Factors Which Affect Erythropoiesis in Partially Nephrectomized and Sham-operated Rats

By A. Anagnostou, G. Vercellotti, J. Borone, and W. Fried

Current concepts of the pathogenesis of anemia in uremic animals are derived mainly from the results of studies performed either in vitro or in bilaterally nephrectomized animals. These data may not be applicable to the situation which exists in more chronically uremic animals. In 1932, Chanutin and Ferris showed that removal of five-sixths of the renal mass caused rats to become uremic and to remain so for a prolonged period of time. Rats made uremic in this manner were utilized as models for studying the pathogenesis of the anemia of uremia. Removal of five-sixths of the renal mass of rats caused their BUNs to rise to over 100 mg/100 ml and to remain at this level for over 3 wk. The hematocrits of these uremic rats fell from 42% to 30% in 3 wk. Erythropoietin (Ep) production immediately fell to a barely detectable level postoperatively and did not increase significantly in 3 wk, although the renal remnant hypertrophied. Extrarenal Ep production also remained at a low level and did not increase during the 3-wk observation period. The response of plethoric uremic rats to 2 units of Ep was as great (in some experiments greater) as that of sham-operated ones. A surprising finding was that plethoric uremic rats, injected with saline rather than with Ep, incorporated more ⁵⁹Fe into their red blood cells than did sham-operated ones. This finding suggested that in uremic rats erythropoiesis was less markedly suppressed by plethora than it was in non-uremic rats.

The association of anemia with renal failure was first reported by Richard Bright in 1836 and has been amply documented since then. The anemia is usually caused primarily by a lack of sufficient erythropoietin (Ep) production, although multiple factors may contribute to its severity. Inhibitors of Ep and toxic effects of uremic serum on heme synthesis are among the factors that have been implicated in the etiology of the anemia of uremia, based on results of studies carried out in vitro, and in acutely uremic animals (bilaterally nephrectomized).

Ep is produced primarily by extrarenal sites in neonatal mammals, and a small but significant amount of Ep is produced in extrarenal sites of nephrectomized adult animals, including man. Two anephric patients have been observed to develop an increase in their plasma Ep level several months postnephrectomy. However, no information is available regarding the degree to which extrarenal sites can be expected to hypertrophy following nephrectomy.

Chanutin and Ferris have described a procedure whereby five-sixths of the renal mass of rats could be removed. Rats operated in this way remain uremic for several months, and therefore they provide a potentially useful model for studying the causes of the anemia of chronically uremic animals.

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The purpose of the studies to be reported here was to investigate some aspects of the pathogenesis of the anemia of uremic rats (five-sixths renal mass removed), particularly their ability to produce Ep from renal and extrarenal sites and their ability to respond to Ep.

MATERIALS AND METHODS
The following two-stage surgical procedure described by Chanutin and Ferris was used for the production of uremia: Rats were lightly anesthetized with ether and the upper and lower poles of the left kidney were removed through a midline abdominal incision, leaving the pelvis and the hilum intact. In this manner, two-thirds of the left kidney was excised during the first stage of the procedure. One week later, the right kidney was removed, leaving the animal with only one-sixth of the original renal mass. Sham-operated controls had their abdomens opened and closed twice, at the same times as the partially nephrectomized groups. At the end of each operation, all animals were injected intraperitoneally with 5 ml of an isotonic saline solution containing 1 mg/ml of streptomycin and 100 units/ml of penicillin G. They also received 2 mg of lmferon intramuscularly. Hypoxia was produced in an airtight steel chamber attached to a vacuum pump, with a release valve designed to open when the desired vacuum was achieved. Hematocrits were determined using the microcapillary method. Plasma BUN levels were determined individually by the urease (conductivity) method in a Beckman Analyzer. Plasma Ep levels were assayed in exphypoxic polycythemic mice by the method of Gordon and Weintraub (at least 6-8 assay mice were used for each sample). Ep levels are expressed both in terms of the 48-hr per cent 59Fe incorporation into the mouse RBC and in equivalent units of the International Reference Preparation (IRP) of Ep. To determine the response of the bone marrow to exogenous Ep, rats were made plethoric by injection of 4 ml of isologous RBC intravenously (i.v.) on each of 2 consecutive days. Three and four days after the last transfusion, they were injected subcutaneously with 1 unit of Ep t and the following day they all received 2 μCi of 59Fe i.v. Twenty-four hours later, the animals were bled by cardiac puncture. The plasma was separated by centrifugation, and the radioactivity in both the cells and plasma obtained from 1 ml of blood was counted in a well-type scintillation counter. The plasma did not contain a significant amount of radioactivity. Radioiron incorporation into the rat RBC was calculated using the formula:

