Limited red blood cell (RBC) regeneration often prevents collection of sufficient blood from autologous donors. We studied the effects of subcutaneous recombinant erythropoietin (rEPO) in subjects making frequent blood donations. Six healthy iron-replete male subjects took rEPO (200 U/kg) subcutaneously daily, and donated blood (450 mL) twice a week for 3 weeks. During a control study, these subjects also attempted twice-weekly blood donations without rEPO. Four other males given rEPO, including one with idiopathic hemochromatosis, waited until day 8 to begin blood donations. All healthy subjects took oral ferrous sulfate. Subcutaneous rEPO given with blood donations resulted in a marked reticulocytosis (mean peak value 568 ± 159 × 10^6/L v 235 ± 77 × 10^6/L, control study; P < .05), and enhanced RBC production at 28 days (1,208 ± 227 mL v 719 ± 161 mL, P < .05). rEPO in advance of blood donations was slightly less effective in normal subjects (941 ± 139 mL, P < .05); however, the subject with hemochromatosis produced substantially more RBCs (1,764 mL) than any normal subject. rEPO-treated normal subjects (but not the rEPO-treated patient with hemochromatosis or untreated controls) produced iron-deficient RBCs with elevated zinc protoporphyrin levels and low hemoglobin content. These cells appeared within 1 week of rEPO administration and before laboratory confirmation of depleted iron stores. Thus, subcutaneous rEPO is an effective stimulant of erythropoiesis in nonanemic blood donors. However, in addition to eventual depletion of iron stores, early functional iron deficiency affects response to the drug. © 1993 by The American Society of Hematology.

Materials and Methods

Study Design and Patient Selection

All studies were performed following approval by the Committee on Clinical Investigations of Beth Israel Hospital and in accord with an assurance filed with, and approved by, the Department of Health and Human Services.

Simultaneous rEPO and blood donations. Six adult male volunteers (subjects 1 to 6) in good health, with normal blood counts and iron stores, participated in a two-armed study (rEPO and control). In the rEPO arm, each subject self-administered rEPO (Procrit, 200 U/kg; kindly supplied by Ortho Biotech Division, Raritan, NJ) by subcutaneous injection daily for the first 21 days. During this 3-week period, each subject also attempted to donate a unit of blood (450 mL ± 10%) twice a week—may have contributed to the rather modest response to rEPO. The plasma half-life of rEPO is longer when administered subcutaneously, and subcutaneous drug is more effective in patients with the anemia of renal failure.

The current study, therefore, tested whether the effect of rEPO on autologous donors could be increased with a daily subcutaneous administration schedule. In the process, we studied characteristics of RBCs produced under stimulation by rEPO and the effects of iron stores on the erythroid response.
period was delayed 1 week, beginning on day 8 of the study, and there was no control arm. Four subjects enrolled in this study; three were adult male volunteers in good health, with normal blood counts and iron studies at enrollment (subjects 7 to 9). The fourth subject (subject 10) was a 44-year-old man with untreated idiopathic hemochromatosis, diagnosed by iron studies (serum ferritin 5,080 ng/mL) and a liver biopsy demonstrating prominent iron deposition (measured iron 49,952 μg/g dry weight; normal range 530 to 900 μg/g dry weight). This patient also had controlled hypertension and had suffered a transient ischemic attack 9 months before participation in the study.

**Laboratory Measurements**

Complete blood counts were measured using the H1-Hemalog (Technicon Instruments Co, Tarrytown, NY) on blood samples anticoagulated with potassium EDTA. This instrument determines RBC volume and hemoglobin concentration on a cell-by-cell basis using flow cytometry; arbitrarily established gates (hemoglobin concentration <28 and >41 g/dL; cell volume <60 and >120 fL) were used to define hypochromic and hyperchromic cells, and microcytic and macrocytic cells, respectively. Reticulocytes were identified using new methylene blue-stained slides and reported as the absolute number per microliter. Daily RBC phlebotomy losses for each subject were measured: these losses consisted of the volume of whole blood collected at each donation plus blood collected for laboratory testing and multiplied by that day’s hematocrit. Each subject’s RBC mass was determined based on body weight, an estimated blood volume of 70 mL/kg, and the hematocrit. RBC production (in excess of baseline) over the first 4 weeks of the study was then calculated using the following formula:

\[
\text{RBC Production} = \sum \text{RBC Phlebotomy Losses}_{\text{day 1-28}} + (\text{RBC mass}_{\text{day 2}} - \text{RBC mass}_{\text{day 1}})
\]

