) of ABO-matched human bone marrow cells and have attempted to partially separate the inhibitor(s) of erythropoiesis from uremic serum by gel filtration.

Methods. *Patients.* Thirty patients of blood type A undergoing maintenance hemodialysis for chronic renal failure were studied after informed consent had been obtained. Their ages ranged from 28 to 72 years (mean 52). Eleven patients were female and 19 were male. They had been on hemodialysis for periods ranging from 10 to 132 months (mean 78). Most were on dialysis 5 hr three times per week using a standard 1-m^2 dialyzer.

Blood was drawn immediately before hemodialysis, and the serum was heat inactivated at 56°C from 30 min and stored at −30°C until use. Sera from 10 normal volunteers of blood type A served as the control.

Gel filtration of sera. Sera of five patients with chronic renal failure and five normal subjects were chromatographed on a Sephadex G-15 column (142 × 2.2 cm, i.d.; Pharmacia, Inc., Piscataway, NJ). Three milliliters of each serum was loaded and eluted with 0.05 *M* NH_4HCO_3 (pH 7.8), 24 ml/hr, at 4°C and monitored by uv spectroscopy at 230 nm. The columns were calibrated with cytochrome *c* (13,000 Da), insulin (5800 Da), angiotensin I (1297 Da), and creatinine (113 Da). Samples obtained by gel filtration were adjusted to 3 ml of volume and passed through a Millipore

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0.45-μm membrane before they were added to culture.

Bone marrow culture techniques for CFU-E and CFU-GM assays. Bone marrow was aspirated into syringes containing preservative-free heparin from the sternum or iliac crest of normal volunteers of blood type A after their consent had been obtained. Mononuclear cells were collected after density centrifugation over Ficoll–Paque (Pharmacia).

Erythroid colony-forming units (CFU-E) were assayed by the semisolid agar culture method described elsewhere (9); 10^5 cells were suspended in 0.5 ml of α-medium containing 0.3% agar, 20% fetal calf serum (FCS), 1 U/ml of erythropoietin (Toyobo, Osaka, Japan), 1% bovine serum albumin (BSA), and 10^{-4} *M* 2-mercaptoethanol. The mixtures were gently placed on 35-mm plastic dishes (Lux Scientific, Newbury Park, CA) and allowed to gel at room temperature. Then, 0.5 ml of the growth medium was poured around each of the agar gels and incubated at 37°C in 5% CO_2 in air for 7 days. After incubation, the agar disks were transferred onto a glass slide and covered with filter paper to remove excess water. The dried preparations were fixed with 5% glutaraldehyde solution and stained with benzidine. Aggregates consisting of eight or more benzidine-positive cells were counted as CFU-E colonies. The culture condition for CFU-GM was set up identically to that for CFU-E except for the absence of BSA and 2-mercaptoethanol and the use of 10% human placental conditioned medium (HPCM) instead of erythropoietin as a source of colony-stimulating activity as previously described (9). Colonies of 50 or more cells were counted with an inverted microscope on Day 10. All cultures were performed in triplicate.

Test sera or fractionated samples were added to culture at a final concentration of 10%. Mixed samples of uremic and normal sera in various proportions were also added to CFU-E culture to clarify whether nutritional deficiency or the presence of toxic substances plays an important role in CFU-E colony growth inhibition by uremic sera. The concentration of FCS was set at 20% in this experiment

Results. Sera from 30 patients of blood type A on hemodialysis for chronic renal failure were tested in cultures of normal bone marrow of the same blood type (Fig. 1). Control cultures containing 10% normal sera produced 431 ± 29 (mean ± SD) erythroid colonies in the CFU-E assay and 95 ± 11 granulocyte–macrophage colonies in the CFU-GM assay, respectively. Of all the uremic sera, 20 inhibited colony growth of CFU-E below 70% of that of control ($P < 0.01$), although there was a wide range of inhibiting effects among these sera. On the other hand, only one serum inhibited colony growth of CFU-GM as compared with control sera. Moreover, 13 sera stimulated CFU-GM colony growth above 150% of that of control ($P < 0.01$).

Table I shows the CFU-E assay data in three different experiments in which mixed samples of uremic and normal sera in various proportions were added to culture. Uremic sera from patients with chronic renal failure inhibited CFU-E colony growth in a dose-dependent manner. Normal sera did not reduce the inhibitory effect of uremic sera.