\[
\% \text{ RBC } 59\text{Fe uptake} = \frac{0.06 \times \text{weight } \times 100 \times \text{cpm/ml of rat blood}}{\text{cpm } 59\text{Fe injected}}
\]

The rate of 59Fe uptake into RBC of nonpolycythemic rats was determined by injecting 2 μCi of 59Fe i.v. Twenty-four hours later, 1 ml of blood was obtained by cardiac puncture, and the amount of radioactivity in the specimen was counted in a well-type scintillation counter. The per cent RBC 59Fe incorporation was calculated by the formula shown above, except that the blood volume was estimated at 0.05 × body weight. The statistical significance of the various results was determined using Student’s t test.

RESULTS
Body Weight, BUN, and Hematocrit (Table 1)
Uremic rats lost an average of 35 g during the 3 wk following completion of five-sixths nephrectomy, whereas sham-operated rats gained about 10 g. The
Table 1. Effects of Removal of Five-Sixths of the Kidney on the Body Weight, BUN, and Hematocrit (Mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>24-hr Uremic Rats (n = 10)</th>
<th>1-wk Uremic Rats (n = 6)</th>
<th>2-wk Uremic Rats (n = 6)</th>
<th>3-wk Uremic Rats (n = 12)</th>
<th>Sham-operated Rats* (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodyweight (g)</td>
<td>210 ± 5.5</td>
<td>208 ± 16</td>
<td>187 ± 13</td>
<td>175 ± 11.6</td>
<td>220 ± 9.3</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42 ± 0.2</td>
<td>38 ± 0</td>
<td>37 ± 2.2</td>
<td>30.6 ± 2.2</td>
<td>44 ± 0.5</td>
</tr>
<tr>
<td>BUN (mg/100 ml)</td>
<td>98 ± 3.5</td>
<td>200 ± 20</td>
<td>160 ± 42</td>
<td>120 ± 33</td>
<td>20.1 ± 2.7</td>
</tr>
</tbody>
</table>

*Values determined at some time as those of 3-wk uremic rats.

Hematocrits of uremic rats dropped from about 42% to about 30% in 3 wk. Their BUN rose to a maximum of about 200 mg/100 ml 1 wk postnephrectomy, after which it gradually fell to about 120 mg/100 ml 3 wk postnephrectomy.

Ep Production by the Kidney Remnant (Table 2)

Rats that had five-sixths of their renal mass removed in a two-stage procedure 1 hr, 7, 14, and 21 days earlier, and sham-operated rats were made hypoxic (either 0.5 or 0.46 atmosphere) for 7 hr. They were bled immediately afterward. Plasma was collected, pooled, and 0.5 ml was injected into plethoric mice for assay of Ep. Table 2 shows that rats with only one-sixth of their renal mass remaining had undetectable Ep levels in experiment 1 and only barely detectable amounts of Ep in experiment 2 after exposure to 0.5 atmosphere. In experiment 2, there was a small but significant difference between the Ep level of rats operated 3 wk prior to hypoxia and those in which partial nephrectomy was completed 1 hr prior to hypoxia. The plasma Ep level of partially nephrectomized rats exposed to 0.46 atmosphere was greater than that of rats exposed to 0.50 atmosphere; however, the plasma Ep level of partially nephrectomized rats exposed to 0.46 atmosphere 1 hr postoperatively did not differ significantly from that of rats exposed to 0.46 atmosphere 3 wk postoperatively.

