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The Anemia of Chronic Renal Failure: *In Vitro* Response of Bone Marrow To Erythropoietin[1] (38931)

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Patients with chronic renal failure (CRF) maintained on hemodialysis have a relatively normal histological appearance of the bone marrow (1). However, the normal quantity of erythroid tissue is inappropriate for the degree of anemia and, in general, correlates with inadequate levels of erythropoietin (ESF) for the degree of anemia in these patients (2, 3). The presence of a toxic factor(s) suppressing erythropoiesis and/or the ability of ESF to stimulate the erythroid compartment has been suggested by inhibition of erythroblast maturation in cultures prepared with uremic sera (4), the decreased response to ESF in uremic animals (5), the ability of chronic hemodialysis to increase the hematocrit (6, 7), and inhibition of [^{3}H]thymidine-incorporation into nucleated red cells in bone marrow cultures prepared with uremic serum (8). A substance capable of inhibiting ESF activity has been described by Fisher and co-workers (9) and Moriyama, Saito, and Kinoshita (10).

The "ESF responsiveness" of the erythroid stem cell has not been evaluated in the anemia of CRF and the typical inadequate erythropoiesis (6, 11, 12) may reflect to some degree an intrinsic cellular defect in these patients. The ability of ESF to stimulate heme synthesis in cultures of bone marrow has been demonstrated by many workers (13–16). Krantz, Gallien-Lartigue, and Goldwasser (15) described a method of bone marrow culture in which heme synthesis, for periods of incubation longer than 12 hr, was dependent upon the presence of ESF in the culture medium. Using this method, the response of the erythroid stem cell to a standard dose of ESF has been evaluated in patients with polycythemia vera (17), and bone marrow failure (18). The purpose of this study is to compare the ability of normal bone marrow and marrow from patients with CRF to respond to a standard level of ESF by the use of the *in vitro* culture system. In an attempt to define the presence of a toxic serum factor in uremic patients, normal and CRF marrow cells were cultured in both autologous and normal serum of isoantigen type AB (AB serum).

Methods. 1. Patients. The 14 patients with CRF were participating in the chronic hemodialysis program at the Denver Veterans Administration Hospital. All patients were men with a mean age of 46 (range 33–53). Four patients had polycystic kidney disease, eight had chronic glomerulonephritis, one had gouty nephropathy, and one had a multicystic renal disease associated with adenoma sebaceum. The laboratory parameters and bone marrow specimens were obtained immediately before the patients reported for a routine dialysis. The mean hematocrit was 21% (range 13–35%); the mean BUN level at the time of study was 68 mg/100 ml (range 54–105). All patients were anuric. Bone marrow biopsy specimens on these patients showed normal erythroid maturation in 13 of 14 and moderate megaloblastic features in 1 of 14. In general, the CRF marrows could not be differentiated histologically from normal specimens. The control patients were seen in consultation by the hematology service of the Denver VA Hospital and had normal bone marrow biopsies. These seven patients were evaluated for the following reasons: three patients had insignificant mild anemia, one patient had hepatomegaly, one had slightly increased

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IgA, one unexplained leukocytosis, and one leukopenia, most likely of familial origin. The BUN and creatinine levels were normal in all control subjects.

2. Bone marrow culture. The method of Krantz, Gallien-Lartigue, and Goldwasser (15) was used for the marrow cultures. In brief, bone marrow was aspirated from the posterior iliac crest and placed in heparinized sterile tubes. The marrow aspirate was washed three times with NCTC-109 (Colorado Serum Co., Denver, Colo.) and a single cell suspension prepared by repeated passage through a pipette. All marrow cells obtained from each patient were used in the cultures. The variability of final cell counts was due to different numbers of cells obtained from different patients. Differential counts were done on a Wright's stained preparation of each marrow aspirate to determine the percentage of erythroblasts. The cells were resuspended in fetal calf serum (Colorado Serum Co.), NCTC-109, and either autologous or AB serum. Sheep erythropoietin, Step III (Connaught Medical Research Laboratories, University of Toronto, Canada), was used in all experiments at a final concentration of 0.5 U/ml in the system. Four groups of culture plates were prepared with either normal or CRF marrow. The experimental design may be summarized as follows:

Group A	*Group B*
Autologous serum	Autologous serum
saline (control)	ESF stimulated
Group C	*Group D*
AB serum	AB serum
saline (control)	ESF stimulated

