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Heinz W. Radtke, ... , Robert A. Campbell, James W. Fisher

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Research Article

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HEINZ W. RADTKE, ARVIND B. REGE, MAX B. LAMARCHE, DAGMAR BARTOS, FRANTISEK BARTOS, ROBERT A. CAMPBELL, and JAMES W. FISHER, *Department of Pharmacology, Tulane University School of Medicine and Renal Dialysis Unit, New Orleans Charity Hospital, New Orleans, Louisiana 70112; Pediatric Renal-Metabolic Laboratory, The Department of Pediatrics, University of Oregon, Health Sciences Center, Portland, Oregon 97201*

ABSTRACT Fetal mouse liver and normal human bone marrow cell cultures were used for studies on the inhibition of erythroid colony formation (CFU-E) by sera from anemic patients with end-stage renal failure and the polyamine spermine. Sera from each of eight predialysis uremic anemic patients with end-stage renal failure produced a significant ($P < 0.001$) inhibition of erythroid colony formation in the fetal mouse liver cell cultures when compared to sera from normal human volunteers. In vivo or in vitro dialysis of the uremic sera with a 3,500-dalton exclusion limit membrane removed the inhibitor from uremic sera. The uremic serum dialysate provided by the membrane fractionation was significantly inhibitory in the erythroid cell cultures. When this dialysate was applied to gel filtration chromatography (Bio-Gel P-2) the inhibitor was found to be in the same molecular weight range as [^{14}C]spermine. The polyamine spermine produced a dose-related inhibition of erythroid colony formation (CFU-E) in fetal mouse liver and normal human bone marrow cultures. Thus, the following evidence is provided that the in vitro inhibitor of erythropoiesis found in chronic renal failure patients' sera is identical with the polyamine spermine: (a) the inhibitor and radio-labeled spermine appeared in identical Bio-Gel P-2 effluent fractions; (b) when spermine was added to normal human sera at concentrations reported in sera of uremic patients, and studied in both the fetal mouse liver cell culture and normal human bone marrow cultures, a dose-related inhibition of erythroid colony

(CFU-E) formation was noted; and (c) the inhibitory effects of crude uremic serum, uremic serum dialysate, and fractions of uremic serum dialysate from a Bio-Gel column, on erythroid colony formation were completely abolished by the addition of a specific rabbit antiserum to spermine.

INTRODUCTION

Patients with chronic renal failure usually suffer from a hypoproliferative normochromic normocytic anemia (1–3). In addition to hemolysis (4) and blood loss from various sources (5–7), two major factors have been implicated in the mechanism of the anemia of renal insufficiency which may be the cause of the hypoproliferative state of the bone marrow in patients with end-stage renal failure. First, serum erythropoietin concentrations, although often found to be normal (8, 9) or elevated (8–11), may not be high enough in relation to the severity of the anemia. Secondly, metabolic inhibitors accumulating in plasma and body fluids of patients with end-stage renal failure have also been reported to reduce the proliferation and maturation of erythroid cells (12–15).

During the past several years, (an) erythropoiesis inhibiting factor(s) in sera of uremic animals (16) and patients with anemia of chronic renal failure (14, 15) have been described. Uremic sera were found to impair in vitro erythroid progenitor cell growth of both erythroid burst-forming units and erythroid colony-forming units (CFU-E)¹ in rabbit and human marrow cultures (15, 16), as well as heme synthesis in canine bone marrow cultures (14, 17).

Using a fetal mouse liver cell culture system, we have recently demonstrated a serum inhibitor of CFU-E

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¹ Abbreviation used in this paper: CFU-E, erythroid colony-forming units.

TABLE I
*Clinical Data on Eight Predialysis Patients with
Anemia of Chronic Renal Failure*

Patient	Sex	Age	Diagnosis	Blood urea nitrogen <i>mg/100 ml</i>	Creatinine <i>mg/100 ml</i>	Hematocrit <i>%</i>
P.L.	M	67	Glomerulonephritis	167	18.9	17
C.A.	M	55	Nephrosclerosis	141	28.2	16
C.B.	M	33	Glomerulonephritis	190	27.4	16
S.P.	F	42	Pyelonephritis	152	20.5	10
L.J.	M	42	Glomerulonephritis	225	36.6	11
F.S.	M	58	Glomerulonephritis	197	24.3	14
T.I.	M	73	Pyelonephritis	130	9.5	19
A.M.	F	62	Glomerulonephritis	207	26.4	18
Mean		54		177	24.0	16.1
SD		12.9		32.2	7.4	2.4

formation of low molecular weight in sera from severely azotemic patients. This inhibitor was removed completely from the serum by *in vivo* and *in vitro* dialysis (18).

