Effects of Recombinant Human Erythropoietin on Megakaryocytes and Platelet Production in the Rat

By Michael V. Berridge, John K. Fraser, John M. Carter, and Fu-Kuen Lin

The contention that erythropoietin (Epo) affects platelet production was investigated in the rat with recombinant human Epo (rHuEpo). In normal rats, Epo caused a dose-dependent increase in both reticulocyte and platelet numbers, the reticulocyte response preceding that of platelets. Withdrawal of Epo resulted in reticulocytes and platelets returning to control levels. Administration of Epo, an effect not associated with a change in the half-life of circulating Epo. In polycythemic rats a platelet response was observed before an effect on reticulocytes. Erythropoietin caused a 2.4-fold increase in the frequency of small acetylcholinesterase-positive cells within 24 hours, and increased the mean megakaryocyte diameter within 48 hours. Furthermore, the [3H]-thymidine labeling index of megakaryocytes from rats treated for 24 hours with rHuEpo was increased for all stages of megakaryocyte maturation. These results support the proposal of an effect of Epo on rat megakaryocytes causing increased platelet production.

Erythropoietin (Epo) is recognized as the major erythropoietic hormone. However in certain experimental conditions, partially purified Epo has been shown to have an effect on thrombopoiesis in mice,1,2 and more recently these observations have been confirmed using recombinant human Epo.3 Furthermore, severe acute hypoxia, which results in elevated Epo levels, stimulates platelet production in rats,4 mice,5 and in humans.6 In mice, these effects have been attributed to platelet shedding in response to hypoxia,5 but other studies have shown increased [35S]sulphate incorporation suggesting increased platelet synthetic activity.4 Prolonged exposure to hypoxic conditions, however, results in reduced numbers of circulating platelets.4,5,7,8 In vitro experiments have also shown an effect of Epo on megakaryocyte colony formation in plasma clots,9,11 in serum-free culture in the presence of IL-3,11 and in agar culture,12 although this latter effect was dependent on the Epo preparation used. More recently, recombinant Epo has also been shown to stimulate megakaryocyte colony formation in plasma clots,9,11 and to promote the differentiation of murine megakaryocytes in liquid culture.13 The use of recombinant Epo in clinical studies with anemic patients with end-stage renal disease indicated no effect on platelets following long-term administration,14 although other studies have shown significant effects.7,15 It is possible that Epo has short-term effects on megakaryocyte maturation and platelet production that are not observed following long-term administration of Epo, an effect not associated with a change in the half-life of circulating Epo. In polycythemic rats a platelet response was observed before an effect on reticulocytes. Erythropoietin caused a 2.4-fold increase in the frequency of small acetylcholinesterase-positive cells within 24 hours, and increased the mean megakaryocyte diameter within 48 hours. Furthermore, the [3H]-thymidine labeling index of megakaryocytes from rats treated for 24 hours with rHuEpo was increased for all stages of megakaryocyte maturation. These results support the proposal of an effect of Epo on rat megakaryocytes causing increased platelet production.

In this study we investigated the hematopoietic response of rats to acute and chronic administration of recombinant human Epo (rHuEpo). We demonstrate that Epo results in a coordinate, dose-dependent increase in circulating reticulocytes and platelets and that these responses are transient in both the acute and chronic models. Effects of Epo on bone marrow megakaryopoiesis are also described.

MATERIALS AND METHODS

Animals. Female Fischer 344 rats aged 2 to 4 months (120 to 150 g), bred at the Wellington School of Medicine, were used throughout this study. In general, animals were age and size matched between experimental groups and injected and bled under ether anesthesia.