The cultures were incubated for 48 hr at 37° in a water-saturated atmosphere containing 5% CO_2. A volume of 0.2 ml of $Fe^{59}Cl_3$ (Abbott Laboratories, North Chicago, Ill.) preincubated in AB serum was added to each culture plate for a final concentration of 3 μCi/ml. The final plates contained the following volumes

	ml	*% of total*
NCTC-109	1.6	53
Fetal calf serum	0.5	17
Autologous or AB serum	0.5	17
Fe^{59} in AB serum	0.2	6.5
ESF or saline	0.2	6.5
	3.0	100

After 24 hr additional incubation, the heme was extracted from the packed cells with acid methyl ethyl ketone (19) and Fe^{59}-heme counted in a well scintillation counter. Background counts averaged 6% of the total counts. The results are expressed in counts per minute (cpm) of Fe^{59}-heme/1000 erythroblasts. Since varying numbers of erythroblasts were used in the culture system, an experiment was done in which marrow cells from a single normal subject were plated at varying cell counts in a system containing autologous serum. This allowed us to establish that the heme counts and ESF response were relatively constant over the range of erythroblast numbers used in subsequent experiments. This baseline data is given in Table I.

3. Analysis of results. All groups contained two plates, and the mean was used for further calculation. In all experiments, response to ESF was measured by comparison with the patient's own control given by:

$$\text{Response to ESF} = \frac{\text{Fe}^{59}\text{ heme} - \text{ESF (group B or D)}}{\text{Fe}^{59}\text{ heme} - \text{saline (group A or C)}} \times 100\%.$$

ESF response in AB serum was compared with ESF response in autologous serum and defined as:

$$\text{Response to AB serum} = \frac{\text{Fe}^{59}\text{ heme} - \text{AB serum (group D)}}{\text{Fe}^{59}\text{ heme} - \text{autologous serum (group B)}} \times 100\%.$$

TABLE I. HEME SYNTHESIS RATES BY NORMAL BONE MARROW CELLS AT VARYING NUMBERS OF ERYTHROBLASTS.

Number of erythroblasts per mm³	cpm Fe^{59}-heme/1000 erythroblasts NaCl (control)	ESF (stimulated)	% Response
640	4462	7225	162
1600	8346	9506	114
2240	7759	8531	111
3200	8903	11571	130
4480	6466	7287	112
5760	6803	8237	121

Percentage difference between the plates was subjected to standard statistical analysis using the Student's paired or unpaired *t* test.

4. Iron quantities. Since our technique uses Fe^{59}-heme as end point, the amount of iron in the system was measured to be certain that the CRF patients did not have iron-deficient erythropoiesis in the culture. The following amounts of iron were present:

	Measured		*Actual*	
	Vol (*ml*)	*Fe/TIBC* (*μg/100 ml*)	*Iron* (*μg*)	*TIBC* (*μg*)
NCTC-109	1.6	0/0	0	0
Fetal calf serum	0.5	310/500	1.55	2.50
EP or saline	0.2	0/0	0	0
AB serum	0.5	52/186	0.26	0.93
Fe^{59}-AB serum	0.2	85/45	0.19	0.09
	3.0	67/117	2.0	3.52

The number of erythroblasts in the plates varied from 320 to 8200/mm^3. We have found that for this number of cells, the system contains sufficient iron both from the standpoint of total available iron (2.0 μg/plate, resulting in an iron concentration in the plates of 67 μg/100 ml) and iron saturation (Fe/TIBC = 67/117, 57% saturation). Plates prepared with autologous uremic serum had similar quantities of total available iron (mean 2.1 μg/plate resulting in an iron concentration of 70 μg/100 ml) and iron saturation (mean Fe/TIBC = 70/124, 56% saturation). The control patients had normal serum Fe/TIBC and the iron quantities were similar to those in the plates prepared with uremic sera.

Results. 1. Response to ESF. CPM of Fe^{59}-heme and ESF response is shown in Table II. The ESF response of bone marrow cultures obtained from normal subjects and patients with CRF is shown in Fig. 1. Normal bone marrow suspended in autologous serum showed a percentage response of 141 ± 19% (Mean ± SEM), and CRF marrow suspended in autologous serum a response of 170 ± 30%. Normal bone marrow suspended in AB serum showed a response of 160 ± 19% and CRF marrow suspended in AB serum a response of 283 ± 56%.