The present study was undertaken to attempt to identify the low molecular weight inhibitor of erythropoiesis. Inhibition of CFU-E formation in fetal mouse liver cell and in human bone marrow cultures was demonstrated with crude uremic sera, uremic serum dialysates, dialysate fractions, and free polyamines, respectively. Reversal of the inhibition of erythroid colony formation by sera from azotemic patients was seen after preincubation of the uremic sera with anti-serum to spermine.

METHODS

Sera were obtained from eight patients with anemia of advanced chronic renal failure who were markedly azotemic immediately before the onset of regular hemodialysis treatment. The clinical data on each patient giving age, sex, underlying diagnosis, hematocrit, creatinine, and blood urea nitrogen levels are shown in Table I. A pool of sera from 10 normal human volunteers served as the control. Informed consent was obtained from both patients with chronic renal failure and the normal volunteers.

Sera were heat inactivated at 56°C for 30 min and stored in a deep-freeze at -80°C until assayed. Each serum sample was tested before and after *in vitro* dialysis for 48 h against distilled water in a dialysis tubing with an exclusion limit of 3,500 daltons (Spectrophor membrane tubing, Spectra Medical Instruments, Inc., Los Angeles, Calif.). Total dialysate from these pooled uremic sera was freeze-dried and readjusted to the same volume as that of the serum samples. The inhibitory effect of each serum was compared to that of the uremic serum dialysate.

Gel filtration chromatography was carried out with a 60-ml column of superfine polyacrylamide beads (Bio-Gel P-2, -400 mesh, Bio-Rad Laboratories, Richmond, Calif.) with a fractionation range of 100–1,800 daltons. After applying 1 ml of the uremic serum dialysate derived from 2 ml of crude serum, distilled water was passed through the column at a flow

rate of ~4 ml/h using gravity flow. The aliquots of effluent collected were 1.1 ml. Testing of pooled fractions of 3.3 ml, which were freeze-dried and dissolved in 1 ml of normal serum starting with the void fraction, was carried out in the fetal mouse liver cell cultures.

Lyophilized rabbit antiserum to spermine reconstituted in distilled water (1 ml/ampule) were added to (a) 1 ml of a predialysis serum at concentrations of 20–200 μ l/ml, an amount capable of binding ~0.06–0.70 nmol of spermine and (b) to each of eight predialysis sera from patients with end-stage renal failure and to a normal control serum at a concentration of 100 μ l/ml. After a 5-h incubation at 37°C with anti-spermine the sera were centrifuged at 2,000 g for 30 min and the supernate removed and tested in the culture together with the corresponding untreated sera. 1 ml of uremic serum dialysate was preincubated with 200 μ l of antispermine for 5 h before applying to the column. Effluent fractions were tested in the fetal mouse liver cell culture in the same way as the nonpreincubated dialysate samples.

Unlabeled spermine and spermidine (free base, Sigma Chemical Co., St. Louis, Mo.) at concentrations of 0.8–3.3 nM were added to normal human sera and tested in the fetal mouse liver cell cultures in the presence of 200 mU erythropoietin. In addition, spermine was tested in a similar manner in normal human bone marrow cultures.

Fetal mouse liver cell culture technique. Fetal mouse (CD-1) liver cells from fetuses 13-d old or less, known to be predominantly erythroid committed and able to produce very high numbers of erythroid colonies in a semisolid culture medium, were prepared according to the technique of Iscove et al. (19). Liver cells were disaggregated and suspended as single cells at a concentration of 100,000 cells/ml in a culture medium containing Eagle's minimum essential medium in methylcellulose, 30% fetal calf serum, 100 mU/ml of human urinary erythropoietin,² 0.1 μ M of mercaptoethanol, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin. When human

² Human urinary erythropoietin was supplied by the Department of Physiology, University of Northeast, Corrientes, Argentina. The material was further processed and assayed by the Hematology Research Laboratories, Childrens Hospital of Los Angeles, under U. S. Public Health Service research grant HE-10880 (National Heart, Lung and Blood Institute).

serum samples were tested at a concentration of 15%, the amount of fetal calf serum was accordingly reduced to 15% to maintain a total serum concentration of 30% (Figs. 1, 5, and 6).