Animal treatment and blood cell analysis. rHuEpo9 (Amgen Biologics, Thousand Oaks, CA) (4,000 U/mL, 10,000 U/A2a) in phosphate buffered saline (PBS) containing 0.025% bovine serum albumin (BSA)] was diluted in 0.9% saline and injected intravenously (IV) in a volume of 0.4 mL into groups of three to four rats. Where necessary, rats were bled before injecting Epo by inserting a 24-gauge needle into the tail vein and allowing two drops of blood to flow into a tube containing 5 units of heparin (Sigma Chemical Co, St Louis). Red cells and leukocytes were enumerated in a Coulter S (Coulter Electronics, Hialeah, FL), whereas platelets were counted manually in a hemocytometer using a 20-fold dilution of whole blood in 1% sodium oxalate. Reticulocytes were either counted manually on blood smears using standard methods or quantitated by flow cytometry (FACS 420; Becton Dickinson, Sunnyvale, CA) using the thiazole orange method of Lee et al.30

Megakaryocyte purification. Bone marrow cells were prepared from the femurs and tibias of rats killed by cervical dislocation. Cells were flushed into CATCH medium17 without prostaglandin and washed twice. Megakaryocytes were enriched by centrifugation through a 30 mL self-generated gradient of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) prepared by centrifuging 54% Percoll in CATCH medium at 30,000 g for 20 minutes in a JA-20 rotor of a J2-21 Centrifuge (Beckman Instruments Inc, Palo Alto, CA) at 4°C. To this gradient were added 5 mL bone marrow cells (10⁶ nucleated cells/mL) in CATCH medium before further centrifugation at 1,800 g for 15 minutes. The megakaryocyte rich fraction (p = 1.05 to 1.06 g/mL as determined using Pharmacia density calibration beads) was aspirated from the gradient and washed in fresh CATCH medium. This suspension was layered onto a 2% to 4% linear gradient of Ficoll 400 (Pharmacia) in CATCH medium supplemented with 3% fetal calf serum (FCS) and centrifuged at 100 g for seven minutes. The supernatant was discarded, the cell pellet
resuspended in CATCH medium, and the Ficoll gradient separation repeated once or twice more. The final preparation consisted of 20% to 80% pure megakaryocytes as determined by staining with May-Grunwald-Giemsa (MGG) and the recovery was between 30% and 50%. Differential cell analysis of a representative megakaryocyte preparation was 34% megakaryocytes, 23% erythroblasts, 19% lymphocytes, 6% neutrophils, 3% monocytes, 1% to 2% eosinophils, and 12% myeloid blasts and other cells.

$[^{75}\text{Se}]$-selenomethionine incorporation into platelets. The method used to determine $[^{75}\text{Se}]$-selenomethionine incorporation into platelets was similar to the murine thrombopoietin assay described by Clift and McDonald. Groups of three rats were injected IV with saline or with 10 units rHuEpo at time zero and again after eight hours, at which time one set of animals was simultaneously injected with 5 $\mu$Ci $[^{75}\text{Se}]$-selenomethionine (Amersham International, Amersham, UK). A second set of animals treated similarly with saline and Epo was injected with 10 $\mu$Ci $[^{75}\text{Se}]$-selenomethionine 32 hours after initiating the treatment. Sixteen hours after injecting the radioactivity, animals were bled from the tail vein into heparin for platelet counts (see above), then bled by cardiac puncture into 2 volumes of 1% EDTA in 0.9% saline for determination of radioactivity incorporated into platelets. Blood was further diluted with an equal volume of 1% EDTA in saline and platelet rich plasma (PRP) prepared by centrifugation at 200 g for seven minutes. Platelets were obtained from PRP by centrifugation at 800 g for ten minutes, and washed twice with 1% ammonium oxalate. Platelets were quantitated using a hemocytometer and radioactivity determined in a $\gamma$-counter.

Megakaryocyte size measurement. Bone marrow cell suspensions in 50% FCS were smeared onto glass microscope slides, air dried, and stained with MGG. Mean megakaryocyte diameter was measured microscopically, cell diameter being calculated as the square root of the sum of the squares of two diameters measured at right angles. Most megakaryocytes were concentrated at the leading edge of the smear, and all recognizable megakaryocytes in the areas evaluated from the pooled slides were measured. Megakaryocytes were identified and staged according to the criteria of Williams and Levine.

Acetylcholinesterase staining. Acetylcholinesterase (AchE) is a specific cytochemical marker that has been used extensively to identify megakaryocytes and their precursors in rodent bone marrow. The method used followed that of Long and Williams. Bone marrow from both femurs and tibias of pairs of rats injected with saline or rHuEpo was flushed into CATCH medium and washed by centrifugation at 200 g for seven minutes. The cells were resuspended in CATCH medium containing 50% FCS and smears made on glass microscope slides. AchE staining was for four to five hours and small AchE-positive cells were defined as being <20 $\mu$m.