Extrarenal Ep Production (Table 3)

In order to determine whether the decline of renal Ep production in rats with very little remaining renal mass was accompanied by a compensatory increase in the production of Ep by extrarenal sites, the following experiment was conducted. The kidney remnant was removed 3 wk after removal of five-sixths of the renal mass, and 15 hr later the rats were made hypoxic (0.4 atmosphere) for 7 hr. A group of rats which had undergone two sham operations 3 wk previously had both kidneys removed 15 hr prior to hypoxia and served as a control group. Immediately after exposure to hypoxia, the rats were exsanguinated and their pooled plasma was stored and assayed for Ep. Table 3 shows that the plasma Ep levels (as expressed by the 48-hr per cent 59Fe uptake into mouse RBC) were very low in all groups (below the linear portion of the dose response curve), and in three out of four experiments no significant differences were observed between the values in animals which had only one-sixth of their normal renal mass prior to complete nephrectomy and in those with intact kidneys prior to nephrectomy.
### Table 2. Effects of Removal of Five-Sixths of the Renal Mass on Ep Production by the Kidney Remnant

<table>
<thead>
<tr>
<th>Group*</th>
<th>Time After Completion of 5/6 Nephrectomy</th>
<th>Severity of Hypoxia (Fraction of Atmospheric Pressure)</th>
<th>48-hr Per Cent (^{59})Fe Uptake into RBC of Assay Mice (Mean ± SEM)</th>
<th>Units of Ep/0.5 ml of Plasma</th>
<th>48-hr Per Cent (^{59})Fe Uptake into RBC of Assay Mice (Mean ± SEM)</th>
<th>Units of Ep/0.5 ml of Plasma</th>
<th>Size of Renal Remnant (g) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sham operated†</td>
<td>0.50</td>
<td>14.9 ± 2.2</td>
<td>0.18</td>
<td>20.4 ± 4.4†</td>
<td>0.33</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>1 hr</td>
<td>0.50</td>
<td>1.3 ± 0.4</td>
<td>N.D.</td>
<td>3.6 ± 0.5</td>
<td>0.04</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>1 wk</td>
<td>0.50</td>
<td>1.4 ± 0.3</td>
<td>N.D.</td>
<td>Not done</td>
<td>—</td>
<td>0.49 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>2 wk</td>
<td>0.50</td>
<td>1.0 ± 0.2</td>
<td>N.D.</td>
<td>3.0 ± 0.8</td>
<td>0.04</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>3 wk</td>
<td>0.50</td>
<td>2.0 ± 0.5</td>
<td>N.D.</td>
<td>7.7 ± 2.2§</td>
<td>0.08</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>6</td>
<td>Sham operated†</td>
<td>0.46</td>
<td>43.3 ± 2.5</td>
<td>&gt;0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1 hr</td>
<td>0.46</td>
<td>14.1 ± 1.6</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3 wk</td>
<td>0.46</td>
<td>17.8 ± 0.26</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D., Not detectable.

*Four rats in each group.

†Rats were sham operated twice at weekly intervals. The second procedure was completed 3 wk prior to exposure to hypoxia.

‡The second sham operation was completed 1 hr prior to exposure to hypoxia. Assay mice which received no plasma injections had a per cent \(^{59}\)Fe RBC uptake of 0.6% in experiment 1 and 2% in experiment 2.

§p = 0.007 differs significantly from group studied 1 hr after five-sixths nephrectomy.
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*p = 0.0002.

1 3-wk uremic
Sham operated

2 3-wk uremic
Sham operated

3 3-wk uremic
Sham operated

4 3-wk uremic
Sham operated

5 3-wk uremic
Sham operated

<table>
<thead>
<tr>
<th>Group of Rats</th>
<th>48-hr Per Cent Ep Uptake Into RBC of Assay Mice (Mean ± SEM)</th>
<th>Units of Ep/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 3-wk uremic</td>
<td>2.9 ± 0.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Sham operated</td>
<td>4.6 ± 0.9</td>
<td>0.045</td>
</tr>
<tr>
<td>p = 0.113</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 3-wk uremic</td>
<td>2.1 ± 0.9</td>
<td>N.D.</td>
</tr>
<tr>
<td>Sham operated</td>
<td>1.1 ± 0.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>p = 0.249</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 3-wk uremic</td>
<td>2.9 ± 0.06</td>
<td>N.D.</td>
</tr>
<tr>
<td>Sham operated</td>
<td>2.7 ± 0.3</td>
<td>N.D.</td>
</tr>
<tr>
<td>p = 0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 3-wk uremic</td>
<td>5.0 ± 0.8</td>
<td>0.050</td>
</tr>
<tr>
<td>Sham operated</td>
<td>2.7 ± 0.3</td>
<td>N.D.</td>
</tr>
<tr>
<td>p = 0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 3-wk uremic</td>
<td>4.0 ± 0.8</td>
<td>0.043</td>
</tr>
<tr>
<td>Sham operated</td>
<td>3.1 ± 1.4</td>
<td>0.04</td>
</tr>
<tr>
<td>p = 0.63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D., not detectable.

