Response of Nephrectomized Rats to Erythropoietin. (30305)

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Erythropoietic decompensation in renal failure has been attributed to diminished production of erythropoietin(1,2) or decreased marrow sensitivity(3,4) to this substance. The relative significance of diminished stimulation or marrow inhibition remains unknown. In the present experiments erythropoiesis and responsiveness to erythropoietic stimulation were assessed in rats made acutely uremic by bilateral nephrectomy. In addition, the effect of uremic plasma on *in vitro* reticulocyte iron incorporation was studied. The results suggest that red cell production diminished in part because sensitivity to erythropoietin was decreased. *In vitro* incorporation of radioiron by reticulocytes was not depressed by the uremic milieu.

Materials and methods. Female Sprague-Dawley rats weighing 200-300 g were maintained on a regular laboratory animal diet† and provided water *ad libitum.* Erythropoiesis was assessed in normal rats and in nephrectomized rats 48 or 72 hours following removal of the kidneys. Similar assessments were undertaken in normal rats and nephrectomized rats stimulated by injection of exogenous erythropoietin.

The stimulated nephrectomized rats were given injections of active extract of anemic rabbit plasma(5) one, 18, and 24 hours following nephrectomy. Non-operated controls were stimulated in an identical manner and measurements of erythropoiesis were made in both groups at 72 hours. Erythropoiesis was assessed by: determination of microhematocrits, enumeration of reticulocytes(6) and measurement of erythrocyte incorporation of radioiron 24 hours after intravenous injection of 1 μc Fe^{59}/1 μg Fe^{56} citrate(5). Radioiron partition in the spleen, liver and bone marrow(7) was also determined. Urea nitrogen content was determined on pooled plasma samples from each group(8).

Additional experiments tested the effect of uremic plasma on *in vitro* iron incorporation by reticulocytes. Reticulocytosis was produced by serial bleedings each approximating 20% of the rats' blood volume. One week following the initial bleeding, the rats were exsanguinated. A 50% red cell suspension was prepared by adding one volume of packed washed erythrocytes to an equal volume of Krebs-Ringer phosphate buffer containing glucose and streptomycin. Two ml of cell suspension were incubated with normal or uremic plasma of known iron content and percent saturation. A previous report details the *in vitro* procedure(9). Radioiron uptake of washed erythrocytes was measured in a well counter and the total iron incorporation per 0.1 ml of reticulocytes was determined.

Results. In vivo *experiments.* The erythrocyte radioiron incorporation was not altered 48 hours following nephrectomy but decreased from 26.4 $\pm$ 8.1%‡ in the normal controls to 15.7 $\pm$ 5.3%‡ when measured 72 hours after removal of the kidneys (Table I, A). Reticulocyte counts and bone marrow radioiron uptake were not appreciably different at these intervals.

Injections of exogenous erythropoietin produced increases in erythrocyte radioiron incorporation and reticulocytes (Table I, B). Attendant decreases in marrow and hepatic iron were noted. Similar stimulation following nephrectomy resulted in an increase in erythropoiesis but not of the magnitude noted in the non-uremic controls.

In vitro *experiments.* Reticulocytes incubated in Krebs-phosphate buffer and plasma from normal rats incorporated radioiron during the entire incubation period. Results of these experiments are recorded in Table II. Addition of uremic plasma to the incubation system did not produce inhibition of reticulocyte iron incorporation.

Comment. The erythroid decompensation

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† Purina Laboratory Chow.

‡ Standard Deviation

TABLE I. Erythropoiesis in Nephrectomized Rats.

Type	No. of rats	Hematocrit, %	Reticulocytes, %	% Radio-iron uptake in 24 hr			Urea Nitrogen, mg/100 ml
				Erythrocytes	Marrow	Hepatic	
A. Normal	13	44.2 ± 2.2	1.1 ± .8	26.4 ± 8.1	22.3 ± 5.2	17.3 ± 5.8	—
Nephrectomized							
48 hr post Nx.	10	34.9 ± 3.1	.7 ± .4	30.3 ± 6.2	22.5 ± 4.0	11.0 ± 1.9	231
72 hr post Nx.	9	33.7 ± 1.7	1.4 ± .9	15.7 ± 5.3	19.0 ± 7.3	9.4 ± 1.7	336
B. Erythropoietin stimulus							
Controls	8	42.0 ± 1.1	3.9 ± .3	53.9 ± 6.0	15.8 ± 3.2	12.3 ± 6.4	23
72 hr post Nx.	9	34.3 ± 5.3	2.5 ±1.4	31.1 ± 4.2	20.7 ± 7.2	13.2 ± 2.2	309

TABLE II. *In vitro* Reticulocytes Iron Incorporation.

