Administration of Erythropoietin to Newborn Rats Results in Diminished Neutrophil Production

By Robert D. Christensen, Kenneth W. Liechty, Joyce M. Koenig, Kurt R. Schibler, and Robin K. Ohls

Very high concentrations of erythropoietin (epo), in clonogenic cultures, result in reduced production of neutrophils, and fetal progenitors are more sensitive to this effect of epo than are those of adults. However, the significance of this observation is unclear because no evidence of reduced neutrophil production has been presented following administration of recombinant epo to human or animal subjects. In the present study we injected newborn rats, beginning on the first day of life, with 20, 200, or 2,000 U epo/kg body weight, and measured serum epo concentrations after 2, 8, 24, or 48 hours. After selecting a dose that resulted in serum concentrations greater than 1,000 mU/mL (a concentration that resulted in down-modulation of neutrophil production from neonatal rat progenitors in vitro) other newborn rats were treated for 3 days with that dose (1,000 U epo/kg) or a vehicle control. Administration of epo resulted in increased hematocrits ($P < .001$), reticulocyte counts ($P < .001$), normoblasts/femur ($P < .05$), and normoblasts/spleen ($P < .001$). Recipients of epo also had more erythroid colony-forming units (CFU-E) ($P < .001$) and higher CFU-E tritiated thymidine suicide rates ($P < .01$) than did controls. However, femurs and spleens of epo recipients contained fewer postmitotic neutrophils (femur, $P < .01$; spleen, $P < .01$), proliferative neutrophils (femur, $P < .01$; spleen, $P < .02$), granulocyte-macrophage colony-forming units (CFU-GM) ($P < .005$), and lower CFU-GM tritiated thymidine suicide rates ($P < .01$). Seven and nine days after twice-daily administration of 2,000 U epo/kg, blood neutrophil concentrations had diminished ($P < .05$). Thus, administration of high doses of recombinant epo to newborn rats resulted in diminished neutrophil production accompanying accelerated erythropoiesis.

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MATERIALS AND METHODS

Animals. Sprague-Dawley rats with timed pregnancies (Charles River Laboratories, Stone Ridge, NY) were housed at the University of Utah Vivarium. On the day of delivery, litters were culled to 8 to 10 pups per mother and the pups were administered intraperitoneal injections of either recombinant epo or an equivalent volume of the vehicle in which the epo was suspended (sterile phosphate-buffered saline with 0.02% human serum albumin). The epo (gift of Chuquis-Upjohn Inc, Rosemont, IL) had a specific activity greater than $3 \times 10^7$ U/mg and a purity of greater than 99.7% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Preceding all injections, the site was swabbed with 70% alcohol.

In the first studies, groups of three to five newborn rats were injected intraperitoneally with 20, 200, or 2,000 U epo/kg body weight. After 2, 8, 24, or 48 hours, blood was drawn from their external jugular veins for quantification of epo serum concentrations, and the animals were killed by decapitation. In the second studies, one day after three daily injections of 1,000 U epo/kg body weight (or control) to groups of 5 to 10 newborn rats, blood was drawn by severing the jugular vein, after which the animals were killed and the spleen and femurs removed. In the third studies, groups of four to five newborn rats received twice daily intraperitoneal injections of 2,000 U epo/kg body weight (or control). After 5,
granulocytic cells in the blood, marrow, and spleen. Quantification of serum epo concentrations, and erythroid and granulocytic cells in the blood, marrow, and spleen. Serum epo concentrations were quantified by an enzyme immunoassay (EIA) (Amgen Biologics, Thousand Oaks, CA). Hematocrits were determined by centrifugation of heparinized microcapillary tubes (Damon/IEC Division, Needham Heights, MA). Reticulocyte counts were performed on films of blood incubated with methylene blue. Circulating concentrations of nucleated blood cells were determined electronically (Coulter Electronics, Hialeah, FL). Blood smears were stained with Wright stain and subjected to 300 to 500 cell differential counts.

Marrow cells were obtained by flushing the contents of the femurs three times with 1 mL cell differential counts. Concentrations were quantified by enzyme immunoassay (EIA) 7, or 9 days of injections, blood was obtained as described previously and the animals were killed.

Serum epo concentrations.