Serum iron, iron-binding capacity, vitamin B12, and folate were measured by standard techniques. Ferritin levels were determined by a fluorometric immunometric assay and serum erythropoietin levels by radioimmunometric assay (Diagnostic Systems Laboratory, Webster, TX).

Separation of RBCs by density was carried out using the method of Murphy. In brief, anticoagulated whole blood samples were washed twice with a solution containing 130 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L MgCl₂, 1 mmol/L potassium phosphate buffer (pH 7.4), and 10 mmol/L glucose. An 80% cell suspension was prepared and centrifuged for 1 hour at 37°C at 15,000 rpm in a Sorvall RC-5B centrifuge (Dupont Co, Wilmington, DE) using an SS-34 fixed-angle rotor. The height of the column of packed cells was measured, and a top fraction (15%) was collected by gentle pipetting. The middle 35% was discarded, and the lower 50% was collected. Zinc protoporphyrin, a measure of iron status in RBCs, was determined by fluorometry; the results were expressed as micrograms per microliter of blood and corrected for a hematocrit of 42%.

**Statistical Analysis**

Group data are expressed as mean ± SD. Two-tailed Student’s t-tests were used to determine significance (P < .05).

**RESULTS**

**Blood counts and frequency of blood donations.** Of the six subjects enrolled in the two-arm study, four subjects (1 to 4) participated in the rEPO arm first; the other two subjects (5 and 6) participated in the control arm first. All subjects...
were of normal weight (Table 1). Subcutaneous injections of rEPO (at 10,000 U/mL, between 1.3 and 1.8 mL per injection) were associated with mild burning during administration. No other complications related to drug therapy occurred.

During the rEPO arm, each subject was able to donate 6 U of blood during the 3-week donation interval (Table 1); during the control phase, anemia precluded one or more donations in five of the six subjects (mean donations 5 ± 1). During rEPO administration, the peak reticulocyte count (568 ± 159 × 10⁹/L) occurred between days 11 and 14 and was 2.4 times greater than the peak reticulocyte count without drug (235 ± 77 × 10⁹/L), which occurred later (between days 15 and 21). However, the magnitude of reticulocytosis in the rEPO versus control arm was different for each individual (range 1.2 to 4.7 times greater). Mean reticulocyte counts at the end of each week of the study (Fig 1A) were significantly greater with rEPO administration (during weeks 1 to 3) than without drug.

Phlebotomy-induced decreases in hematocrit occurred in these subjects in both arms (Fig 1A); the lowest mean hematocrit occurred during the third week in both arms but was significantly worse in the control arm (0.34 ± 0.01) than in the study arm (0.38 ± 0.03, P < .05). At the end of the control arm study (end of week 7), the mean hematocrit (0.32 ± 0.04) was 0.02 less than the start of this arm (0.42 ± 0.01, P < .05).

Subjects 7 to 9, who began rEPO 1 week in advance of blood donations, were also able to donate all 6 U of blood. The mean peak reticulocyte count (493 ± 51 × 10⁹/L; Table 1) occurred in all three subjects on study day 13 and was lower than the value in subjects 1 to 6 (P < .05). The reticulocyte count was elevated throughout the period of rEPO administration (Fig 1B). However, the hematocrit dropped substantially, and the minimum hematocrit (0.38 ± 0.02) occurred at the end of week 4, concomitant with the conclusion of blood donations.