The gel-filtration profiles of uremic sera on Sephadex G-15 columns were identical to those of normal sera. Figure 2 shows the elution patterns of uremic and normal sera and the effects of the eluates on CFU-E colony growth of normal human bone marrow cells. Similar results were noted in four additional experiments. On gel filtration of the uremic serum, CFU-E colony growth inhibition was

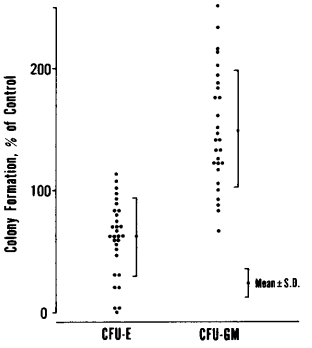

FIG. 1. Effects of sera from 30 patients with chronic renal failure on the colony growth of CFU-E and CFU-GM by normal bone marrow cells. Each serum was heat-inactivated and added to culture at 10%. Values are expressed as percentages of the mean number of colonies formed in control cultures added with normal sera at 10%. All cultures contain 20% FCS, 1% BSA, and 1 U/ml of erythropoietin for CFU-E and 20% FCS and 10% HPCM for CFU-GM.

TABLE I. MIXING EFFECTS OF NORMAL AND UREMIC SERA ON CFU-E COLONY GROWTH

Percentage of serum added to culture			
Normal serum	Uremic serum	FCS	CFU-E colonies/plate[a]
Exp. 1			
10	0	20	431 ± 25
5	5	20	253 ± 17**
0	10	20	98 ± 10***
10	10	20	75 ± 12***
5	10	20	84 ± 7***
Exp. 2			
10	0	20	236 ± 19
5	5	20	142 ± 11**
0	10	20	63 ± 8***
Exp. 3			
10	0	20	313 ± 33
5	5	20	187 ± 14**
0	10	20	158 ± 9**

[a] Normal human bone marrow cells (10^5) were inoculated in each culture. Values are expressed as means ± SD.
** $P < 0.005$.
*** $P < 0.001$.

noted in samples from different fractions: the void volume peak (F1) and the delayed eluants before the second absorbance peak (F3, 4). The inhibition by fraction 1 was noted only in the uremic serum. Fractions 3 and 4 from both uremic and normal sera were inhibitory to CFU-E colony growth. The CFU-GM colony growth was not significantly inhibited by any samples of fractionated eluates in either uremic or normal sera. Moreover, samples from F1 which inhibited colony growth of CFU-E and enhanced CFU-GM colony growth. This colony-enhancing effect was higher in uremic sera than in normal sera.

Discussion. The present results indicate a selective inhibition of erythroid colony growth by uremic sera. Although there are many studies regarding the effects of uremic sera on erythropoiesis *in vitro,* no attempt has been made to match the blood type between test sera and target bone marrow cells, even when human marrow was cultured. There is, however, a possibility that inhibition of erythropoiesis by uremic sera in such a situation results not only from a toxic mechanism but also from a reaction to erythrocyte allo-antigens through an immunologic mechanism, since a substantial number of patients with chronic renal failure on hemodialysis require blood transfusions. In this regard, our present experimental system using ABO-matched donor bone marrow cells seems to be more suitable for evaluating the effects of uremic sera on CFU-E colony growth *in vitro.* The data showing high incidence of CFU-E colony inhibition by uremic sera, therefore, seem to result not from an immunologic mechanism or a lack of nutrients but from the presence of an erythropoiesis inhibitor(s) in uremic sera, since normal sera could not reduce the inhibitory effect of CFU-E by uremic sera.