$[^{3}H]$-thymidine labeling. Pairs of rats were injected with saline or with 20 units rHuEpo at 0, 8, and 24 hours, the third injection being combined with $[^{3}H]$-thymidine (Amersham) at 1 $\mu$Ci/g. Animals received a further injection of saline or Epo six hours later and were killed 48 hours after initiating treatment. Bone marrow cells were flushed from one femur of each animal with CATCH medium, combined, washed, and resuspended in medium containing 50% FCS. Cells were smeared onto glass microscope slides, dried, fixed, and autoradiographed for 28 days using NTB-2 photographic emulsion (Kodak-Eastman, Rochester, NY). Megakaryocytes were stained with MGG and staged using the criteria of Williams and Levine.

RESULTS

Effect of rHuEpo on rat platelets in vivo. To determine the effects of Epo on platelet production, groups of three to four rats were injected six to eight times over three days (Fig 1) with 20 units rHuEpo per injection and blood platelets, reticulocytes, leukocytes, and erythrocytes measured. Figure 1D shows that compared with control animals injected with saline, rats injected with rHuEpo showed elevated platelet counts between days 2 and 6 following the initial injection (peak on day 5) and thereafter rebounded to below control values. No significant change in platelets was observed within the first 30 hours and no significant increase in platelet numbers was observed if BSA (1 $\mu$g per injection), which was used as a carrier in the TC Epo, was injected using the same protocol. Platelet and reticulocyte responses to Epo were qualitatively similar (Fig 1C and D) although increased reticulocytes were observed 24 hours before an effect on platelets. Blood leukocytes were also significantly elevated by 35% to 48% on days 3 and 4 (Fig 1A), but little change in red cell numbers was seen during the 18 days of observation (Fig 1B). However, a small but significant increase in hematocrit (5% to 9%) was observed from day 5 (results not shown). Differential analysis of blood leukocytes at peak response on day 4 showed that circulating nucleated erythroid cells increased from barely detectable levels in control animals to 1.1% ± 0.4% of total cells following Epo treatment. The percentage of circulating neutrophils also increased by 30% ± 14% in response to Epo, an effect not observed in controls injected with saline or with carrier BSA. Other blood cell types were not significantly affected by Epo treatment. Daily bleeding caused a 10% fall in blood hemoglobin concentration by day 5 and this effect was spared in Epo treated animals ($P < .0005$, results not shown).

![Fig 1. Effect of rHuEpo on circulating blood cells. Groups of four to five rats were injected with saline (C) or with 20 units rHuEpo (A) eight times over three days (arrowed) and bled daily for ten to 11 days. Blood cell determinations are presented with the SEM. (A) Leukocytes, (B) erythrocytes, (C) reticulocytes, (D) platelets.](www.bloodjournal.org)
Effect of chronic administration of rHuEpo on rat platelets. To determine the effects of long-term administration of Epo on rat platelets, groups of three rats were injected daily with rHuEpo over 22 days (twice daily on days 1 and 2) and bled every second day. Figure 2C and D shows that platelets and reticulocytes showed qualitatively similar responses to Epo, peak values being observed four to six days after initiating treatment. Both platelet and reticulocyte responses returned to normal after 15 to 17 days, a small rebound effect being observed with platelets. Blood leukocytes were slightly elevated on day 4 and on days 18 to 20 of Epo treatment (Fig 2A), whereas red cells increased steadily between days 4 and 14 reaching a new steady state of 17% to 24% above controls on days 12 to 20 (Fig 2B). The effect of Epo on red cells was paralleled by increases in both the hematocrit and hemoglobin concentration (results not shown).