* Plasma was pooled from four rats per group prior to Ep assay.
† Renal remnant was removed 3 wks after completion of five-sixths nephrectomy and just prior to exposure to hypoxia.
‡ Bilateral nephrectomy performed 3 wk after second sham operation and just prior to exposure to hypoxia.

Per Cent $^{59}$Fe Incorporation Into Rat RBC (Table 4)

Three weeks postoperatively, five-sixths nephrectomized and sham-operated rats received 2 μCi $^{59}$Fe i.v. Twenty-four hours later, the per cent $^{59}$Fe incorporation into their RBC was determined. Table 4 shows that a significantly smaller percentage of the injected $^{59}$Fe was incorporated into the RBC of the uremic rats than into those of sham-operated ones.

Response of Plethoric Uremic and Sham-Operated Rats to Ep Injected 3 Wk Postoperatively (Table 5)

Rats made uremic by five-sixths nephrectomy and sham-operated animals were hypertransfused on days 18 and 19 after nephrectomy. On days 21 and 22, half of the rats in each group were injected subcutaneously with 1 unit of Ep. The rest of the rats received an equal volume of normal saline. On day 23, all

<table>
<thead>
<tr>
<th>Group of Rats</th>
<th>Hct. (%)</th>
<th>BUN (mg/100 ml)</th>
<th>Body Weight (g)</th>
<th>24-hr Per Cent $^{59}$Fe Uptake Into Rat RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-wk uremic</td>
<td>32 ± 4.9</td>
<td>102 ± 14.5</td>
<td>190 ± 7.9</td>
<td>16.7 ± 3.7</td>
</tr>
<tr>
<td>(n = 8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham operated</td>
<td>38 ± 1.02</td>
<td>17 ± 0.8</td>
<td>219 ± 3.2</td>
<td>26.2 ± 3.2</td>
</tr>
<tr>
<td>(n = 8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p = 0.0002.
† Three weeks after completion of five-sixths nephrectomy.
animals were injected i.v. with 2 μCi $^{59}$Fe, and 24 hr later blood was collected for determination of the hematocrit, BUN, and per cent RBC $^{59}$Fe incorporation. (Rats with hematocrits of less than 55% were discarded.) Since the uremic rats had a significant loss of body weight as compared to the sham-operated ones, a second control group was added consisting of rats with body weights of 150–200 g (similar to that of the uremic animals). Table 5 shows the results. Plethoric uremic rats incorporated as much or more $^{59}$Fe into their RBC in response to Ep as did sham-operated ones. The possibility that the uremic animals received a comparatively higher dose of Ep because of their smaller body weight was excluded by the fact that there was no significant difference in the response of the two control groups in experiment 3 to Ep. A somewhat surprising finding was that plethoric uremic animals, which did not receive Ep, incorporated more $^{59}$Fe into their RBC than did sham-operated ones.

**DISCUSSION**

After removal of five-sixths of the renal mass of rats, the BUN rises to over 200 mg/100 ml within the first postoperative week. The BUN then gradually declines to 100–120 mg/100 ml and remains above 100 mg/100 ml for at least 3 wk. The hematocrit of uremic rats falls from 42% to 30% within the first 3 wk postoperatively. Accordingly, these rats probably provide a more suitable model for studying the pathogenesis of the anemia of chronically uremic subjects than either the bilaterally nephrectomized rats or the in vitro models that have been used until now.

