The Anemia of Chronic Renal Failure and Chronic Diseases: In Vitro Studies of Erythropoiesis

By Stephen F. Wallner, John E. Kurnick, Harry P. Ward, Rita Vautrin, and Allen C. Alfrey

The presence of a serum factor in chronic renal failure (CRF) which inhibits erythropoietin-stimulated erythropoiesis was studied, using a technique in which dog marrow cells were stimulated to produce heme in the presence of human serum. In the total series comparing 27 normal sera with 52 CRF sera, less heme was synthesized when the system contained CRF sera (total series, p = 0.0001). There was no evidence of inhibition of heme synthesis by serum from 12 patients with the anemia of chronic diseases (CD). Mixing experiments with normal and CRF sera suggested that this defect in CRF serum was not due to lack of a factor necessary for heme synthesis. Addition of urea, creatinine, and guanidinosuccinic acid to normal serum did not impair its ability to support erythropoiesis in this system. These data demonstrated that serum from patients with CRF contains a material inhibiting erythropoiesis in vitro. We propose that this material is responsible, in part, for the clinically severe anemia seen in these patients.

Anemia almost invariably occurs in patients with chronic renal failure (CRF) and frequently complicates the course of a wide spectrum of other chronic diseases (CD) including chronic infections, inflammatory states, and malignancies. Reticulocytopenia, bone marrow histology, and ferrokinetic data all suggest that these anemias are due primarily to underproduction of red cells. This conclusion is further supported by evidence that serum erythropoietin (EP) levels are low in relation to the degree of anemia in both CD and CRF. Failure to increase serum level of EP is likely to be critical in the failure of the marrow erythroid compartment to expand in response to the anemia.

The presence of a serum factor toxic to erythropoiesis in patients with CRF has been suggested by several lines of evidence, including inhibition of erythroblast maturation and decreased \(^{3}H\)-thymidine incorporation into erythroblasts in tissue cultures prepared with serum from uremic patients, decreased response to EP in uremic animals, and the ability of chronic hemodialysis to increase hematocrit. Recently, Fisher et al. reported that heme synthesis of normal marrow was inhibited by serum from uremic patients (referred to hereafter as "uremic serum"), and we have found that marrow cells from uremic patients synthesized heme better in a normal serum of isoantigen type
AB (AB serum) than in autologous uremic serum. Studying rats with an experimentally induced anemia of chronic disease, Zucker and Lysik reported that bone marrow cells were hyperresponsive to EP in vitro and, in contrast to the anemia of CRF, no evidence of “toxic” marrow suppression was found.

In the present experiments, we have been able to distinguish between the hypoerythropoietinemic anemias of CRF and CD by studying heme synthesis of dog erythroblasts in a system containing human serum. We found no evidence of erythropoietic inhibitors in serum from patients with CD (CD serum) whereas we have been able to confirm the presence of inhibitors of EP-directed heme synthesis in the serum of patients with CRF.

**MATERIALS AND METHODS**

**Samples**

*Normals.* Control sera were taken from 21 physiologically normal volunteers, 19 of whom were male. The mean age was 33 yr (range 18-53).

*Renal failure.* These sera were obtained from 22 stable patients participating in the chronic hemodialysis program at the Denver Veterans Administration Hospital. Blood samples were taken immediately prior to a routine hemodialysis. All were men with a mean age of 44 yr (range 28-61). Most patients were anemic with mean hematocrit of 25%, (range 15%-50%). The patient with hematocrit of 50% had polycystic kidney disease. The remainder of the hematocrits were under 40%. Mean predialysis BUN was 68 mg/100 ml and creatinine 12.0 mg/100 ml (ranges 56-114 and 8.4-22.0, respectively). All patients were oliguric. None of the patients had received blood transfusions in the 6 mo prior to this study, and all patients were receiving ferrous sulfate 900 mg/day.