1 ml of cell culture was plated in 35 × 10-mm petri dishes and incubated for 48 h at 37°C in a humidified atmosphere at 95% air and 5% CO₂. The pH was maintained constant as indicated by the color of the phenol-red-indicator present in the minimal essential medium. After staining of the plates with diaminobenzidine according to the method of Ogawa (20), CFU-E of eight or more cells were counted in three replicate culture plates using an inverted microscope. One-sixteenth of the plate area was scored and considered to be representative of the total plate area since very large numbers of colonies were usually present (1,000–5,000 colonies/plate). To minimize variations in erythroid colony formation only one stock batch of fetal calf serum, pooled normal AB serum, and one batch of erythropoietin (specific activity 9.4 U/mg protein) were used. Testing of the pre- and postdialysis sera, before and after treatment with antiserpentine and the corresponding effluent fractions were always carried out in parallel in the same assay in order to minimize variations in erythroid colony formation in the various groups being compared.

Human bone marrow cell technique. Bone marrow was aspirated from the posterior iliac crest of normal male human volunteers and collected in an equal volume of Hanks' balanced salt solution with 5 U/ml heparin. Under sterile conditions, an equal volume of disaggregated cell suspension was layered over Ficoll-Paque and centrifuged at 400 g 30–40 min at 18°–20°C. After transferring the mononuclear cells to a clean tube, they were washed twice in 2 vol of Hanks' balanced salt solution by centrifuging at 100–400 g for 10 min and the washed pellet resuspended in alpha medium. The final concentration of culture mixtures was: 0.74% methylcellulose, 30% normal human AB serum, 2×10^4 cells/ml, 200 U penicillin/ml, 200 µg streptomycin/ml and 0.1 mM mer-

captoethanol. Human urinary erythropoietin (0.2–2 U/ml) was added to each microtiter well before plating 0.1 ml culture medium. The culture plates were incubated at 37°C in 5% CO₂—95% air for 7 d. CFU-E type colonies were scored directly with an inverted microscope at ×100 after staining with diaminobenzidine (20).

The Student's paired and unpaired *t* test and linear regression analysis were used for the statistical analyses.

RESULTS

Sera from five patients with end-stage renal failure significantly inhibited ($P < 0.01$) CFU-E formation in fetal mouse liver cell cultures when compared to the effect of 10 pooled normal human sera. In vitro dialysis (48 h) of the uremic sera resulted in a significant ($P < 0.01$) reduction of inhibition. Pooled dialysate of the uremic sera after freeze-drying and readjusting to the original volume of the uremic serum samples demonstrated approximately the same inhibitory effect on CFU-E formation as the predialysis sera (Fig. 1).

Samples of uremic serum dialysate when applied to a Bio-Gel column which separates substances according to molecular weight exhibit their inhibitory effects on CFU-E formation only in fraction 7 and 8 (Fig. 2B). To determine if the polyamine spermine would elute with these fractions, ¹⁴C-labeled spermine was applied to the Bio-Gel column. As illustrated in Fig. 2D, the appearance of radiolabeled spermine in the column effluent is congruent with that of the uremic inhibitor of erythropoiesis. The inhibitory