Effect of rHuEpo on platelets of rats with induced polycythemia. In an attempt to amplify the response of platelets to Epo, groups of rats were made polycythemic by intraperitoneal injection with homologous red cells, the rationale being that lowered endogenous Epo levels may heighten the platelet response to Epo. Rats were bled daily and rHuEpo injected over a period of three days beginning five days after initiating red cell treatment. Although platelet numbers were depressed by about 45% in polycythemic rats, an effect directly attributable to the increased hematocrit and blood volume (Fig 3), a significant effect on platelet numbers was observed 24 hours after the first Epo injection (Fig 3D). This effect was sustained for four days during which time platelet counts returned to near normal in both Epo-treated and control rats. In contrast, in polycythemic rats, reticulocyte response to Epo was delayed for 48 hours (Fig 3C) at which time circulating nucleated erythroid cells comprised 3.6% ± 0.5% of total cells (control, 0.3% ± 0.2%). Elevated red cell numbers were observed by day 4 after initiating Epo treatment (Fig 3B), and this increase was maintained for the duration of the experiment while red cell numbers returned toward normal.

Dose response of rat platelets to rHuEpo. Figure 4 compares the dose response of platelets and reticulocytes to a single IV injection of rHuEpo over a dose range to 1 to 90 units per rat (6.5 to 660 U/kg). The dose response curves at peak were linear, no plateau being observed over the dose range tested. Peak response occurred on day 2 with platelets and day 3 with reticulocytes. A significant increase in reticulocytes was observed within 24 hours of Epo injection even at the lowest Epo dose tested (1 unit per rat), whereas platelet responses were not observed until 48 hours with a minimum dose of 3.3 units per rat required for significant response.
Fig 4. Concentration dependence of rHuEpo effect on rat reticulocytes and platelets. Groups of three rats were injected with increasing amounts of rHuEpo and (A) reticulocytes and (B) platelets measured over four to five days. ○ 1.1 units per rat; △ 3.3 units per rat; ▲ 10 units per rat; □ 30 units per rat; ■ 90 units per rat. Cell response was measured relative to a control group injected with saline and the results presented with the SEM. The dose response at peak value is presented in the inset of each panel.

Effect of rHuEpo on \([^{75}\text{Se}]\)-selenomethionine incorporation into platelets. Thrombopoietin is frequently measured by determining \([^{75}\text{Se}]\)-selenomethionine incorporation into platelets.\(^{13,20}\) To determine whether increased platelet production in response to Epo is associated with an effect on protein synthesis, groups of rats were injected with \([^{75}\text{Se}]\)-selenomethionine eight or 32 hours after initiating Epo treatment, and incorporation into platelets measured 16 hours later. Table 1 shows that rHuEpo resulted in a 29% increase in \([^{75}\text{Se}]\)-selenomethionine incorporation into platelets eight to 24 hours after initiating Epo treatment \((P < .005)\), and an 82% stimulation between 32 and 48 hours \((P < .0005)\).

Half-life of rHuEpo determined with bioactive \(^{125}\text{I}\)-Epo. To determine the effective circulating concentration of Epo in the preceding experiments, rHuEpo was radioiodinated\(^{26}\) under conditions that resulted in full retention of bioactivity as determined in a CFU-E assay. Pairs of normal and polycythemic rats were injected with \(^{125}\text{I}\)-rHuEpo \((11.5 \text{ units per rat})\) and loss of circulating radioactivity monitored over 2.5 hours (Fig 5). The overall half-life of Epo was 122 minutes for control rats and 108 minutes for polycythemic animals. A two phase curve was observed in both determinations, the first component having a half-life of 37 to 40 minutes and the second a half-life of 233 to 240 minutes. These results differ from data obtained using an in vivo assay for Epo\(^{27}\) but are similar to those obtained using biologically inactive \(^{125}\text{I}\)-Epo.\(^{28}\) Animals chronically injected with saline or Epo for 22 days before injecting \(^{125}\text{I}\)-Epo showed similar initial loss of circulating \(^{125}\text{I}\)-Epo, but significantly increased second order decay (half-life, 167 to 190 minutes) that was not significantly greater in Epo-injected animals. These results indicate no major immunological response of rats to rHuEpo.

To validate the use of radioactivity in whole blood to measure the serum half-life of Epo, serum from rats injected 2.5 hours previously with \(^{125}\text{I}\)-Epo was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. All radioactivity was recovered as undegraded \(^{125}\text{I}\)-Epo with a molecular weight of 34 kd.