*Chronic diseases.* These sera were obtained from 12 patients at the Denver Veterans Administration Hospital and the American Medical Center at Denver. Nine were men. The mean age was 57 yr (range 40-82). All patients fulfilled the usual criteria for anemia of chronic diseases, including anemia (mean hematocrit 32%, range 24%-38%), hypoferremia and depressed iron-binding capacity, absence of blood loss, reticulocytopenia, and the clinical presence of a chronic disease. Seven patients had malignant disease, and five had chronic infections or inflammatory conditions. Bone marrow biopsy specimens in five patients showed quantitatively normal erythroid compartments and normal erythroid maturation. All five had increased iron stores. None of the patients with malignant conditions had clinical evidence of marrow involvement or had been on chemotherapeutic agents. All patients had normal renal function.

All sera used in these studies were inactivated at 56°C for 45 min before use in the tissue cultures.

**Tissue Cultures**

The bone marrow cells used in the system were obtained from healthy mongrel dogs weighing 15-17 kg. The animals were anesthetized with sodium pentobarbital and sacrificed by exsanguination in an attempt to limit as much as possible the number of mature erythrocytes which subsequently entered the culture plates. Immediately after death, the ribs were removed from the animal, split lengthwise in two directions, placed in a sterile bottle containing approximately 100 ml of NCTC-109 (Microbiological Associates, Bethesda, Md.) and shaken slowly on a shaking table for 15 min. After removal of the bones the marrow cells were centrifuged (180 g) at 4°C for 10 min and washed twice with 50 ml of NCTC-109. In this manner, an average of $3 \times 10^8$ nucleated marrow cells could be extracted from one dog. This quantity of cells made it possible to prepare enough culture plates to study up to 30 sera with the cells obtained from a single animal. Sections of bone marrow from each dog showed histologically normal appearing erythroid tissue.

The technique of growing dog marrow cells in EP-stimulated cultures containing human serum has been described by Ward. The cells were resuspended in NCTC-109, fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), and the human serum to be tested. Sheep erythroid-
poietin, Step III, (Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont., Canada) was used in all experiments at a final concentration of 0.3 U/ml of culture media. The non-EP-stimulated plates contained an equal volume of normal saline. Our preliminary work with this technique had shown that $1 \times 10^7$ nucleated cells/ml of culture fluid would give maximum heme synthesis. If this number of cells was exceeded, heme synthesis decreased suggesting "media exhaustion." Using $1 \times 10^7$ cells/ml, a dose-response curve to EP showed that 0.3 U/ml would give near maximum heme synthesis. The tissue culture dishes used were 60 x 15 mm (Falcon Plastics No. 3002, Oxnard, Calif.). The final plates contained the volumes shown in Table 1. Each plate, therefore, contained $3 \times 10^7$ nucleated cells. Aqueous penicillin G and streptomycin were added to a concentration of 100 U/ml and 100 g/ml, respectively. In addition, each milliliter of tissue culture mixture was supplemented with 0.3 mg of glutamine.

The cultures were incubated for 48 hr at 37°C in a water saturated atmosphere containing 5%, CO2. A volume of 0.2 ml of $^{59}$FeCl$_3$ (International Nuclear Co., Irvine, Calif.) preincubated in normal human AB serum to assure transferrin binding, was added to each culture plate with a final concentration of 2.5 $\mu$Ci/ml of culture medium. After 4 hr of additional incubation, the marrow cells were removed from the plates, $^{59}$Fe-heme extracted from the cells using acid methylethylketone,$^{15}$ and $^{59}$Fe counted in a well scintillation counter (Picker Autowell 2).