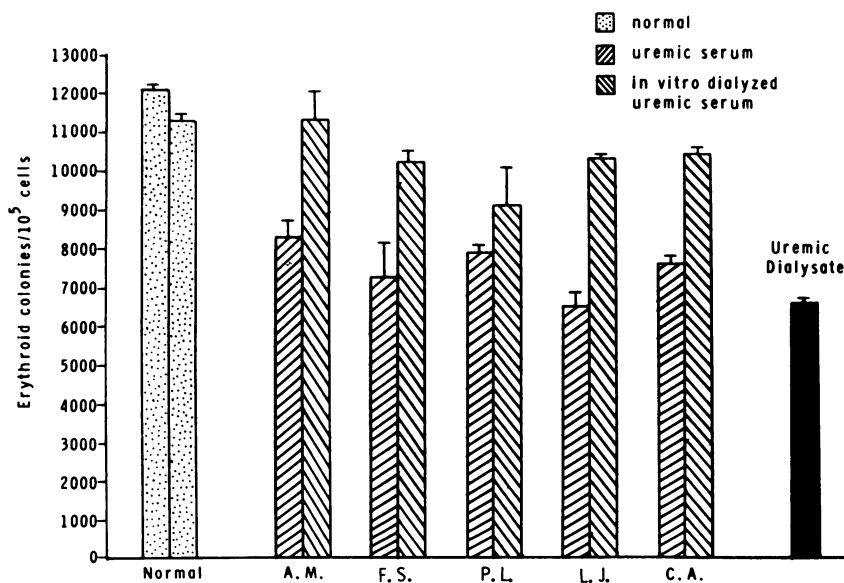


FIGURE 1 The effects of normal and uremic human sera before and after in vitro dialysis and the uremic dialysate itself (solid bar) on erythroid colony formation (CFU-E) in fetal mouse liver cultures containing 100 mU erythropoietin/ml. The uremic sera produced significantly ($P < 0.01$) less inhibition of erythroid colony formation after in vitro dialysis. Bars indicate SEM of three replicates of 15% human serum plus 15% fetal calf serum.

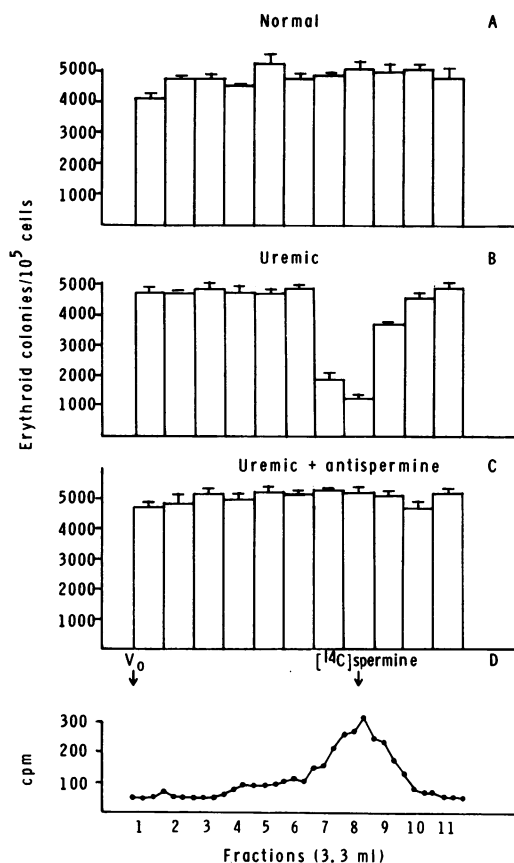


FIGURE 2 Effects of uremic serum dialysate fractions derived from gel filtration chromatography (Bio-Gel P-2) on erythroid colony formation (A–C). Enumeration of the fractions (3.3 ml) begins with the void volume (V_0). A illustrates the effects of Bio-Gel fractions from a normal serum dialysate, B is a serum dialysate from an azotemic patient, and C a serum dialysate from an azotemic patient, which was preincubated with spermine antiserum. D shows the radioactivity (counts per minute) of 1.1-ml fractions, when [¹⁴C]spermine tetrahydrochloride was added to a uremic serum dialysate before fractionation. Bars indicate SEM of three replicates.

effect of the uremic fractions 7 and 8 on erythroid colony formation in the fetal mouse liver cell culture was completely blocked when the dialysate was preincubated for 5 h with antispermine antiserum (200 μ l/ml dialysate) before applying to the column (Fig. 2C).