Table 1. Serum Concentrations (mU/mL; mean ± SEM) of epo in Groups of Newborn Rats at Various Intervals After Injections of epo

<table>
<thead>
<tr>
<th>Dose of epo (U/kg)</th>
<th>2</th>
<th>8</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>6 ± 1</td>
<td>9 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>200</td>
<td>57 ± 9</td>
<td>130 ± 2</td>
<td>28 ± 6</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>2,000</td>
<td>1,112 ± 62</td>
<td>2,012 ± 155</td>
<td>1,208 ± 72</td>
<td>85 ± 13</td>
</tr>
</tbody>
</table>

Cell-cycle analysis of hematopoietic progenitors. Tritiated thymidine suicide studies were performed as described by Lu et al. Briefly, aliquots of cells were incubated with either α MEM alone, α MEM containing "cold" (nonradioactive) thymidine, α MEM containing tritiated thymidine of high specific activity (80 Ci/mmol/L; New England Nuclear, North Billerica, MA), or with α MEM containing tritiated thymidine plus excess "cold" thymidine. After a 20-minute incubation, with agitation every 5 minutes, cells were washed twice and placed into clonogenic cultures as described previously.

Data analysis. Statistical comparisons were performed using the Student t-test, the paired t-test, or the Mann-Whitney U-test, as applicable.

RESULTS

Serum epo concentrations. Serum concentrations of epo before and after administration of 20, 200, and 2,000 U epo/kg body weight are shown in Table 1. Eight hours after administration of 2,000 U/kg, epo concentrations were 2,012 ± 155 mU/mL (mean ± SEM). A dose of 1,000 U/kg was selected for the subsequent series of studies, based on our previous observation that a concentration of 1,000 mU/mL was sufficient to reduce neutrophil production from progenitors of rats in vitro.

Effect of epo administration on concentrations of circulating cells. Daily administration of 1,000 U epo/kg, for 3 days, resulted in increased hematocrits (0.43 ± 0.02 L/L v 0.36 ± 0.03 L/L in controls, mean ± SD, P < .001) and reticulocyte counts (58% ± 8% v 22% ± 6% in controls, P < .001, Fig 1).

Effect of epo administration on marrow and spleen cell populations. As shown in Fig 2, newborn rats injected with 1,000 U epo/kg, for 3 days, had fewer postmitotic neutrophils flushed from their femurs (464 ± 55 × 10^3/femur,
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**Fig 2.** Cells were flushed from the femurs of 4-day-old rat pups which, beginning on the first day of life, received three daily injections of either epo (1,000 U/kg/injection) (n = 5) or a vehicle control (n = 5). Shown are the mean ± SEM numbers of postmitotic neutrophils (segmented neutrophils + band neutrophils + metamyelocytes), proliferative neutrophils (myeloblasts + promyelocytes + myelocytes), macrophages, normoblasts, and lymphocytes recovered/femur. CONT, injected with the control; EPO, injected with erythropoietin; *, indicates P < .01 versus control; **, indicates P < .05 versus control.

mean ± SEM) than did control recipients (803 ± 74 x 10³/femur, P < .01). Recipients of epo also had fewer proliferative neutrophils (231 ± 17 x 10³/femur) than controls (377 ± 37 x 10³/femur, P < .01). However, epo recipients had more normoblasts (2,462 ± 280 x 10³/femur) than controls (1,894 ± 248 x 10³/femur, P < .05). No differences were observed in the number of macrophages or lymphocytes flushed from femurs of epo versus control recipients.

The spleens of epo recipients contained fewer postmitotic neutrophils (1,821 ± 239 x 10³/spleen) than did spleens of control recipients (4,560 ± 690 x 10³, P < .01). Spleens of epo recipients also contained fewer proliferative neutrophils (4,620 ± 990 x 10³/spleen) than controls (7,880 ± 124 x 10³, P < .02). However, spleens of epo recipients contained more normoblasts (75.9 ± 0.4 x 10⁶/spleen) than controls (22.2 ± 0.2 x 10⁶, P < .0001).

**Hematopoietic progenitor cell concentrations.** As shown in Fig 3, clonogenic cultures from spleens of epo recipients produced more CFU-E colonies (1,910 ± 36/10⁶ plated cells, mean ± SEM) than did cultures from controls (449 ± 43/10⁶ plated cells, P < .0001). However, epo recipients developed fewer CFU-GM colonies (1,174 ± 176/10⁶ plated cells) than did controls (2,389 ± 216/10⁶ plated cells, P < .005).

To determine the effect of epo administration on the concentration of hematopoietic progenitors in the circulating blood, clonogenic cultures were performed on light-density blood cells from epo and control recipients. Circulating concentrations of BFU-E were higher in epo recipients (203 ± 87/10⁶ plated light-density blood cells, mean ± SEM) than in control recipients (5 ± 2/10⁶, P < .05). No differences were noted, however, in circulating concentrations of CFU-GM (135 ± 49/10⁶ in epo recipients vs 123 ± 25/10⁶ in control recipients) or CFU-MIX (43 ± 13/10⁶ in epo recipients vs 15 ± 2/10⁶ in control recipients, P = .08).