Iron Quantities

Since our method used $^{59}$Fe-heme as its endpoint, the amounts of iron in the system were measured in order to be certain that there was no significant difference in the iron quantities in the culture plates prepared with serum from each of our three groups of subjects. The Fe/TIBC of the fetal calf serum and the AB serum/$^{59}$FeCl$_3$ mixture were 221/264 and 117/250 $\mu$g/100 ml, respectively. The iron concentrations shown in Table 2 were present in the human serum samples and in the final 3.0 ml of culture fluid which was calculated from all iron present in the culture plates. We have found that this was sufficient iron for the number of cells in the system. The final culture media contained almost identical iron quantities for the three groups of subjects. Therefore, differences in $^{59}$Fe-heme synthesis could not be due to variations in the sizes of the labeled and nonlabeled iron pools with differential uptake of the $^{59}$Fe tracer. In addition, there was no correlation between a patient's $^{59}$Fe-heme counts and serum iron levels in any of the three subject groups.

Data Analysis

Duplicate saline (control) and EP-stimulated culture plates were prepared for each human serum studied, and the mean of the two plates was used for further calculation. For the six individual dog experiments, differences in the $^{59}$Fe-heme counts among the three groups of human subjects were tested using Student's t test. For the total series of six dog experiments, differences were
Fig. 1. Erythropoietin-directed $^{59}$Fe-heme synthesis by marrow cells in the titration experiment in which serum from five different pairs of normal (NL) and chronic renal failure (CRF) subjects was mixed. The total amount of serum was held constant at 0.5 ml per culture plate. The data indicate that less heme is synthesized as the percentage of renal failure serum is increased. Vertical bars represent the mean ± SEM of the five data points from the erythropoietin-stimulated plates and is expressed in counts per minute (cpm) of $^{59}$Fe-heme synthesized by $3 \times 10^6$ nucleated marrow cells. The slope of the line is given by $Y = 1816X + 1329$. The regression is significant (by F test, $p < 0.001$).

tested using a two way analysis of variance technique. Linear regression analysis was used to calculate the line shown on Fig. 1. The significance of this line was determined using the F test. Response to EP was calculated by the following formula which compares saline and EP-stimulated plates for each serum:

$$\% \text{ EP response} = \frac{^{59}\text{Fe-heme, EP-stimulated plates}}{^{59}\text{Fe-heme, saline plates}} \times 100.$$  

RESULTS

Four separate studies were performed:

(1) Sera from normal subjects and from patients with CD and CRF were assayed for their ability to support erythropoiesis. Six experiments were performed. In each experiment, the marrow cells from one dog were cultured in the presence of a number of serum samples from different human subjects. No serum sample was studied more than once against the marrow cells of a given dog although serum from some subjects was studied in more than one of the six experiments. The number of different sera studied in each experiment is shown in Table 3. The $^{59}$Fe-heme counts reported in Table 3 represent the mean of all the serum samples studied in each patient group for each of the six dog experiments. No significant differences were noted in the saline (control) culture plates between normal sera and CD or CRF sera. The $p$ values shown in Table 3 were obtained by comparing the EP-stimulated $^{59}$Fe-heme counts of normal and CD or CRF sera. In each experiment, the mean $^{59}$Fe-heme counts in the uremic group were lower than the normal group. For the entire series of six dog experiments, this difference was highly significant ($p = 0.0001$, analysis of variance). No differences were found when comparing the normal and CD groups in either individual experiments (Student's t test) or for the two experi-
ERYTHROPOIESIS

Table 3. Ability of Normal, Chronic Renal Failure, and Chronic Disease Serum to Support Erythropoiesis by Dog Bone Marrow Cells