To directly demonstrate the inhibitory effects of polyamines on CFU-E formation in the fetal mouse liver cell culture, spermine and spermidine were added to normal human sera at concentrations which are often found in sera from patients with chronic renal failure (21). As shown in Fig. 3, spermine and spermidine demonstrated a dose-related inhibition of CFU-E formation similar to that of Bio-Gel fraction 7 added to normal human sera. To prove the specificity of this polyamine-induced inhibition spermine dissolved in

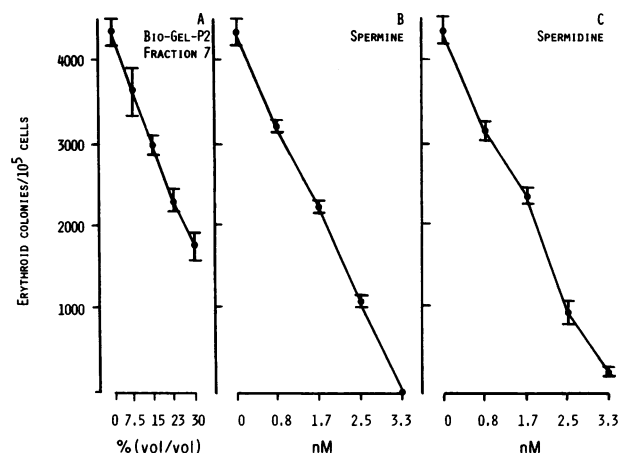


FIGURE 3 Inhibitory effects of Bio-Gel fraction 7 in comparison to that of spermine and spermidine on erythroid colony formation in fetal mouse liver cell cultures. Bars indicate SEM of three replicates.

normal human sera was added to normal human bone marrow cultures at the same concentrations. As shown in Fig. 4, spermine produced a dose-related inhibition of normal human CFU-E formation ($r = 0.94$, $P < 0.001$).

To determine whether the inhibition is due to endogenous spermine present in the crude serum of azotemic patients, one serum was incubated for 5 h with antispermine antiserum at increasing concentrations (20–200 μ l/ml). As shown in Fig. 5, 80 μ l of anti-spermine completely abolished the inhibitory effect of

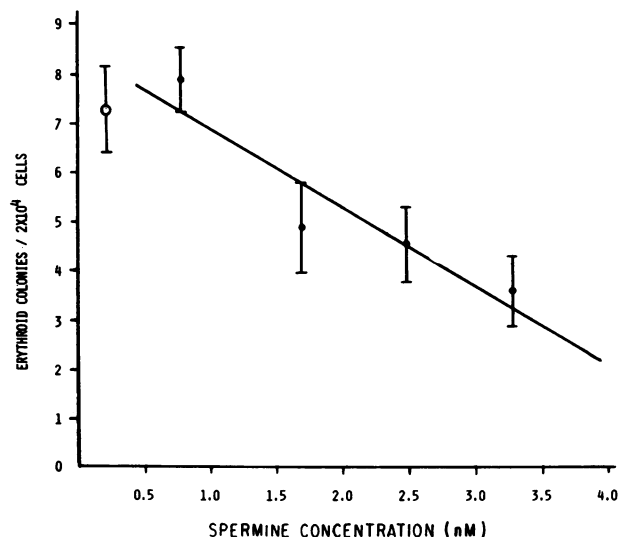


FIGURE 4 Inhibitory effects of spermine added to normal sera on human erythroid colony (CFU-E) formation in normal human bone marrow cultures containing 200 mU erythropoietin. (0) = 200 mU erythropoietin alone in normal human sera. ($n = 4$).

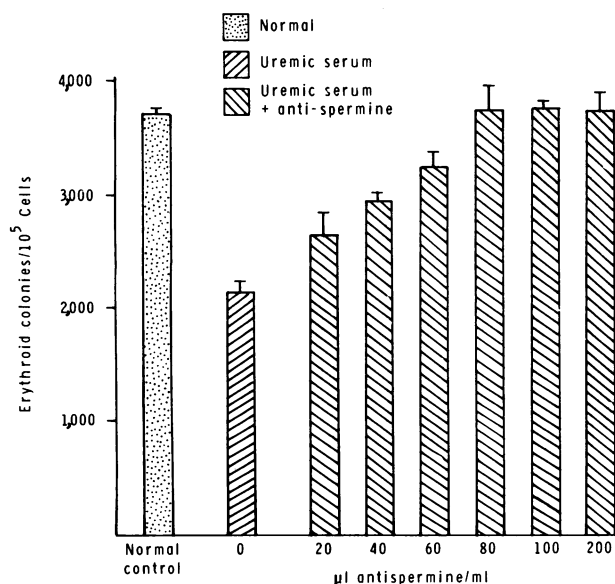


FIGURE 5 Effects of preincubation of a uremic serum with increasing concentrations of spermine antiserum on the inhibition of erythroid colony formation. Bars indicate SEM of three replicates.