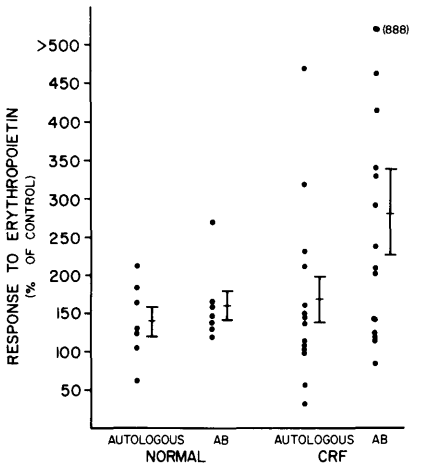

FIG. 1. Response of bone marrow cells from normal and chronic renal failure patients to erythropoietin when cultured in autologous and AB serum. Vertical bars represent the standard error of the mean (SEM).

When marrow cells were suspended in autologous serum there was no significant difference in the percentage response to ESF between normal and CRF marrow (P = NS, not significant). In AB serum the percentage response to ESF between normal and CRF marrow was not significant (P = 0.14, Student's t test). Normal marrow responded to ESF similarly in both autologous and AB serum (P = NS). However, CRF marrow appeared to respond to ESF better in AB than autologous serum (P = 0.08, Student's paired t test).

2. Effect of uremic serum on general heme synthesis. The presence of a factor(s) in uremic serum that is toxic to general heme synthesis was evaluated by comparing total heme synthesis in the cultures prepared with autologous serum and cultures prepared with AB serum. In these experiments, a standard 0.5 U/ml of ESF was added to all cultures in order to reduce the effect of variable amounts of endogenous ESF in the sera as outlined in Methods. As shown in Fig. 2, bone marrow from normal subjects always showed greater heme synthesis when cultured in autologous serum than in AB serum (response to AB serum = 41 ± 12%). In sharp distinction, bone marrow from patients with CRF usually showed greater

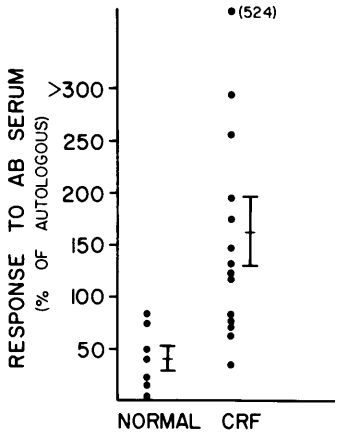

FIG. 2. Response of bone marrow cells from normal and chronic renal failure to AB serum in erythropoietin-stimulated cultures (mean ± SEM).

total heme synthesis in AB serum than in autologous serum with a response to AB serum of 165 ± 34%. The difference was significant ($P = 0.03$).

Discussion. The ability of ESF to stimulate heme synthesis in this culture method has been confirmed by numerous investigators (13–16) and, in fact, can be used as an *in vitro* assay for ESF (16). Recently, by reversing the concept of the method and keeping the ESF concentration constant, this system has been used to evaluate the erythropoietin-responsive cell. "Responsiveness" must be measured by comparison to identical cultures prepared without ESF. The mathematical problems of using a "ratio" as a measurement are well recognized and variations in the "percentage" of ESF responsiveness reported by investigators (17, 18) largely reflects technical decisions regarding the duration of culture, and specifically, the degree to which the denominator of the ratio (cultures without ESF is allowed to approach background counts). In addition, the type of ESF preparation used in the culture media influences the degree of response. In general, highly purified human ESF has a greater stimulating activity than the commercial, sheep-derived preparations (20). For these reasons, each laboratory must carefully standardize techniques in order to compare the ratio of response between their patients, and it is doubtful that a meaningful comparison can be made between laboratories using slight, but significant variations in technique.

In these studies, the percentage of response to ESF was similar in normals and patients with CRF (Table II, Fig. 1). This finding suggests an intrinsically normal ESF-responsive cell in CRF patients. The applications of this observation to the *in vivo* status of the patient must remain speculative. The degree of response to ESF of CRF marrow in autologous serum was 170 ± 30% and in AB serum was 283 ± 56% ($P = 0.08$, Fig. 1). A larger series might have showed better statistical separation. In addition, our system contains only 17% human serum. Culture mixtures using higher percentages of human serum might also have made the difference more striking. The lack of greater separation suggests that an antierythropoietin factor was not present in the autologous CRF serum. However, the large amount of ESF added to the cultures (0.5 U/ml) may have overwhelmed an inhibitor and, thereby, masked a meaningful difference. It is doubtful that the renal inhibitor substance described by Fisher and co-workers (9) and Moriyama, Saito, and Kinoshita (10) using *in vivo* methods could be demonstrated by the *in vitro* technique used in these studies. In addition, all patients with CRF were being maintained on chronic hemodialysis and it is possible that an ESF inhibitor had been removed by dialysis (11). Thus, a definite conclusion regarding an ESF inhibitor in CRF must await additional studies.