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Patient Group</th>
<th>No. Sera Studied</th>
<th>cpm $^{59}$Fe-Heme/$3 \times 10^7$ Cells*</th>
<th>p vs. NL</th>
<th>% EP Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NL</td>
<td>3</td>
<td>$1368 \pm 291$</td>
<td>$6710 \pm 2286$</td>
<td>$349 \pm 117$</td>
</tr>
<tr>
<td></td>
<td>CRF</td>
<td>9</td>
<td>$1700 \pm 274$</td>
<td>$3686 \pm 1229$</td>
<td>$0.08 \pm 62$</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>6</td>
<td>$2270 \pm 429$</td>
<td>$5114 \pm 1324$</td>
<td>$228 \pm 38$</td>
</tr>
<tr>
<td>2</td>
<td>NL</td>
<td>5</td>
<td>$3775 \pm 220$</td>
<td>$8450 \pm 439$</td>
<td>$227 \pm 20$</td>
</tr>
<tr>
<td></td>
<td>CRF</td>
<td>7</td>
<td>$3567 \pm 280$</td>
<td>$5891 \pm 432$</td>
<td>$0.003 \pm 5$</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>6</td>
<td>$3607 \pm 182$</td>
<td>$6908 \pm 573$</td>
<td>$194 \pm 21$</td>
</tr>
<tr>
<td>3</td>
<td>NL</td>
<td>4</td>
<td>$1897 \pm 548$</td>
<td>$8009 \pm 1503$</td>
<td>$486 \pm 96$</td>
</tr>
<tr>
<td></td>
<td>CRF</td>
<td>7</td>
<td>$2026 \pm 271$</td>
<td>$5058 \pm 664$</td>
<td>$0.06 \pm 44$</td>
</tr>
<tr>
<td>4</td>
<td>NL</td>
<td>6</td>
<td>$1356 \pm 164$</td>
<td>$4501 \pm 885$</td>
<td>$354 \pm 81$</td>
</tr>
<tr>
<td></td>
<td>CRF</td>
<td>8</td>
<td>$815 \pm 41$</td>
<td>$2461 \pm 256$</td>
<td>$0.006 \pm 27$</td>
</tr>
<tr>
<td>5</td>
<td>NL</td>
<td>4</td>
<td>$1287 \pm 281$</td>
<td>$4992 \pm 882$</td>
<td>$426 \pm 66$</td>
</tr>
<tr>
<td></td>
<td>CRF</td>
<td>8</td>
<td>$949 \pm 117$</td>
<td>$2305 \pm 400$</td>
<td>$0.002 \pm 27$</td>
</tr>
<tr>
<td>6</td>
<td>NL</td>
<td>5</td>
<td>$660 \pm 118$</td>
<td>$2228 \pm 231$</td>
<td>$270 \pm 70$</td>
</tr>
<tr>
<td></td>
<td>CRF</td>
<td>5</td>
<td>$620 \pm 62$</td>
<td>$1292 \pm 116$</td>
<td>$0.007 \pm 19$</td>
</tr>
</tbody>
</table>

*The values represent the mean ± SEM.
†Student’s t test comparing EP-stimulated $^{59}$Fe-heme counts of normal and CRF or CD groups.

Abbreviations: CD, chronic disease subjects; cpm, counts per minute; CRF, chronic renal failure subjects; EP, erythropoietin; NL, normal subjects; NS, not significant (p > 0.1).

The apparent defect in CRF serum could be due to either an inhibitor of erythropoiesis or lack of a factor necessary to support erythropoiesis. In order to evaluate these possibilities a serum titration experiment was performed using marrow cells from one dog. In this experiment five normal subjects and five CRF patients were paired and their sera mixed. The amount of human serum was kept constant at 0.5 ml/culture plate. The ratio of CRF/normal serum is shown in Fig. 1. Each of the means ± SEM represents the mean of the five data points obtained from the serum mixtures. The line shown

Table 4. Effect of Addition of Urea and Creatinine to Four Normal Sera (cpm $^{59}$Fe-Heme/$3 \times 10^7$ Cells)

<table>
<thead>
<tr>
<th>Untreated Sera</th>
<th>Urea and Creatinine Added (Final Concentrations)</th>
<th>(cpm $^{59}$Fe-Heme/$3 \times 10^7$ Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urea Nitrogen 10 mg/100 ml</td>
<td></td>
</tr>
<tr>
<td>Saline (control) plates</td>
<td>$1287 \pm 281$</td>
<td>$1273 \pm 294$</td>
</tr>
<tr>
<td>EP-stimulated plates</td>
<td>$4992 \pm 882$</td>
<td>$5169 \pm 1061$</td>
</tr>
</tbody>
</table>

The values represent the mean ± SEM of the observations on four normal sera before and after addition of urea and creatinine. There was no demonstrable effect of the addition of these materials on either baseline or EP-stimulated heme synthesis.