the crude uremic serum on CFU-E formation when compared to a normal human control serum. As further noted in Fig. 5, increasing concentrations of anti-spermine in the uremic serum produced a graded increase in CFU-E formation, reaching a plateau between 80–200 μ l of antispermene, where virtually no inhibition could be observed. As shown in Fig. 6, each of eight crude uremic sera lost their inhibitory effect on CFU-E formation in the fetal mouse liver cell culture completely when preincubated with sufficient amounts of antispermene antiserum (100 μ l/ml). On the other hand, when antispermene serum was added to normal human serum no effect on CFU-E formation was seen on erythroid colony formation (Fig. 6). This suggests that the inhibition of *in vitro* erythropoiesis is due to spermine.

In order to determine the specificity of the rabbit antiserum to spermine, normal rabbit serum was added to sera from three different predialysis patients, normal human serum and fetal calf serum and studied on erythroid colony formation in fetal mouse liver cultures. When normal rabbit serum was added to sera from these three predialysis patients with severe anemia, no effect was seen on the inhibitory effects of these uremic sera on erythroid colony formation in fetal mouse liver cultures. In addition, no effect was seen on erythroid colony formation with normal rabbit serum in the presence of normal human serum or fetal calf serum. Thus, the effects of the spermine antiserum in blocking the inhibition of the uremic serum on erythroid colony formation is not due to some com-

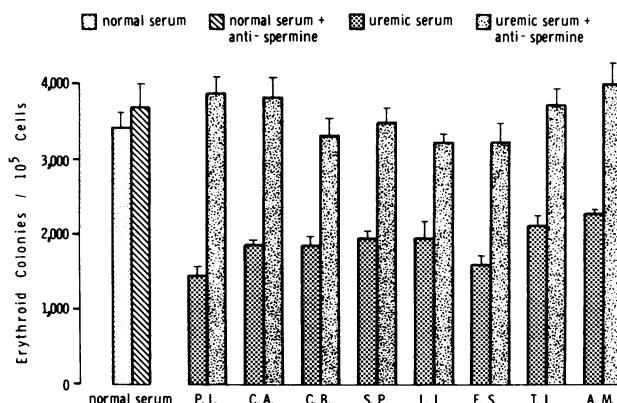


FIGURE 6 Effects of preincubation of eight uremic sera and a normal control serum with and without antispermene antiserum on erythroid colony formation in fetal mouse liver cell cultures containing 100 mU erythropoietin/ml. Bars indicate SEM of three replicates.

ponent of normal rabbit sera other than the antibody to spermine.

DISCUSSION

Sera from anemic patients (22) in severe states of uremic intoxication immediately prior to regular hemodialysis therapy contain an inhibitor of erythropoiesis which can be removed by *in vitro* and *in vivo* dialysis (18). In the present studies serum dialysates from azotemic patients have proven a good source of the erythropoiesis inhibitor. Thus, it was possible to recover the inhibitor, at least in part, from the *in vitro* dialysate. Applying this uremic serum dialysate to a polyacrylamide gel column, which permitted molecular weight fractionation down to 100 daltons, the inhibitor was found to be in a low molecular weight range. Among the compounds of ~200 daltons found to be significantly elevated in the serum of uremic patients (22) the polyamines were selected for further study, since they have been reported previously to suppress cell proliferation (23). In addition, the inhibitor of erythropoiesis was found to elute with the same polyacrylamide gel fractions as did radiolabeled spermine added prior to separation (Fig. 2).

Disturbances in polyamine metabolism have been described in patients with different stages of renal insufficiency (21, 24). Polyamines have also been found to suppress the activity of such crucial endogenous enzymes as adenylate cyclase (25) and Na-K-ATPase (26). Furthermore, because of the high avidity of these polycationic compounds for anionic sites, the surface charge of erythroid cells was found to be reduced in the presence of elevated polyamine concentrations (27). Therefore we tested the major endogenous mammalian polyamines spermine and spermidine in the fetal mouse liver cell culture to determine whether they

might elicit an inhibitory effect on erythroid colony formation. As indicated in our present studies, both spermine and spermidine produced a dose-related inhibition of erythroid colony formation.