The reticulocyte and hematocrit values for the subject with idiopathic hemochromatosis (subject 10) are shown in Fig 1C. By protocol, a unit of blood was collected from this patient twice a week during weeks 2 through 4. However, because of this patient’s history of hypertension and transient ischemic attack, additional units of blood were collected (one each during weeks 2, 4, 5, and 7) to keep his hematocrit less than 0.40. As a result, he donated 8 U of blood during the 21-day donation period and 10 U during the entire 7-week study. His peak reticulocyte count (868 × 10⁹/L) also occurred on day 12. Despite the frequent phlebotomies, his hematocrit never fell below 0.40.

RBC production. RBC production at the end of 4 weeks, expressed as volume of packed RBCs, is shown for each subject in Table 1. The volume of RBCs generated by subjects 1 to 6 on rEPO (1,208 ± 227 mL) was 68% higher than in the same subjects without rEPO (719 ± 161, P < .05). However, results in individual subjects varied; for example, subject 3 made 2.4 times more RBCs with rEPO (1,439 mL) than without (605 mL), whereas subject 5 had very little increase in RBCs with rEPO (800 mL versus 750 mL without rEPO).

![Fig 1. Absolute reticulocyte counts (left) and hematocrits (right), baseline and at the end of each week of the study. (A) Subjects 1 to 6, simultaneous rEPO and blood donations. (B) rEPO study; (C) control study. (B) Subjects 7 to 9, rEPO starting 1 week ahead of blood donations. (C) Subject 10, with idiopathic hemochromatosis, rEPO 1 week ahead of blood donations. The shaded area corresponds to the rEPO administration period.*P < .05 (rEPO v control).](www.bloodjournal.org)
Although subjects 7, 8, and 9 were each able to complete six blood donations during the 21-day interval, RBC production was lower (941 ± 139 mL) than for subjects 1 to 6 (P < .05). RBC production was highest (1,764 mL) in the subject with hemochromatosis (subject 10).

Serum EPO levels. Serum EPO levels were determined twice a week during the study. During the rEPO administration period, all subjects maintained elevated serum EPO levels (range 190 to 803 mU/mL). In the six subjects who also participated in the control arm, maximum serum EPO levels occurred at the end of the third week of the study (range 28 to 60 mU/mL).

Vitamin B₁₂ and folic acid. Vitamin B₁₂ and folate levels were measured at the start of the study and at day 28 (following blood donations). Each subject maintained constant levels of each vitamin throughout this period. B₁₂ levels were normal in all subjects, and 7 of 10 subjects had normal folate levels. Three subjects had borderline or low folate levels (normal value ≥ 2.0 ng/mL): subject 1 with rEPO: 1.2 ng/mL (day 1), 1.1 ng/mL (day 28); without rEPO: 1.4 ng/mL (day 1), 2.4 ng/mL (day 28); subject 5 with rEPO: 2.2 ng/mL (day 1), 1.9 ng/mL (day 28); without rEPO: 2.1 ng/mL (day 1), 2.4 ng/mL (day 28); and subject 9 with rEPO: 1.4 ng/mL (day 1), 1.6 ng/mL (day 28).

Iron stores. Percent saturation of transferrin (iron saturation) and serum ferritin levels for the study groups are shown in Table 1 (baseline values) and Fig 2 (values over time). Baseline iron stores were normal at the beginning of each study arm (iron saturation >15%; ferritin >20 ng/mL) in subjects 2 to 9. Subject 1 had borderline iron saturation (14%) but a normal ferritin level (86 ng/mL) at the start of the rEPO arm and normal values during the control study. Abnormal values for iron saturation and ferritin occurred in subjects 1 to 6 during rEPO administration by the end of the third week (Fig 2A). Without rEPO, mean iron saturation and ferritin levels fell but were never below normal. In subjects 7 to 9, mean iron saturation was abnormal by the end of the first week and mean ferritin levels by the end of week 2 (Fig 2B). Iron saturation in the patient with hemochromatosis (subject 10) was markedly elevated at the start of the study (80%) and rose to 100% following discontinuation of rEPO (Fig 2, panel C). His baseline ferritin level (5,080 ng/mL) fell by 30% (to 3,470 ng/mL) during rEPO administration and then rebounded (6,120 ng/mL at the end of week 4; 4,681 ng/mL at the end of week 7).