Plasma	% reticulocytes	% PI/TIBC*	μg Fe/ml/plasma	0 min	30 min	1 hr	2 hr	3 hr	24 hr
Normal	14.3	30.0	1.85	.02	.33	.52	.54	.71	.98
Uremic†		48.0	1.89	.05	.49	.80	1.00	1.05	1.10
Normal	9.3	55.5	1.93	.02	.22	.36	.52	.60	.91
Uremic†		58.6	1.66	.03	.42	.65	.97	1.20	1.41
Normal	4.2	56.5	2.94	.12	.12	.28	.38	.62	.94
Uremic†		58.6	1.66	.09	.16	.28	.48	.71	.96

* $\text{PI/TIBC} = \frac{\text{Plasma iron}}{\text{Total iron binding capacity of plasma}}$

† 1.6 mg urea nitrogen/1.00 ml plasma.

of renal failure undoubtedly results from multiple abnormalities and delineation of the composite mechanism remains incomplete. Demonstration of a renal origin for erythropoietin in certain species was a salient achievement(10) and withdrawal of stimulation of red blood cell production due to deficient renal erythropoietin has been suggested by inappropriately low levels of plasma erythropoietin in patients with renal insufficiency and severe anemia(1,2). Naets'(3) observations on erythropoiesis following nephrectomy in dogs support this contention. However, a true separation of the effect of withdrawal of stimulation from suppression by the uremic state has never clearly been accomplished. Moreover, Nathan(11) and his co-workers have recently shown that chronically dialyzed anephric humans were able to maintain hemoglobin levels of 7 to 9 g/100 ml in the presence of mild hemolysis. These and other observations(12) indicate the need for continued pursuit of this problem.

In an attempt to evaluate the relative importance of erythropoietic suppression and diminished stimulation, erythropoiesis was assessed in nephrectomized uremic rodents. Diminished erythropoiesis manifested by decreased erythrocyte radioiron incorporation and reticulocytes was apparent 72 hours post nephrectomy in non-stimulated animals. An altered response of the anephric rat to stimulation by exogenous erythropoietin administration was also evident at 72 hours. The sustained higher level of erythropoiesis in the stimulated animals 72 hours after nephrectomy compared to the non-stimulated group with a similar degree of uremia indicate that the total erythropoietic decompensation is not due to effects of the uremic state *per se*. If such were the case the manifestations of uremic suppression would be expected to be equal regardless of the antecedent rate of red cell production prior to nephrectomy or the degree of stimulation. Conversely marrow suppression or lack of sensitivity is indicated by the differences noted in the uremic and non-uremic animals given exogenous erythropoietin and confirms the earlier report of Reismann *et al*(4). *In vitro* experiments which demonstrated no impairment in reticulocyte radioiron assimilation when reticulo-

cyte suspensions were incubated with uremic plasma and the studies of Markson and Moore (13) which demonstrate that hemoglobin synthesis by normoblasts is not inhibited by uremic serum suggest that diminished iron incorporation into erythrocytic precursors is not the explanation for the altered ferrokinetics noted in these experiments.

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Activities Associated with Thrombokinase Derived from Bovine Plasma.* (30306)

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Thrombokinase has been isolated from bovine plasma in a state approaching electrophoretic homogeneity(1), and it has given a single boundary in the ultracentrifuge(2). Yet this evidence does not prove that such preparations are free from significant impurities. The purest preparations have displayed the following activities: 1. activation of prothrombin in the presence of oxalate; 2. rapid activation of prothrombin in the presence of ionic calcium, phospholipid and accelerator (factor V); 3. hydrolysis of p-toluenesulfonyl-L-arginine methylester (TAMe); 4. activation of chymotrypsinogen(3).

The TAMe esterase activity has already been found to parallel thrombokinase closely (1,4). One purpose of the present study was to learn whether gel filtration easily separates the other activities. Since it was also desirable to explore the usefulness of gel filtration in purification, thrombokinase was used after the seventh step in the 8-step procedure now used for its isolation(2). That is, it had been chromatographed on DEAE-cellulose, but it had not been fractionated electrophoretically.

Materials and methods. Thrombokinase concentrate. 86 ml of Step-7 thrombokinase (2), the product from about 200 liters of bovine plasma, was brought to 0.7 saturation with ammonium sulfate, and the resulting precipitate was dissolved in 10 ml 0.4 M phosphate buffer, pH 8.13. A small amount of undissolved light brown material was removed by centrifugation. The solution had 2849 TAMe units and 9.01 mg protein per ml.

Barium carbonate-treated bovine serum. Frozen bovine serum was thawed and mixed with 50 mg barium carbonate per ml for 15 minutes at room temperature. After removal of the barium carbonate by centrifugation, the treated serum was stored at —23°C.

Prothrombin. Prepared by a procedure involving adsorption on barium sulfate, elution with 0.3 M phosphate buffer, pH 6.6 and passage through a flat column of Standard Super-Cel(5).

Chymotrpysinogen. Chymotrypsinogen A. 5× crystallized, chromatographically homo-

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