It should be emphasized that the fetal mouse liver CFU-E culture used in our experiments served as a convenient assay system to detect and follow variations in inhibitor levels in sera and different serum fractions derived from anemic azotemic patients, and does not necessarily reflect all of the effects of the inhibitor on the patients' erythroid progenitor cells. To establish the effects of spermine on normal human CFU-E formation an experimental series was performed using human bone marrow cultures. Again, a dose-related inhibition of human CFU-E formation could be observed when increasing concentrations of spermine were added to normal human sera (Fig. 4). Since we have shown previously that crude uremic serum inhibits normal human CFU-E formation in the same way as fetal mouse liver erythroid colony formation (18), it seems very likely that it is the same inhibitor or a family of chemically related inhibitors which affect human (18), rabbit (15, 16), and murine erythroid progenitor cells of both BFU-E and CFU-E type, as well as the heme synthesizing nucleated erythroid cell compartment (14, 17).

In an attempt to prove that spermine molecules that are present in sera from azotemic patients were the only inhibitors of erythroid colony formation, specific anti-spermine antiserum was added to the *in vitro* test system. As shown in Fig. 6, spermine antiserum completely neutralized the inhibitory activity of each of eight different uremic sera. For comparison, when added to a normal human serum, spermine antiserum did not influence CFU-E formation in the fetal mouse liver cell culture. The same observation was made when spermine antiserum was added to the uremic serum dialysate 5 h before passing it through a polyacrylamide gel column. As shown in Fig. 2c, there was no inhibition of CFU-E formation by any of the Bio-Gel fractions of uremic serum following the addition of spermine antiserum. The addition of normal rabbit serum did not interfere with the inhibitory effects of uremic serum on erythroid colony formation.

These observations provide suggestive evidence that spermine may be responsible for the inhibition of the erythroid progenitor cell (CFU-E) compartment in the bone marrow of patients with anemia of chronic renal failure. Our studies do not completely discount the possibility of other inhibitors of erythropoiesis as yet unidentified; however, if other substances are involved in the inhibition of erythropoiesis in uremia, their effects on the CFU-E compartment would appear to be minimal, i.e., below the level of inhibition detectable in our fetal mouse liver cell culture assay system.

Four criteria were recently proposed by Bergstrom and Furst (22) which should be fulfilled for a compound to be considered as a uremic toxin. Our present data provide evidence that spermine meets all four of these criteria for an inhibitor of erythropoiesis in chronic renal failure: (a) spermine has been chemically identified in uremic sera and can be measured quantitatively in biological fluids (21, 24); (b) the serum concentrations of spermine in uremic patients have been shown to be elevated above normal controls (21); (c) inhibitory effects of spermine on *in vitro* erythroid colony growth can clearly be demonstrated at concentrations in the range of serum polyamine levels found in uremic patients (Figs. 3 and 4); and (d) high polyamine concentrations have been shown to be associated with anemia of chronic renal failure, dysfunction of cellular immunity, and uremic neuropathy (21). Furthermore, there is suggestive evidence that serum concentrations of a heretofore unknown inhibitor of erythropoiesis is directly correlated with the severity of anemia of chronic renal failure. This is derived from the observation that anemia of uremia progresses relentlessly in the course of renal insufficiency up to the point where hemodialysis support is essential (9), and that anemia improves after several weeks of adequate dialysis therapy, in spite of a significant drop in the patients' serum erythropoietin concentration (9, 28). Thus, it is highly likely that it is an inhibitory factor which accumulates in uremia and is removed, at least in part, by hemodialysis, that correlates with the degree of anemia. The inhibitor of erythropoiesis that we have previously shown to be removable from predialysis sera of azotemic patients by both *in vivo* and *in vitro* dialysis (18) may, indeed, be spermine, since the inhibitory effect of uremic sera could be mimicked by increasing concentrations of pure spermine added to normal sera; and since the effect of the uremic sera on erythroid colony formation could be completely abolished by the addition of specific spermine antiserum. On the other hand, systematic studies to determine an inverse correlation between the patients' hematocrit values and serum spermine concentrations directly have not yet been performed in patients with end-stage renal failure.

Knowledge of the nature and characteristics of erythropoiesis inhibitors should enable us to treat renal anemia more effectively. Therapeutic interventions such as the combination of hemodialysis and measures which remove this polycationic toxin more directly from the blood of uremic patients could prove useful in treating the anemia of chronic renal failure.

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