The plasma Ep level of uremic rats exposed to 0.5 atmosphere was barely detectable, and that of uremic rats exposed to 0.46 atmosphere was significantly elevated, yet much less than that of sham-operated rats exposed to 0.46 atmosphere. The plasma Ep titer of uremic rats made hypoxic 3 wk after the second surgical procedure was slightly higher in only one experiment than was that of uremic rats made hypoxic immediately postoperatively. Taking into
consideration the fact that the rats were more anemic 3 wk postoperatively, this finding indicates that little, if any, increase in Ep production occurred as the rats adapted to their relatively renoprival condition. Although Ep production did not apparently increase substantially, the renal remnant hypertrophied in 3 wk, to about twice the weight that it had immediately postoperatively. Also, there was no consistent increase in extrarenal Ep production within 3 wk after removal of five-sixths of the kidneys. Accordingly, as was anticipated, lack of Ep production contributed significantly to the pathogenesis of the anemia of partially nephrectomized rats.

It has been suggested that either the retention of toxic substances or the production of Ep inhibitors by animals with renal failure results in suppression of erythropoiesis by a direct effect on the bone marrow. This concept has been supported by reports that heme synthesis by marrow cells, cultured in vitro, is inhibited by addition of uremic plasma to the culture media, as well as by the results of studies showing that bilaterally nephrectomized animals respond poorly to Ep. The observations reported in this paper, on the other hand, indicate that plethoric, chronically uremic rats respond to injection of Ep at least as well as do plethoric nonuremic ones. This apparent discrepancy suggests that the factors in uremic plasma which inhibit heme synthesis in vitro are either neutralized, or overcome, by some compensatory mechanism in vivo. Bilaterally nephrectomized animals are very ill after 2 days when their response to Ep is measured. They, accordingly, may fail to respond to Ep because of the existence of conditions related to their generally debilitated state, or because of conditions to which the chronically uremic rat eventually accommodates. The improvement in the anemia of some patients on chronic dialysis programs has also been attributed to the removal of factor(s) which suppress the bone marrow. However, this assumption requires direct confirmation. Another possibility is that dialysis, by correcting the hyperphosphatemia and acidosis, results in a left shift in the hemoglobin oxygen dissociation curve. This latter assertion has not been corroborated experimentally. Additionally, the observation by Van Dyke et al. that injection of a crude extract of Ep to a patient with renal failure failed to produce a significant reticulocytosis has been interpreted as evidence favoring the concept that uremia inhibits erythropoiesis in man. Essers et al. on the other hand, have observed a rise in the reticulocyte count of one of five uremic patients after injection of a large amount of Ep-rich plasma. They also have concluded that the response of uremic patients to Ep is less marked than is that of nonuremic patients. These observations, if confirmed in a larger group of patients under more controlled circumstances, do suggest that uremic humans, unlike rats, may be less responsive to erythropoietin than nonuremic ones. It is also possible that inhibition of the marrow response to Ep occurs only in subjects who are more severely uremic than are the rats with subtotal nephrectomies.

A consistent and unexpected finding was that the 24-hr per cent $^{59}$Fe uptake into RBC of hypertransfused uremic rats was greater than that of sham-operated rats. This finding suggested that the rate of erythropoiesis of uremic rats was not as markedly suppressed by plethora, as was that of nonuremic
controls. The observations that the relative response of marrow from uremic animals to Ep in vitro was greater than was that of nonuremic marrow,9,25 and that the marrow from uremic animals contained more CFU-E than did that from nonuremic ones,26 suggested that erythropoiesis was less markedly suppressed in hypertransfused uremic rats than in hypertransfused normal rats, because the erythropoietin-responsive cells of uremic rats were either more numerous or more sensitive to endogenous Ep than were those of nonuremic ones. Another possibility, which will be tested, is that extrarenal sites of Ep production are suppressed to a lesser degree by plethora than are those in the kidneys.

Finally, one must exclude the possibility that the differences in the per cent $^{59}$Fe uptake into RBC of plethoric uremic and nonuremic rats reflects an aberration in the manner of utilizing $^{59}$Fe, rather than a real difference in erythropoiesis. These three interpretations of this intriguing phenomenon are currently under investigation in our laboratory.

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18. Chanutin A, Ferris EB: Experimental


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