RBC size and hemoglobin distribution. RBC size and hemoglobin concentration histograms generated by the Technicon H-1 blood analyzer were obtained daily in 8 of the 10 subjects (histograms were not available for subjects 1 and 4). In normal subjects taking rEPO, but not in the subject with hemochromatosis (subject 10), an elongated shoulder caused by markedly hypochromic cells with hemoglobin concentrations as low as 20 g/dL was identified on the hemoglobin concentration histogram by day 7 or 8. Representative RBC size and hemoglobin histograms from a subject with normal iron stores (subject 6), both with and without rEPO, and subject 10, with hemochromatosis, are shown in Fig 3. In subject 6, the hypochromic shoulder developed at a time when the ferritin (46 ng/mL) and iron saturation (22%) were still normal. In each subject, the shoulder became a distinct second peak over the second week of the study, and maximum size was attained by the end of the fourth week. The two peaks merged by the end of the sixth week. The RBC volume histogram retained a unimodal shape, although a tail of macrocytic cells emerged during the rEPO administration period.

The hypochromic cell peak was not detected in subjects who donated blood without rEPO. Subject 10, with iron overload, developed a prominent macrocytic tail on the red cell volume histogram and a smaller hypochromic tail on the hemoglobin concentration histogram, but a unimodal distribution was maintained.

Correlation of the volume and hemoglobin concentration of individual RBCs on a two-dimensional plot is shown in Fig 4 for a representative subject with normal baseline iron stores (subject 9) and the subject with idiopathic hemochromatosis (subject 10). The normal distribution of RBCs on this plot resembles a teardrop. Reticulocytes are present in the tail, in the top left side of the middle quadrant. Older cells, which become progressively normocytic and normochromic because of dehydration and loss of potassium and membrane surface in excess of hemoglobin, are more centrally located in this quadrant. This pattern is seen in both subjects at baseline. In subject 9, on day 15, the new cell population was present as a bulge on the left side of the teardrop (left middle quadrant); the majority of these cells were hypochromic (hemoglobin concentration <28 g/dL) but not macrocytic (volume <120 fl). By day 29, the proportion of hypochromic cells was greater and the bulge had shifted downward as cells became more microcytic. Remnants of the hypochromic population were identifiable on these plots throughout the remainder of the 7-week study. By contrast, subject 10 developed a large increase in cells in the upper left and middle quadrants (macrocytic, hypochromic cells) consistent with reticulocytes by day 15; however, the normal teardrop outline of the histogram was preserved here and on day 29.

Density separation of hypochromic RBCs. To determine whether the underhemoglobinized RBCs produced during rEPO treatment were caused by iron-deficient erythropoiesis, fractionation of whole blood samples by density separation was carried out in subjects 7 to 9 and the patient with hemochromatosis (subject 10). The lightest concentrations of reticulocytes were found during the first 4 weeks of the study. In the three normal subjects (subjects 7 to 9), ZPP levels in unfractionated whole blood and bottom fraction (dense) cells were normal (<79 μg/dL) at all time points. ZPP levels in the light fraction were greater than 100 μg/dL on days 16, 19, 23, and 26 but were lower on subsequent assays (days 32, 43, and 60). No elevations in ZPP levels were seen at any time point in whole blood or
density-separated fractions in the patient with idiopathic hemochromatosis (subject 10).

**DISCUSSION**

The effect of daily subcutaneous rEPO (1,400 U/kg/week) on erythropoiesis was substantial, with very high peak reticulocyte counts during the rEPO administration period. Although phlebotomy-induced anemia can increase the erythropoietic rate, the additional effect of rEPO was demonstrated in subjects 1 to 6, who served as their own controls. In this group, the mean peak reticulocyte count during the rEPO study was 2.4 times greater than during the control arm (blood donations without rEPO). Mean RBC production was also significantly better with rEPO (1,208 ± 277 mL versus 719 ± 161 mL). Thus, subcutaneous rEPO is effective in stimulating erythropoiesis in nonanemic donors. Further studies are needed to determine whether in this setting, as in the anemia of renal failure, equivalent dosages of rEPO are more effective when administered by the subcutaneous as opposed to intravenous route.