Effects of rHuEpo on bone marrow megakaryocytes in vivo. The responses of rat platelets to rHuEpo (Figs 1 through 4, Table 1) suggest that Epo may exert a direct effect on megakaryocytes and their precursors in bone marrow. Several approaches were used to examine this question. First, the mean diameter of marrow megakaryocytes from animals injected once or on two consecutive days with rHuEpo (20 units per rat) was measured. Figure 6 shows that the mean diameter of all megakaryocytes increased by 17% to 18% on days 2 \((P < .005)\) and 3 \((P < .0005)\) following Epo injection. This increase in megakaryocyte diameter is seen in a shift in size distribution of the population and is particularly evident with large megakaryocytes with diameters greater than 42 \(\mu\)m, which increased in numbers 3.5-fold, 8-fold, and 10.5-fold on days 1, 2, and 3, respectively, following Epo treatment. Quantitatively similar results were obtained with splenic megakaryocytes from rats treated three days previously with rHuEpo \((P < .0005)\) (results not shown). To investigate the DNA-synthetic response of megakaryocytes to rHuEpo, pairs of rats were injected with saline or Epo and \([^{3}\text{H}]\)-thymidine incorporation into megakaryocytes was measured by determining \([^{3}\text{H}]\)-thymidine incorporation into megakaryocytes, as undegraded \(^{125}\text{I}\)-Epo with a molecular weight of 34 kd.

Table 1. Effect of rHuEpo on \([^{75}\text{Se}]\)-Selenomethionine Incorporation in Rat Platelets

<table>
<thead>
<tr>
<th>Labeling Period*</th>
<th>Percent Incorporation ((\times 10^{-2})) ± SE</th>
<th>Percent Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Epo</td>
</tr>
<tr>
<td>(A) 8-24 h</td>
<td>1.82 ± 0.15</td>
<td>2.34 ± 0.03</td>
</tr>
<tr>
<td>(B) 32-48 h</td>
<td>2.94 ± 0.22</td>
<td>5.36 ± 0.22</td>
</tr>
</tbody>
</table>

*Groups of three rats injected with saline or rHuEpo (10 units per rat at time zero and after eight hours) were injected with (A) 5 \(\mu\)Ci or (B) 10 \(\mu\)Ci \([^{75}\text{Se}]\)-selenomethionine as indicated. Platelet numbers were elevated by 18% on day 2. Statistical analysis was by the Student’s \(t\) test.
cytes determined between 24 and 48 hours. Table 2 shows that [H]-thymidine incorporation as determined autoradiographically was enhanced by Epo treatment at all stages of megakaryocyte maturation. The observation that this effect was more pronounced with immature megakaryocytes was further investigated by examining small AchE-positive cells in bone marrow. Thus, rats were injected with rHuEpo and bone marrow smears stained for AchE activity. Small (<20 μm) positively staining cells were enumerated and expressed as a percentage of total AchE cells. Table 3 shows that the frequency of small AchE+ cells increased transiently on day 1 following Epo treatment (P < .005).

**Effect of rHuEpo on megakaryocytes in vitro.** The effects of Epo on platelets, megakaryocytes, and small AchE+ cells in bone marrow may have been indirectly mediated. To examine this problem, megakaryocytes were purified from rat bone marrow and cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% FCS in the presence or absence of rHuEpo (1 U/mL). On day 1 of culture a difference in megakaryocyte size was detected in response to rHuEpo (P < .005) with control megakaryocytes having an average diameter of 21.4 ± 0.39 μm (n = 105; range, 12 to 37) and Epo-treated megakaryocytes 24 ± 0.5 μm (n = 91; range, 13 to 25). By day 3, few recognizable megakaryocytes were observed in control cultures, whereas megakaryocytes cultured with Epo for three days showed retention of morphological characteristics.

**DISCUSSION**

In the present study we show that rHuEpo exerts a reproducible, dose-dependent stimulatory effect on rat platelets under several different experimental conditions, and present evidence to suggest that these effects most likely result from a direct effect of Epo on bone marrow megakaryocytes and their immediate precursors. This interpretation is supported by other experiments that show an effect of recombinant Epo on the clonal growth of megakaryocyte colonies in plasma clots and on megakaryocyte differentiation in vitro. Furthermore, our results provide considerable support for the contention of a close in vivo relationship between erythropoiesis and thrombopoiesis. In contrast, McDonald...
Table 3. Effect of rHuEpo on the Frequency of Small AchE-positive Cells in Rat Marrow In Vivo