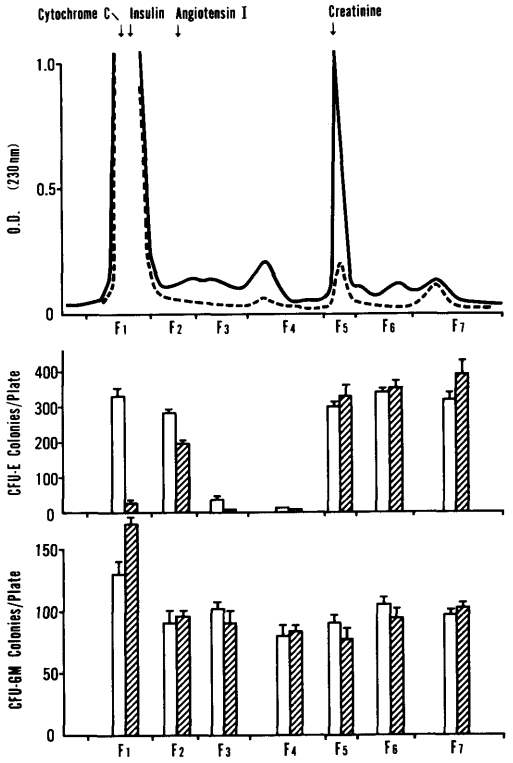

FIG. 2. Gel-filtration profiles of uremic and normal sera on a Sephadex G-15 column and the effects of fractionated eluates on the colony growth of CFU-E and CFU-GM in a typical case of five experiments. The solid line denotes an elution pattern of uremic serum, and the broken line denotes that of normal serum. Each sample from the fractions was added to a culture of 10^5 normal bone marrow cells at 10%. The hatched columns denote the number of colonies formed when fractionated samples of uremic serum were added, and the open columns denote that of normal serum. Values are expressed as the means ± SD of triplicated cultures.

The results of the present study on the CFU-GM inhibitory effect of uremic sera differ from those of Vincent *et al.* (10) showing inhibition by uremic sera. On the other hand, McGonigle *et al.* (8, 11) reported that uremic sera supported CFU-GM colony growth more efficiently than sera from normal subjects although cultures containing human sera produced fewer colonies in comparison with controls. Delwiche *et al.* (12) also have recently reported the inhibitory activity of uremic sera on CFU-GM colony growth. They speculate that this inhibitory activity appears to be nonspecific because of the lack of association with leukocytopenia in uremic patients. The reason for the discrepancy in the hemopoiesis inhibitory activities of uremic sera seems to result from the difference in assay systems. Some investigators used a double-layer method in which the production of colony-stimulating activity by feeder cells might be easily affected by test sera added to culture. Others used xenogeneic bone marrow cells as target cells. Our results may help to explain the reason that granulocytopenia is not seen in uremic patients. The results of the present study using human bone marrow cells suggest that inhibition of granulopoiesis is not a major feature of uremia and that the uremic serum inhibitor(s) is more specific to erythroid cell lineage.

On gel filtration of uremic sera, we found some different fractions showing inhibition of erythropoiesis in bone marrow cultures. This Sephadex G-15 resin column excludes molecules of more than 1500 Da. Of these fractions, one in the molecular weight range of the void volume fraction (>1500 Da) appears to be specific for uremic sera. This fraction may consist of proteins and polypeptides which are minimally dialyzed by conventional membranes. The results seem to be compatible with the clinical observation that continuous ambulatory peritoneal dialysis (CAPD), which may be related to a more efficient removal of "middle-molecule" uremic toxins (molecular size 500–5000 Da), produces an improvement in the anemia associated with chronic renal failure (13). Another fraction in the molecular weight range of 100 to 500 Da may contain spermine and spermidine, which are also considered to be uremic toxins (14, 15), although inhibition of erythropoiesis by these fractions was noted in both uremic and normal sera. Excessive low-molecular erythropoiesis-inhibiting substances might be conjugated with higher molecules such as peptides and proteins in uremic sera. It is of interest that the samples of fraction 1 which inhibit *in vitro* erythropoiesis in fact enhance the colony growth of CFU-GM, suggesting that colony-stimulating factors as well as inhibitors accumulate in sera and body fluids of uremic patients. Recent clinical trials of recombinant human erythropoietin for treatment of anemia associated with end-stage renal failure clearly indicate that supplementation by exogenous erythropoietin is able to correct the anemia (16). However, compared to physiological conditions, a large amount of erythropoietin is necessary to correct this type of anemia. When we can remove erythropoiesis inhibitors from patients more efficiently, it would seem possible to reduce the requirement of erythropoietin for treatment of this anemia. Further characterization and identification of the serum inhibitor(s) specific for erythropoiesis are now under investigation in our laboratory.

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