Abbreviations: cpm, counts per minute; EP erythropoietin

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Table 5. Effect of Addition of Guanidinosuccinic Acid to Five Normal Sera

<table>
<thead>
<tr>
<th></th>
<th>Untreated Sera</th>
<th>Guanidinosuccinic Acid Added (Final Concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.0 mg/100 ml</td>
</tr>
<tr>
<td>Saline (control) plates</td>
<td>1143 ± 132</td>
<td>1161 ± 201</td>
</tr>
<tr>
<td>EP-stimulated plates</td>
<td>2846 ± 267</td>
<td>2446 ± 207</td>
</tr>
</tbody>
</table>

The values represent the mean ± SEM of the observations on five normal sera before and after addition of guanidinosuccinic acid. Addition of this material did not effect control or EP-stimulated heme synthesis.

Abbreviations: cpm, counts per minute; EP, erythropoietin.

on this figure was calculated as described in Methods. The regression was significant by F test, p < 0.001.

(3) Urea and creatinine were added to four normal sera to levels comparable to those seen in uremia (Table 4). These urea and creatinine treated normal sera were tested against the marrow cells of one dog. No significant differences in 59Fe-heme counts were observed when adding urea and creatinine to normal sera.

(4) Guanidinosuccinic acid (GSA) was added to five normal sera to levels previously reported to occur in uremia. These sera were tested against the marrow cells of one animal as shown in Table 5. No difference was found between the control and GSA-treated sera.

DISCUSSION

The ability of EP to stimulate heme synthesis in cultures of bone marrow has been demonstrated by numerous investigators. Krantz et al. 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 EP in the culture medium. Using this method, the response of human erythroblasts to a standard dose of EP has been evaluated in a number of disease states. The existence of an EP toxin in CRF has been proposed for many years. Markson and Rennie have reported that erythroblast maturation was inhibited in vitro in cultures of marrow prepared with uremic sera. Saito has shown an inhibition of 1H-thymidine incorporation into erythroblasts in cultures prepared with uremic sera. In vivo work by Bozzini et al. suggested that there was a decrease in response to exogenous EP in uremic animals. Several groups have shown that the hematocrit of patients with CRF will rise when maintenance hemodialysis is commenced, suggesting removal of material somehow toxic to erythropoiesis. Using a system similar to ours, Fisher et al. have reported that heme synthesis by normal human marrow is inhibited by uremic sera. This observation is in line with our findings that CRF marrow appears to synthesize heme better in a normal serum than in autologous uremic serum. This finding suggests that the erythropoietic defect in uremia is not intrinsically to the erythroblasts but reflects a “conditioned” abnormality of the erythroid precursors due to alterations in uremic serum.

In this report, we have adapted the basic method of Krantz et al. to use dog bone marrow as a target organ as proposed by Ward. The technique has been fully standardized in our laboratory as outlined in Materials and Methods.
The two parameters generated with this method are heme synthesis in saline (control) plates and in EP-stimulated plates. Since a normal bone marrow organ has been used, we believe that EP-stimulated heme synthesis is the more important parameter. The calculation of per cent EP response uses a ratio which reflects changes in either the numerator (EP-stimulated cultures) or the denominator (cultures without added EP). Variations in heme synthesis in the saline (control) plates, which may be due to differences in endogenous EP, render the per cent EP response an inaccurate measure of maximal EP-stimulated erythropoiesis. For this reason, interpretation of our results is based solely upon $^{59}\text{Fe}$-heme synthesis in EP-stimulated cultures. Per cent EP response as conventionally reported is presented in Table 3. However, the $p$ values shown in this table were obtained by comparing the raw $^{59}\text{Fe}$-heme counts in the EP-stimulated plates between the normal and CRF or CD patient groups, not by comparing per cent EP response.