Despite increased RBC production with subcutaneous rEPO administration, the hematocrits of subjects 1 to 6 still fell during the donation period. Because a lag of 4 or more days is seen between institution of rEPO therapy and appearance of increased numbers of reticulocytes in humans,28 we reasoned that an administration schedule in which rEPO was given before blood donation might reduce or eliminate the anemia. However, even when rEPO was started 7 days in advance of the blood donation period, RBC production did not improve, and anemia occurred by the end of the donation period (end of week 4). On the other hand, the patient with hemochromatosis (subject 10) never developed anemia. This individual was placed on the same rEPO dosage schedule as subjects 7 to 9, although without any supplemental iron administration. His peak reticulocyte count (868 × 10⁹/mL) was higher than that in any other subject except subject 3, and he produced nearly twice the volume of RBCs (1,764 mL) made by other subjects. His exuberant response to rEPO necessitated the removal of four extra units of blood over the study period to keep his hematocrit from rising.

Based on the differences in RBC production between subjects with normal iron stores and this subject with hemochromatosis, we looked closely at factors that might have affected erythropoiesis. B₁₂ and folate levels did not change in individual subjects during the blood donation period. However, despite adequate iron stores in our subjects at the start of each study, and supplemental ferrous sulfate, we found that the blood donation protocol resulted in lower iron stores and that rEPO hastened this depletion. Only the subject with iron overload had no drop in iron saturation levels throughout the study, although his ferritin level fell 30% during rEPO administration. These results are consistent with previous observations that iron stores are critical to the rate of RBC production.29
**REPO AND FUNCTIONAL IRON DEFICIENCY**

The development of iron deficiency occurs in stages: first, body iron stores are depleted, reflected by a fall in serum ferritin; then transferrin saturation becomes abnormal; and finally anemia develops. RBCs with a low mean corpuscular hemoglobin concentration (MCHC) are usually not seen until this final stage. Although our normal subjects taking REPO eventually became iron deficient from the many blood donations, we were surprised to identify markedly underhemoglobinized RBCs promptly following REPO administration and in advance of laboratory evidence of depleted iron stores. Reticulocytes have slightly reduced hemoglobin concentrations on account of greater amounts of cell water and potassium, which are lost with aging. However, the hypochromic cells that appeared with REPO treatment were normocytic and, therefore, undersized for normal reticulocytes, and they formed distinct separate peaks on the RBC histograms, suggesting an abrupt decrement in hemoglobin synthesis. Furthermore, the hypochromic cells in REPO-treated normal subjects were iron deficient, as demonstrated by elevated ZPP levels in the light fraction cells, but not whole blood, of subjects 7 to 9. Protoporphyrins chelated to zinc instead of iron are normally present in the RBC (including reticulocytes) in only very low concentrations; the concentration of ZPP increases in the setting of iron deficiency. In contrast to our normal subjects, only the patient with idiopathic hemochromatosis (subject 10) had normal ZPP levels in reticulocyte-enriched fractions.

The presence of iron-deficient RBCs in iron-replete blood donors taking REPO has not previously been reported. The effect of erythropoietin on RBC production is highly dependent on iron stores, which affect the size of the RBC and the amount of hemoglobin per cell, in addition to the rate of erythropoiesis. The quantity of iron suitable for the needs of basal RBC production may be, or become, inadequate when the erythropoietic rate increases, and has been described as a functional or relative iron deficiency. Thus, despite normal iron saturation and normal or even elevated serum ferritin, elevated whole blood ZPP levels have been measured in clinical situations where shortened RBC survival necessitates rapid erythropoiesis, such as hereditary spherocytosis and thalassemia. Furthermore, in occasional REPO-treated patients with renal failure or lymphoproliferative diseases,
dwindling responses to rEPO over time despite adequate or increased iron stores have been reported to improve with iron supplementation.17,40-43 In our rEPO-treated subjects receiving very large doses of drug to stimulate rapid erythropoiesis, physiologically normal amounts of mobilizable iron proved at the onset insufficient to meet the needs of the expanded pool of transferrin receptors on red cell precursors. Thus, iron-deficient RBCs were produced very quickly after institution of rEPO therapy and before the iron stores were depleted by blood donations. Relative iron deficiency was not seen when the same subjects donated blood in the absence of rEPO even though iron saturation and ferritin levels fell over the course of the donation period. In this setting, the modest increases in endogenous serum erythropoietin levels resulting from phlebotomy-induced anemia did not increase erythropoiesis sufficiently to outstrip the available iron stores at any time point. Relative iron deficiency was also not observed in the patient with hemochromatosis; here the demand for iron associated with the marked increase in erythropoiesis was presumably amply met by his increased iron stores.