<table>
<thead>
<tr>
<th>Day</th>
<th>Percent sAchE+ Cells* % SE</th>
<th>Percent Control (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.17 ± 0.31</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>2.26 ± 0.01</td>
<td>4.77 ± 0.38</td>
</tr>
<tr>
<td>2</td>
<td>2.20 ± 0.36</td>
<td>1.84 ± 0.60</td>
</tr>
</tbody>
</table>

*Pairs of rats were injected with saline or 10 units rHuEpo at zero and eight hours (day 1 group) and additionally at 24 and 32 hours (day 2 group) and the percentage of sAchE-positive cells determined at 24 hours and 48 hours respectively. Statistical analysis was by the Student’s t-test. Greater than 220 AchE-positive cells were counted from each rat at each time point.

et al3 failed to show increased platelet levels in mice following administration of recombinant Epo, although increased 35S-sulfate incorporation was noted.

Comparison of the effects of Epo on reticulocytes and platelets showed that although circulating reticulocyte numbers were elevated within 24 hours of administering Epo (Figs 1 and 4), the earliest time at which a platelet effect was seen, even after repeated injections of Epo, was 48 hours (Figs 1 and 4). However, use of a more sensitive assay for platelet production, ie, [3H]-selenomethionine incorporation, demonstrated a significant platelet response to Epo in normal rats within 24 hours (Table 1). In addition, an effect of Epo on AchE+ cells and on megakaryocyte size was seen by 24 hours (Table 3 and Fig 6), and an effect on [1H]thymidine incorporation into megakaryocytes between 24 and 48 hours. Furthermore, an earlier effect of Epo on platelets was observed in polycythemic rats, in which a 32% elevation of platelet numbers was observed 24 hours after Epo treatment. Interestingly, in this situation the reticulocyte response to Epo was delayed for 48 hours, presumably due to depletion of marrow pools of mature Epo-sensitive cells in response to low endogenous Epo. In this regard a 73% decrease in marrow CFU-E has been shown in transferrin-induced polycythemia18 supporting the argument for a delayed reticulocyte response to Epo in polycythemia.

Chronic adnministration of Epo resulted in coordinate paralysis of the stimulatory effect of Epo on both platelets and reticulocyte numbers. Thus, 15 to 16 days after initiating daily injections of Epo, platelet and reticulocyte numbers had returned to control values. This effect was not caused by an immunological reaction against Epo because after 22 daily injections, the serum half-life of Epo was not significantly decreased compared with saline injected controls. Assuming a constant red cell half-life it can be argued that for erythrocytes to be maintained in a steady state at 20% above control levels, precursor cell levels need not necessarily remain elevated after establishing the new steady state providing that progenitor cell proliferation and differentiation are coordinately stimulated in response to Epo. However, because platelets are end cells with a relatively short half-life, this argument cannot be used to explain the loss of effect of Epo on platelet levels after 15 to 16 days. Rather, it appears that other overriding regulatory controls or feedback mechanisms may act to return platelets to pretreatment levels despite continued administration of Epo. For example, putative Epo receptors on megakaryocytes and their precursors may be down-regulated by Epo resulting in decreased sensitivity to Epo. In contrast, studies that have examined circulating Epo concentrations in human subjects exposed to high altitude hypoxia have observed that following an initial rise, Epo levels return to normal under conditions where erythropoiesis remains enhanced.3033 This implies that under sustained erythropoietic stress normal concentrations of circulating Epo can be associated with enhanced erythropoiesis, implying increased progenitor cell sensitivity to Epo.

Reticulocyte and platelet responses to Epo under different experimental conditions were remarkably similar, with both cell types returning to normal levels five to seven days after initiating short-term Epo treatment (in normal and polycythemic rats) and 15 to 16 days after initiating chronic Epo treatment. The apparent dissociation of these responses in polycythemic rats where the reticulocyte response was delayed and the platelet response advanced is interesting. The delayed reticulocyte response to Epo in polycythemia can be explained by decreased endogenous Epo resulting in a decreased pool of Epo-sensitive precursors (erythroblasts and CFU-E). In contrast, the advanced response of platelets to Epo may have been caused by red cell injection resulting in increased blood volume, a fall in the concentration of circulating platelets (Fig 3), and consequently increased sensitivity of thrombopoiesis to Epo.