The data from our experiments showed that EP-directed heme synthesis by dog bone marrow cells was impaired in a milieu of CRF serum. Heme synthesis in the normal and CD plates was nearly identical. This finding suggested that the inhibition by CRF serum was not an effect that might be found in the serum of any chronically ill patient and reinforces our argument that CRF serum contains a uremic erythropoietic toxin. It should be noted, however, that the mean hematocrit of the CD patients was somewhat higher than that of the CRF patients. Thus, we cannot completely exclude the possibility that the CD sera contained an inhibitor in lower concentration. The lack of correlation between hematocrit and heme synthesis in both CRF and CD groups argues against this possibility.

The results of the titration experiment are presented in Fig. 1. These data lead us to propose that uremic serum contains an inhibitor of EP-stimulated heme synthesis and does not lack a factor necessary to support erythropoiesis, since small amounts of normal serum fail to correct the abnormality of the uremic serum. It is possible that the normal serum may have progressively replaced a material lacking in the uremic serum, but this seems unlikely since the system contains the multifactorial media NCTC-109 and fetal calf serum, and these would be expected to furnish any factors which might be lacking in the uremic serum. Further experiments are being done to verify this finding.

The results of the third experiment show that neither urea nor creatinine can be considered to be inhibitory to heme synthesis in vitro. It has been proposed that guanidinosuccinic acid is a uremic toxin and is responsible for the platelet functional defect seen in uremic patients. The data from our fourth experiment (Table 5) show that this material does not inhibit erythropoiesis.

The data presented here do not allow us to propose a mechanism of action for the inhibitory effects of serum from uremic patients. In an experiment in which mice were injected with EP and sera from either normal or uremic subjects, Fisher et al. showed a decreased EP response and suggested that the CRF sera contained an anti-EP material. Other conclusions might be drawn from these observations, and this finding requires further experimental documentation. In addition to the possibility of an anti-EP material, a true toxin, inhibiting heme synthesis and/or erythroblast maturation would fit our data. This
could be either a pathologic substance or a physiologic substance present in excess, as is the case with guanidinosuccinic acid.

Several lines of evidence suggest that middle molecules of approximate molecular size 300–1500 are responsible for some of the manifestations of the uremic syndrome. This material has been reviewed by Babb et al.24 These substances would be of intermediate dialyzability on current hemodialysis equipment, and this would be consistent with the slow progressive rise in hematocrit seen in patients on chronic hemodialysis. We are currently studying patients immediately before and after hemodialysis to determine the effect of a single dialysis on inhibitor levels. We are also studying dialysis patients longitudinally in order to follow inhibitor levels as their hematocrits rise.

Based on our results and the material briefly reviewed here, we propose the following hypothesis. An important part of the mechanism for development of the reticulocytopenic anemia seen in CD and CRF is the inappropriately low levels of serum EP in both CD and CRF. The serum of patients with CD is free of demonstrable erythropoietic toxins. The serum of patients with CRF contains a material other than urea, creatinine, or guanidinosuccinic acid capable of inhibiting EP-stimulated erythropoiesis in vitro. This inhibitor, which by indirect evidence may be of middle molecular size, could explain why the anemia of CRF is more severe than the anemia of CD when both have inappropriately low levels of EP.

In this report, we have demonstrated that serum from patients with CRF is inhibitory to erythropoiesis in vitro. We have developed and standardized a technique which uses a readily available bone marrow target organ for the study of erythropoiesis in the anemia of CRF. We have shown that this method can be used to screen suspect uremic erythropoietic toxins. We believe that the dog marrow system can be used to draw conclusions concerning the chemical nature and mode of action of our proposed toxin. Using this technique, studies directed at these issues are currently in progress.

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