Total heme synthesis in the ESF stimulated cultures of normal subjects (Fig. 2) was always higher when the normal bone marrow cells were cultured in autologous serum rather than AB serum. Although measurable erythropoietic factors, e.g., iron and folic acid were similar between the two types of serum, it is likely that many factors in an autologous "self" serum support heme synthesis better than a "foreign," albeit normal, AB serum. In striking contrast, total heme synthesis was better in AB serum in 9 of 14 patients with CRF and, for the total group, there was a significant difference when compared to normal bone marrow (Normal = 41 ± 12% vs CRF

TABLE II. HEME SYNTHESIS RATES BY NORMAL AND CRF BONE MARROW CELLS IN AUTOLOGOUS AND AB SERUM.

	cpm FE^{59}-heme/1000 erythroblasts						
	Autologous serum					AB serum	
Controls	Group A NaCl	Group B ESF	% ESF response	Group C NaCl	Group D ESF	% ESF response	% AB response (D/B)
RH	3991	7406	186	2508	3436	137	46
TC	5623	11947	212	169	220	129	2
OF	3016	3179	105	1079	1279	119	40
JK	1015	642	63	69	100	144	16
MB	734	923	125	128	203	158	22
FM	2916	3700	127	1184	3194	270	86
FG	212	351	166	163	266	163	76
Mean ± SEM			141 ± 19			160 ± 19	41 ± 12
CRF							
SA	1056	1725	163	2438	5097	209	295
CB	1947	2953	152	1060	4380	413	148
RB	1076	612	57	676	823	122	135
LC	3330	3414	103	671	5967	889	175
EC	440	2070	471	1609	5296	329	256
RF	15656	19333	123	11521	23512	204	122
DG	3150	3396	108	3490	4179	120	123
WK	1073	2494	232	1750	2044	117	82
RM	1296	1809	135	330	1121	340	62
EM	1393	4463	320	326	1507	463	34
JM	2214	717	32	1655	1408	85	197
LN	1454	2159	149	695	1649	237	76
DP	784	1669	213	6102	8740	143	524
MS	3777	4594	122	1206	3529	293	77
Mean ± SEM			170 ± 30			283 ± 56	165 ± 34

= 165 ± 34%, $P = 0.03$). It is very unlikely that this marked difference in heme synthesis is due to variations of iron and TIBC in the autologous and AB serum since the concentrations of iron were very nearly equal as outlined in Methods. This finding suggests a toxic factor(s) in uremic serum that suppresses total heme synthesis in the cultures. The ability of autologous serum to impair marrow responsiveness could not be correlated with measurable serum factors such as BUN or degree of anemia. It is possible that AB serum corrects a deficiency of CRF serum. However, the fact that the ESF response of CRF marrow could not be distinguished from normal marrow in autologous serum makes this seem unlikely (Fig. 1).

Obviously, the effect of uremic serum on normal marrow would be of interest. Studies are now in progress to determine whether serum from patients with CRF will suppress the heme synthesis of normal marrow.

Using a similar culture system and experimental design, Fisher *et al.* (21) have recently reported findings identical to ours, a reduction of heme synthesis by normal marrow in uremic mileau. In addition, they demonstrated that uremic serum will suppress heme synthesis by a normal marrow.

The presence of a substance(s) in uremic serum that inhibits or "dampens" erythropoiesis has been suggested by several investigators (4–7, 9, 10) using *in vivo* techniques and by Saito (8) using *in vitro* studies. In the latter study, uremic serum appeared to inhibit the proliferative capacity of erythroblasts, as measured by [^{3}H]thymidine incorporation. The findings of decreased heme synthesis in our studies is consistent with decreased erythroid proliferation in cultures with uremic serum, but this pa-

rameter was not measured specifically. It is noteworthy that in all experiments uremic serum was obtained from patients on chronic hemodialysis and, therefore, the toxic factor(s) inhibiting erythropoiesis might be expected to be even greater in patients with uncontrolled renal failure.

Summary. Bone marrow cells of patients with chronic renal failure were studied in short-term *in vitro* cultures to determine erythropoietin responsiveness. Seven normals and fourteen patients on hemodialysis were studied. Bone marrow cells of normal subjects and of patients with chronic renal failure responded similarly to erythropoietin. Total heme synthesis was significantly lower in cultures prepared with uremic serum than normal serum. We conclude that there is a substance in the serum of uremic patients which suppresses general heme synthesis and that this "uremic toxin" may be responsible, in part, for the clinically severe anemia seen in these patients.

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