Injecting rats with red cells that had been depleted of leukocytes to induce polycythemia resulted in a reduction in platelet numbers that correlated inversely with increased blood volume and with increased red cell numbers (Fig 3). No corresponding decrease in blood leukocytes was observed suggesting rapid compensatory mechanisms controlling the concentration of circulating leukocytes.

A transient increase in circulating WBCs was observed in each of the three different experimental protocols used two to four days after initiating Epo treatment. This effect, which was primarily neutrophilic, and apparently not endotoxin-associated, as determined by the Limulus amebocyte assay (F-K Lin, unpublished observation) was not seen in control animals injected with saline or PBS or in animals injected on several occasions with 1 μg BSA according to the protocol described in Fig 1. These results could be explained by an inflammatory response to the non-homologous human Epo (0.1 to 0.2 μg per injection) or by other cellular mechanisms involved in the feedback control of Epo-induced erythropoiesis in non-stressed animals under normal oxygen tension.

Effects of Epo on megakaryocytes were observed as early as 24 hours after initiating Epo injections (Fig 5, Table 3), small AchE-positive cells showing the greatest response to Epo. However, because Epo is capable of enhancing AchE activity in megakaryocytes,14 it is possible that the Epo effect observed by this method resulted from improved identification rather than increased numbers of these cells. That the Epo effect on megakaryocytes is probably not due to an indirect effect is suggested by in vitro results where Epo induced increases in megakaryocyte size within 24 hours of culture (cf, in vivo results). The possibility remains that the Epo effect on cultured megakaryocytes could be mediated by primary effects of Epo on other contaminating cell types such as cells of the lymphoid or myeloid lineages, which are known to elaborate many different hematopoietic growth factors.
following appropriate stimulation. This possibility seems unlikely because neither lymphoid nor myeloid cells express erythropoietin receptors\textsuperscript{15} (JK Fraser, MV Berridge, unpublished results). Furthermore, nonspecific stimulation of lymphoid and myeloid cells is discounted by the absence of endotoxin from rHuEpo. On the other hand, a direct effect of Epo on megakaryocytes is supported by studies demonstrating stimulation of clonal growth of megakaryocyte colonies and isolated megakaryocytes with rHuEpo.\textsuperscript{13-15}

The biological significance of injecting relatively large amounts of Epo into normal and stressed animals is worth considering in terms of the range of circulating Epo concentrations that may be encountered under physiological conditions, and that have been used clinically. In the present experiments, peak circulating Epo concentrations of 2.0 U/mL (20 units Epo injected three times over eight hours) and 1.0 U/mL (10 units Epo injected twice over eight hours) are attained as determined from the serum half-life of \textsuperscript{125}I-Epo (Fig 5). In comparison, Abbrecht and Littell\textsuperscript{16} observed circulating Epo concentrations exceeding 1.5 U/mL in mice exposed to hypoxia for 20 hours, and human subjects have attained >0.8 U/mL at high altitude.\textsuperscript{31} Furthermore, patients with aplastic anemia and simple anemias not involving kidney disease, have been shown to have Epo levels of up to 10 U/mL, which are believed to be within the normal physiological response range.\textsuperscript{33} In other studies, a normal human subject injected with Epo reached a serum concentration of 1.4 U/mL Epo\textsuperscript{a} and recent clinical studies with anemic patients have used up to 7 U/mL rHuEpo.\textsuperscript{18}

In summary, although evidence suggests a direct effect of Epo on megakaryocytes and consequently on platelet numbers, the biological significance of Epo in controlling blood platelets remains unknown. The possibility that Epo and thrombopoietin may compete for the same receptors on different responsive cell types is worth considering in this regard.

ACKNOWLEDGMENT

The authors wish to thank Christine Morris for autoradiography of [\textsuperscript{3}H]-thymidine-labeled cells, Peter McCaffery for gel electrophoresis of \textsuperscript{125}I-Epo in serum and urine, and An Tan for technical assistance.

REFERENCES

Effects of recombinant human erythropoietin on megakaryocytes and on platelet production in the rat

MV Berridge, JK Fraser, JM Carter and FK Lin