**Effect of epo administration on cell cycle status of hematopoietic progenitors.** The effect of epo injections on susceptibility of hematopoietic progenitors to tritiated thymidine of high specific activity is shown in Tables 2 and 3. Fewer BFU-E colonies were observed (Table 2, upper panel) after incubation with tritiated thymidine than after incubation with media alone, or after incubation with “cold” thymidine, or after incubation with the combination of tritiated thymidine plus excess “cold” thymidine. The effect of epo injections on cycling status of CFU-GM is shown in the lower panel of Table 2. Fewer CFU-GM colonies were observed after incubation with tritiated thymidine than after incubation with media alone, or after incubation with “cold” thymidine, or with the combination of tritiated thymidine plus a great excess of “cold” thymidine. In addition, similar to the experiment shown in Fig 3, epo administration resulted in the growth of fewer CFU-GM colonies. No effect was observed on CFU-MIX colonies.

The tritiated thymidine suicide rates of CFU-E, BFU-E, CFU-GM, and CFU-MIX after 3 days of epo or control injections are shown in Table 3. Injections of epo resulted in a higher percentage of CFU-E killed by incubation with
tritiated thymidine. However, administration of epo resulted in a lower percentage of CFU-GM killed by incubation with tritiated thymidine. No significant effect of epo administration was observed on the tritiated thymidine suicide rates of BFU-E or CFU-MIX.

Effect of twice-daily injections of 2,000 U epo/kg body weight on circulating normoblast and neutrophil concentrations. These studies were performed to determine whether injecting newborn rats with even higher doses of epo would result in a diminution in blood neutrophil concentration. After 9 days of twice-daily injections of 2,000 U epo/kg body weight (or control), no difference was observed in body weight of recipients (0.41 ± 0.01 L/L) than in controls (0.36 ± 0.01, \( P < .01 \)). As shown in the upper panel of Fig 4, after 5 days of injections, circulating concentrations of normoblasts (72,290 ± 8,850 \( \times 10^3 \) cells/mL, mean ± SEM) were markedly increased, compared with controls (709 ± 250 \( \times 10^3 \) cells/mL, \( P < .001 \)). Lower concentrations of circulating normoblasts were seen after 9 days (17,070 ± 2,280 \( \times 10^3 \) cells/mL) than after 5 days of epo injections (\( P < .01 \)); however, concentrations were still markedly elevated compared with control recipients (1,270 ± 950 \( \times 10^3 \) cells/mL, \( P < .01 \)). Blood monocyte concentrations in the epo recipients (not shown), pooled from days 7 and 9 (1,450 ± 330/\text{mm}^3), did not differ from controls (780 ± 150/\text{mm}^3).

Shown in the lower panel of Fig 4, neutrophil concentrations after 5 days of twice-daily epo injections did not differ from controls. After 7 days of injections, however, epo recipients had lower blood neutrophil concentrations (1,240 ± 410 neutrophils/\text{mm}^3) than did controls (2,280 ± 130/\text{mm}^3, \( P < .03 \)). After 9 days of injections, neutrophil concentrations in the epo recipients were 1,630 ± 130/\text{mm}^3 and in the control recipients were 2,660 ± 500/\text{mm}^3 (\( P = .08 \)). Blood neutrophil concentrations in the 10 epo recipients, pooled from days 7 and 9 (1,430 ± 310/\text{mm}^3), were lower than in the 10 control recipients (2,490 ± 790/\text{mm}^3, \( P < .05 \)).

**DISCUSSION**

Studies by Bradley et al,10 Hellman and Grate,16 Van Zant and Goldwasser,11,12 Mayani et al,23 Ulich et al,24 and our group14,15 suggest that if erythropoiesis is accelerated sufficiently, neutrophil production will decrease. Despite these suggestions, no direct evidence of reduced neutrophil production has been presented after the administration of high doses of recombinant epo to animals or humans.

We hypothesized that if recombinant epo administration is capable of resulting in reduced neutrophil production in vivo, it might do so only under very limited conditions, such as following administration of very high doses to neonatal subjects. Our reasoning was based, in part, on the observa-

### Table 2. BFU-E Colonies (upper panel) and CFU-GM Colonies (lower panel)

<table>
<thead>
<tr>
<th></th>
<th>BFU-E Colonies (mean ± SEM) per 10⁶ cells plated</th>
<th>CFU-GM Colonies (mean ± SEM) per 10⁶ cells plated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>epo Recipients</td>
<td>Control Recipients</td>
</tr>
<tr>
<td>Media alone</td>
<td>126 ± 11*</td>
<td>131 ± 13*</td>
</tr>
<tr>
<td>Media + &quot;cold&quot; thymidine</td>
<td>69 ± 12*</td>
<td>123 ± 22*</td>
</tr>
<tr>
<td>Media + &quot;hot&quot; thymidine</td>
<td>43 ± 11</td>
<td>94 ± 10†</td>
</tr>
<tr>
<td>Media + &quot;cold&quot; + &quot;hot&quot; thymidine</td>
<td>85 ± 7†</td>
<td>140 ± 7†‡</td>
</tr>
<tr>
<td>Media alone</td>
<td>1,297 ± 169*</td>
<td>2,218 ± 420‡</td>
</tr>
<tr>
<td>Media + &quot;cold&quot; thymidine</td>
<td>1,309 ± 89*</td>
<td>2,172 ± 360‡</td>
</tr>
<tr>
<td>Media + &quot;hot&quot; thymidine</td>
<td>790 ± 20</td>
<td>729 ± 81</td>
</tr>
<tr>
<td>Media + &quot;cold&quot; + &quot;hot&quot; thymidine</td>
<td>1,306 ± 158*</td>
<td>2,213 ± 313‡</td>
</tr>
</tbody>
</table>

Before culturing, the spleen cells were incubated in either media alone, or media plus nonradiolabeled ("cold") thymidine, tritiated thymidine of high specific activity ("hot"), or the combination of "cold" plus "hot" thymidine.

*\( P < .05 \) v "hot" thymidine.

†\( P < .05 \) v epo recipients.

### Table 3. Tritiated Thymidine Suicide Rates of Progenitors

<table>
<thead>
<tr>
<th></th>
<th>BFU-E Colonies (percent, mean ± SEM)</th>
<th>CFU-GM Colonies (percent, mean ± SEM)</th>
<th>CFU-MIX (percent, mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>epo</td>
<td>73 ± 3†</td>
<td>39 ± 11†</td>
<td>18 ± 9†</td>
</tr>
<tr>
<td>Control</td>
<td>39 ± 4‡</td>
<td>67 ± 3‡</td>
<td>12 ± 7‡</td>
</tr>
</tbody>
</table>

*Calculated as follows:

\[
\text{Tritiated Thymidine Suicide Rate} = \frac{\text{Media + Cold + [hot + cold]}}{3} \times 100
\]

†\( P < .01 \) v control.
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However, in the present study, using rats on the first day of life, we observed evidence of decreased neutrophil production.

The evidence for diminished neutrophil production in these animals included: (1) reduction in the absolute number of postmitotic neutrophils in femurs and spleens; (2) reduction in the absolute number of proliferative neutrophils in femurs and spleens; (3) reduction in concentrations of CFU-GM; (4) reduction in CFU-GM thymidine suicide rate; and (5) decreased concentrations of circulating neutrophils after 7 and 9 days of epo injections.

The present studies do not clarify whether the epo administration resulted in a transient downregulation of neutrophil production, or a reduction that would continue as long as the epo was administered. Also, the studies do not clarify the mechanism by which epo resulted in diminished neutrophil production. However, previous studies suggest several possibilities. One, originally referred to by Van Zant and Goldwasser as "stem-cell competition," involves diversion of multipotent progenitors into erythroid production and, thus, away from granulocytopoiesis. A second possibility is that epo acts, directly or indirectly, to reduce clonal maturation of CFU-GM. Indeed, this effect has been demonstrated with human fetal CFU-GM in vitro. Studies by Chen et al., Nicola et al., and Walker et al. suggest that, although none of the hematopoietic growth factors directly compete for the same variety of receptor, the binding of certain growth factors can down-modulate the availability of receptors for others. Therefore, if fetal CFU-GM express epo receptors, perhaps the binding of epo to these receptors results in down-modulation of receptors for neutrophilic growth factors. A third possibility is that high concentrations of epo result in reduced elaboration, or activity, of granulocytopoietic growth factors. No evidence has been presented in support of this latter hypothesis.

Administration of recombinant epo to certain anemic neonates has been suggested as a potentially attractive alternative to erythrocyte transfusion. In view of the already reduced antibacterial defense capability of neonates, we suggest additional investigations into the effect of epo on neutrophil production before its widespread implementation as a treatment for anemic neonates.

REFERENCES


Administration of erythropoietin to newborn rats results in diminished neutrophil production

RD Christensen, KW Liechty, JM Koenig, KR Schibler and RK Ohls