Fig 4. Two-dimensional histograms of hemoglobin concentration (HC, X-axis) v RBC volume (V, Y-axis) in subjects 9 and 10 at three time points. The vertical lines represent hemoglobin concentration gates (left line, 28 g/dL; right line, 41 g/dL); the horizontal lines depict volume gates (bottom line, 60 fL; top line, 120 fL). Subject 9 had donated 3 U of blood by day 15, and 6 by day 29; subject 10 had donated 3 U of blood by day 15, and 8 by day 29.

Fig 5. Zinc protoporphyrin levels in whole blood and density-separated fractions. (A) Subjects 7 to 9 (shown as means ± SD). (B) Subject 10. (■) Whole blood; (□) top 15% (light) fraction; (■) bottom 50% fraction. The proportion of reticulocytes (mean percentage ± SD in [A]) in each sample are shown in the inserts.
The limitations of iron on rEPO-driven erythropoiesis thus occur even before laboratory criteria for iron deficiency are met. However, increasing iron stores to above physiologically normal levels to meet the needs associated with rEPO-driven rapid RBC regeneration may be difficult to accomplish. Ferrous sulfate, the best absorbed of oral iron preparations currently available, was inadequate in our study. Intravenous iron may be more effective, but iron dextran, the only compound in use in the United States, has a risk of anaphylaxis and other side effects.

In conclusion, subcutaneous rEPO administration amply enhanced RBC production in nonanemic blood donors. However, in comparison to RBCs generated by subjects without rEPO, the cells produced following rEPO were iron-deficient despite iron stores still sufficient for basal erythropoiesis. Furthermore, the magnitude of the response to rEPO, measured by the amount of RBCs produced, may have been blunted in our subjects, as judged by the larger volume of cells produced by the patient with iron overload; studies of additional patients with hemochromatosis are necessary to confirm this. Thus, in addition to eventual depletion of iron stores, an early functional iron deficiency affects the response to rEPO. The advantages and disadvantages of rEPO-stimulated erythropoiesis in autologous blood donation, and the utility of new regimens to augment iron stores, require further study.

ACKNOWLEDGMENT

We are indebted to the volunteers who participated in this study; to Drs Lucia De Franceschi, Justine Carr, Gary Horowitz, Jim Faix, the staff of the hematology and chemistry laboratories at Beth Israel Hospital, and the hematology laboratory at Brigham and Women’s Hospital for their expert technical assistance with aspects of testing; to Dr Alan Moses and the Clinical Research Center at Beth Israel Hospital for help with portions of the clinical protocol; and to Drs H. Franklin Bunn and Stephen H. Robinson for their advice and critique of the manuscript.

REFERENCES


32. Come SE, Shohet SB, Robinson SH: Surface remodeling vs. whole-cell hemolysis of reticulocytes produced with erythropoietin in iron-deficiency anemia. Blood 44:817, 1974


35. Weed RJ, Reed C: Membrane alterations leading to red cell destruction. Am J Med 41:681, 1966


Red blood cell regeneration induced by subcutaneous recombinant erythropoietin: iron-deficient erythropoiesis in iron-replete subjects [see comments]

C Brugnara, LA Chambers, E Malynn, MA Goldberg and MS Kruskall

Updated information and services can be found at:
http://www.bloodjournal.org/content/81/4/956.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml