Erythropoietin-Dependent Primary Pure Erythrocytosis

By Nicholas Dainiak, Ronald Hoffman, Alan I. Lebowitz, Lawrence Solomon, Louise Maffei, and Kim Ritchey

We investigated the pathogenesis of isolated erythrocytosis of 14 yr duration in a 28-yr-old man. The increase in red cell mass was attributed to increased erythropoietin production. An extensive search for recognized causes of secondary erythrocytosis was unrevealing. Family members were found to be hematologically normal. After reduction of the circulating red cell mass by 20%, erythropoietin activity nearly quadrupled, thus suggesting a normal erythropoietin response to phlebotomy. When bone marrow cells of the patient were cultured in plasma clots in the absence of added erythropoietin, endogenous erythroid colony formation was observed, a pattern previously believed to be specific for polycythemia vera bone marrow cells. Our observations suggest that the erythrocytosis in this individual is best explained by an abnormal "servoregulatory" mechanism of erythropoietin production. In addition, this is the first instance in which the rule that endogenous erythroid colony formation is correlated with the diagnosis of polycythemia vera has not held.

POLYCYTHEMIA VERA and the secondary polycythemias are accompanied by an absolute increase in the measured red cell mass. Secondary polycythemia denotes a group of disorders in which the increased red cell mass is caused by and is maintained by increased erythropoietin production. Normal red cell production through the humoral mechanism of erythropoietin is believed to be controlled primarily by the balance between tissue oxygen supply and demand. Relatively small changes in hematocrit in healthy subjects, either lowered by phlebotomy or increased by transfusion, lead to predictable changes in daily urinary excretion of erythropoietin. Individuals with hypoxia-induced secondary erythrocytosis and increased erythropoietin levels also respond to hematocrit reduction by phlebotomy with further increases in urinary erythropoietin production as well as plasma erythropoietin production. In polycythemia vera, erythropoietin output is markedly decreased at high hematocrit levels, but here also hematocrit reduction to a normal or anemic level results in an increase in measurable plasma and urinary erythropoietin levels, even though this response is blunted.

Erythrocytosis is occasionally associated with benign or malignant neoplasias, many of which are believed to produce erythropoietin-like substances. In tumor-associated erythrocytosis, urinary erythropoietin excretion remains constant over a wide range of hematocrit and is truly autonomous. Several families have
recently been reported in which erythrocytosis has been diagnosed in several members. In these erythrocytotic individuals, erythropoietin production is increased and is unrelated to tissue oxygen requirements, thus suggesting that erythropoietin production is independent of the usual regulatory factors. This report describes a young man with isolated erythrocytosis of 14 yr duration whose increased red cell mass is attributed to increased erythropoietin production in the absence of any detectable secondary causes and in whom a reciprocal relationship of hemoglobin concentration to erythropoietin activity is demonstrated. We believe that this patient's hematologic findings are best explained by an inappropriate upward readjustment of a physiologic "set point" for erythropoietin production.

**CASE REPORT**

A 28-yr-old man presented at age 14 yr with complaints of fatigue. A spleen tip was palpable, and marked plethora was noted. Laboratory data revealed a hemoglobin of 20.8 g/dl, a hematocrit of 59%, a white blood cell count of 7100/cu mm, a platelet count of 160,000/µl and a reticulocyte count of 2.0%. Erythrocyte morphology was unremarkable. The patient was admitted to a community hospital in March, 1964, where bone marrow aspiration demonstrated erythroid hyperplasia. The 51Cr-labeled red cell mass was 57.5 ml/kg, and the plasma volume was 40.0 ml/kg, as determined by the 131I-labeled serum albumin technique. Because of the radiographic prominence of the pulmonary vasculature, angiography of the pulmonary vessels was performed, and no cardiac or pulmonary abnormality was observed. Serum uric acid, plasma iron, total iron-binding capacity, and calcium and liver enzyme determinations were all within normal limits. Radiographic examination of the upper gastrointestinal tract and intravenous pyelography were unrevealing, and chest fluoroscopy and x-rays of the long bones were unremarkable. A liver-spleen scan confirmed the presence of moderate splenomegaly. The patient was said to have polycythemia vera and was phlebotomized two units of whole blood, with resolution of fatigue, and a program of monthly phlebotomies was begun.

The patient has been a smoker of one package of cigarettes daily since 1965. He denies a history of renal colic, gouty arthritis, hypertension, pruritus, peptic discomfort, headaches, bleeding, or thrombotic disease. There is no family history of malignancy, polycythemia, leukemia, or other hematologic dyscrasias. Both parents and 4 siblings (3 sisters and 1 brother) had unremarkable complete blood counts, platelet and reticulocyte counts, and physical examinations.

Over the last 14 yr, the patient has continued to receive monthly phlebotomies because of increasing irritability, fatigue, and malaise when his hematocrit rises above 55%. It is noteworthy that phlebotomy results in a decrease in the size of the spleen on physical examination and that thrombocytosis and leukocytosis have never been observed. In July, 1977, the patient was reevaluated. Physical examination again revealed only a palpable spleen tip. Bone marrow aspiration and biopsy disclosed a hypercellular specimen with mild erythroid hyperplasia and an absence of iron stores. There was no evidence of fibrosis of the bone marrow; cytogenetic analysis of the marrow cells employing the Giesma banding technique revealed no abnormalities. A liver-spleen scan confirmed again the presence of splenomegaly. Arterial blood gas analysis revealed a pH of 7.44, an oxygen saturation of 97.3%, an oxygen tension of 99 mm Hg, and a carbon dioxide tension of 28 mm Hg at rest. Repeat arterial blood gas analysis during sleep, 4 hr after placement of an indwelling intraarterial catheter, revealed an oxygen tension of 87 mm Hg and an oxygen saturation of 95.8%. In addition, the patient was exercised dynamically on a treadmill for 20 min, reaching a heart rate of 185–190 beats/min, and repeat blood gas analysis was immediately performed, revealing an oxygen tension of 112 mm Hg and an oxygen saturation of 97.1%.

Hemoglobin electrophoresis disclosed the presence of hemoglobin AA, and 1.0% hemoglobin F and 1.7% hemoglobin A2 were observed by quantitative analysis. Isoelectric focusing of hemoglobin performed by Dr. H. F. Bunn revealed no abnormal hemoglobin, and the O2 affinity of hemolysates stripped of organic phosphates was also normal. Carboxyhemoglobin levels were less than 0.5% on three occasions. Other laboratory data included a serum iron level of 45 µg/dl, a total iron-binding capacity of 545 µg/dl, a serum lysozyme level of 9 µg/ml (normal, 7–14 µg/ml), a leukocyte alkaline phosphatase score of 63 (normal, 13–130), a serum phosphorus level of 3.9 mg/dl (normal, 3.1–4.5 mg/dl), and quantitatively normal vitamin B12 and B12-binding protein determinations. Serum lactate determinations
obtained before and after inflating a blood pressure cuff above systolic pressure for 10 min were 18 mg/dl and 22 mg/dl, respectively (normal range, 16–20 mg/dl). Similarly obtained samples of blood were assayed for pyruvate, and the levels obtained were 0.5 mg/dl and 0.1 mg/dl, respectively (normal range, 0.3–0.9 mg/dl).

The patient was electively hospitalized in January, 1978, when repeat intravenous pyelography, chest x-ray, abdominal ultrasound examination, brain scan, ACTA of the brain, and pulmonary function testing were entirely unremarkable. A renal arteriogram demonstrated no mass or abnormalities of the renal macrovasculature or microvasculature.

**MATERIALS AND METHODS**

### Red Cell Function Studies

By use of an Aminco Hem-O-Scan apparatus (American Instrument, Silver Spring, Md.) oxygen dissociation curves were constructed at 37°C, and the percentage oxyhemoglobin was determined. Red cell 2,3-diphosphoglycerate (2,3-DPG) levels were spectrophotometrically determined by a modification of the method of Lowry et al.15 on fresh, iced, heparinized peripheral blood. Erythrocyte adenosine triphosphate (ATP) determination was made according to the method of Adams.16 All red cell enzyme determinations were performed as described by Beutler.17

### Erythropoietin Studies

Serum erythropoietin determinations were kindly performed by Drs. James F. Camiscoli and Albert S. Gordon of New York University using the exphypoxic polycythemic mouse assay.18 Serum samples submitted for assay were sterilely collected immediately prior to and 4 days following a 1000-ml phlebotomy calculated to be a 20% reduction in red cell mass. All serum specimens were collected in the fasting state between 8 and 9 A.M. on the day of collection. The hematocrit was 56% prior to phlebotomy and was 48% when the second specimen was taken. In addition, 200 cc of plasma collected in acid-citrate dextrose when the patient's hematocrit was 54% were analyzed for erythropoietin activity by Dr. Allan J. Ersliev of the Cardeza Foundation of the Jefferson Medical College of Thomas Jefferson University, Philadelphia, who used a polycythemic mouse assay technique employing a low-protein concentrate of patient plasma.18 Rabbit antierthropoietin serum with a potency of 1 ml capable of neutralizing 20 units of erythropoietin was added to test plasma, and analysis of erythropoietin activity was repeated. Plasma erythropoietin determinations when the hematocrit was 58% were also performed by radioimmunoassay by Dr. Joseph F. Garcia.20 Antiserum for this assay was obtained from rabbits repetitively immunized from relatively crude human urinary erythropoietin in addition to Freund's adjuvant. Additional details of this radioimmunoassay have been reported.20

### Bone Marrow Culture Studies

Bone marrow cells were obtained by aspiration from the posterior iliac crest of 3 hematologically normal donors, 2 patients with polycythemia vera, 3 patients with iron-deficiency anemia, and our patient. In each case where the diagnosis of polycythemia vera was made, the criteria of the Polycythemia Vera Study Group were fulfilled.21 In all cases informed consent was obtained.

The in vitro plasma-clot culture technique for measuring growth of erythroid colonies from human bone marrow has been described by Tepperman et al.24 After Ficoll-Hypaque separation of the nucleated cell fraction, dispersed bone marrow cells, in final concentrations of 2–8 × 10⁴ cells per 1.1 ml, were washed thrice in Eagle's minimum essential medium and cultured in duplicate in the presence of human erythropoietin (pool M-12-TaLSL) at concentrations of 0, 0.25, 0.5, 1.0, 2.0, and 4.0 IU/ml and in the presence and absence of 0.1 ml of patient and normal sera. Cultures were maintained in a humidified atmosphere of 3% carbon dioxide in air at 37°C. At 7 days of incubation the clots were removed, transferred to glass slides, fixed in glutaraldehyde, and stained with benzidine and hematoxylin to determine the number of hemoglobinized colonies that had formed. Each clot was examined under ×100 magnification, and erythroid colonies consisting of eight or more benzidine-positive cells were counted. These colonies are derived from erythroid progenitor cells called colony-forming units, erythroid (CFU-E). CFU-E are believed to be erythroid-committed precursors that proliferate and differentiate into proerythroblasts in response to erythropoietin.
RESULTS

Red Cell Function Studies

The whole-blood value of hemoglobin affinity for oxygen ($P_{50,O_2}$) was 30.8 mm Hg (normal range, 25.0–27.0 mm Hg). Erythrocyte 2,3-DPG determination revealed 21.7 μmoles/g hemoglobin (normal range, 12.8 ± 2.3 μmoles/g hemoglobin). Red cell ATP determination showed a value of 3.58 μmoles/g hemoglobin (normal range, 3.65–4.45 μmoles/g hemoglobin). Quantitative erythrocyte enzyme determinations revealed the following values: glutamic oxaloacetic transaminase 8.5–10.3 international units (IU) per gram of hemoglobin (normal range, 3.0–5.0 ± 0.67–0.90 IU/g hemoglobin); 6-phosphogluconate dehydrogenase 13.41 IU/g hemoglobin (normal range, 8.78 ± 0.78 IU/g hemoglobin); glucose-6-phosphate dehydrogenase 18.17 IU/g hemoglobin (normal range, 8.34 ± 1.59 IU/g hemoglobin); hexokinase 2.29 IU/g hemoglobin (normal range, 1.16 ± 0.17 IU/g hemoglobin); lactate dehydrogenase 291 IU/g hemoglobin (normal range, 200 ± 26.5 IU/g hemoglobin); phosphoglycerate kinase 384 IU/g hemoglobin (normal range, 320 ± 26 IU/g hemoglobin); pyruvate kinase 16.2 IU/g hemoglobin (normal range, 15 ± 2 IU/g hemoglobin). Repeat pyruvate kinase determination by a separate laboratory was again within the normal range.

Erythropoietin Studies

The results of serum erythropoietin assays before and after a 1000-ml phlebotomy are shown in Table 1. These assays were performed by Drs. Camiscoli and Gordon. Increased erythropoietin activity was observed prior to phlebotomy, and the reduction in red cell mass by approximately 20% resulted in a nearly fourfold increase in erythropoietin activity. A second biologic assay of erythropoietin activity employing a plasma concentrate of patient's plasma performed by Dr. Erselev also revealed dramatically enhanced erythropoietin activity of 160 mU/ml. Plasma similarly concentrated from patients with polycythemia vera consistently has erythropoietin activity less than 5 mU/ml, whereas that from patients with secondary polycythemia regularly has activity of 5–50 mU/ml as assayed by this method. The addition of 0.05 ml of rabbit antierythropoietin serum to 0.4 ml of patient plasma completely eliminated measurable erythropoietin activity. Furthermore, when radioimmunoassay for erythropoietin was performed, a marked increase in activity of 181 mU/ml was determined. When sera of hematologically

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<tr>
<th>Table 1. Patient's Serum Erythropoietin Activity As Determined by an Exhypoxic Polycythemic Mouse Assay Before and After a 1000-ml Phlebotomy</th>
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<tbody>
<tr>
<td>Material Assayed</td>
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<tr>
<td>------------------</td>
</tr>
<tr>
<td>Saline control</td>
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<tr>
<td>Normal human serum</td>
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<tr>
<td>Erythropoietin, 0.05 U/ml</td>
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<td>Patient before phlebotomy</td>
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normal patients are assayed by this technique, values are most often observed to be between 20 and 30 mU/ml.

**Bone Marrow Cultures**

As shown in Fig. 1, the patient's bone marrow cells and those from 2 individuals with polycythemia vera were capable of forming significant numbers of erythroid colonies in the absence of added erythropoietin (endogenous erythropoiesis). The formation of "endogenous" colonies has been reported to occur in cultures containing marrow cells collected from patients with polycythemia vera, but not with cells from hematologically normal persons. Normal human bone marrow cells and those obtained from patients with iron-deficiency anemia were unable to form endogenous colonies in our laboratory. However, when patient serum (but not normal serum) was added to cultures of normal marrow cells in the absence of added erythropoietin, colony formation was observed in quantities similar to those scored in cultures of normal cells in the presence of erythropoietin at 1.0 IU/ml (Table 2). This suggests that the large quantities of erythropoietin present in patient serum are active in vitro. When increasing cell concentrations from polycythemia vera and patient bone marrow were cultured in the absence of added

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**Table 2. Influence of Patient and Normal Sera in the Presence and Absence of Erythropoietin on Erythroid Colony Formation by Normal Bone Marrow Cells**

<table>
<thead>
<tr>
<th>Serum*</th>
<th>Erythropoietin (U/ml)</th>
<th>Number of Colonies ± SEM</th>
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<tbody>
<tr>
<td>Patient</td>
<td>None</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Normal</td>
<td>0.5</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>Normal</td>
<td>1.0</td>
<td>95 ± 18</td>
</tr>
<tr>
<td>Normal</td>
<td>2.0</td>
<td>290 ± 60</td>
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*Volume of 0.1 ml of serum was added to culture and adjusted to a final total volume of 1.1 ml.
PRIMARY PURE ERYTHROCYTOSIS

Fig. 2. Effects of varying concentrations of erythropoietin on patient and normal human colony formation. Each value represents mean ± 1 SEM of result obtained.

erythropoietin, a linear increase in the number of endogenous colonies formed was observed. The patient's bone marrow formed greater numbers of these colonies than did the polycythemia vera bone marrow (Fig. 1). In addition, the numbers of erythroid colonies formed by the patient's bone marrow cells in the presence of varying concentrations of erythropoietin were significantly increased as compared with those formed by normal marrow cells (Fig. 2); these findings are similar to those observed in marrow cells from patients with polycythemia vera.

DISCUSSION

In this young man with a 14-yr history of pure erythrocytosis, no abnormality of hemoglobin function was detected, and an extensive search for causes of secondary erythrocytosis has been unrevealing. Family members are hematologically normal. The patient's marked increase in erythropoietin levels and his clinical course are distinctly unusual for polycythemia vera. The absence of leukocytosis, thrombocytosis, progressive splenic enlargement, or transition to either acute leukemia or the spent phase of this disease after 14 yr of observation supports our contention that the patient does not have polycythemia vera.

The increase in 2,3-DPG level observed in this patient requires explanation. An increased amount of this glycolytic intermediate is observed in individuals who reside at high altitudes or who have chronic hypoxia. It is also increased in patients known to have chronic anemia, pyruvate kinase deficiency, and hyperphosphatemia. It has been shown that erythropoietin induces premature denucleation of red cell precursors and release of immature reticulocytes into the blood. It has also been observed that young red cells have higher levels of many enzymes than do aged red cells, but the evidence for age-dependence of 2,3-DPG is less substantial. We suggest that the increases in 2,3-DPG and in erythrocyte enzyme concentrations measured in this patient in part reflect the presence of a young population of red cells and increased red cell turnover secondary to the stimulus of increased circulating erythropoietin and repeated phlebotomy. In addition, the activity of each red cell enzyme measured was approximately twice normal, except for that of pyruvate kinase. Together with the
observed mild depression of the level of erythrocyte ATP, this observation suggests that the patient may have a mild deficiency of pyruvate kinase and that the increase in 2,3-DPG may in part be attributed to this enzymopathy.

An inverse relationship between hematocrit and erythropoietin production is well established in normal man. The regulation of erythropoiesis involves a complex interplay of factors relating to erythropoietin production, oxygen delivery, and an adequate pool of erythropoietin-responsive stem cells in the bone marrow. When phlebotomized, patients with hypoxia-induced erythrocytosis and those with polycythemia vera increase measurable erythropoietin production. In tumor-associated erythrocytosis and rare forms of recessive familial erythrocytosis, on the other hand, erythropoietin production appears to be independent of the oxygen content of the blood. In our patient erythropoietin production persisted at an increased hematocrit level and increased nearly fourfold following phlebotomy. This response clearly demonstrates that erythropoietin production in our patient is not autonomous and that it is different from the pattern of hormone production in tumor-related erythrocytosis. In light of the normal renal arteriography, excessive erythropoietin activity in our patient cannot be attributed to an abnormality of the renal microvasculature. Rather, our patient's hematologic abnormality may be a reflection of a defective servoregulatory mechanism of (or feedback response to) erythropoietin production, resulting in excessive release of erythropoietin and enhanced stimulation of erythroid precursors of the bone marrow. In this disorder the "thermostat" is reset upward, but the response of erythropoietin production to regulatory factors is maintained.

Greenberg and Golde recently described a form of familial pure erythrocytosis associated with an abnormality analogous to the functional erythropoietic defect in typical polycythemia vera. This entity is believed to be a disorder of the committed erythroid progenitor cell. Unlike the observations made in our patient, erythropoietin production was not increased in these individuals.

Interestingly, the results of in vitro culture studies of our patient's marrow cells also resemble what has been recorded in patients with polycythemia vera. Both endogenous erythropoiesis and enhanced erythropoietin-stimulated erythropoiesis were observed in vitro. Evidence has accumulated that bone marrow cells from patients with polycythemia vera are sensitive to small amounts of erythropoietin present within culture medium constituents and that this may permit endogenous erythropoiesis. It is possible that erythropoietin attached to red cell precursors or erythroid-committed stem cells already activated in vivo by erythropoietin give rise to large numbers of endogenous erythroid colonies. The documentation of in vitro endogenous erythropoiesis in a patient with markedly increased erythropoietin activity and a clinical course atypical for polycythemia vera suggests that this finding is not entirely specific for polycythemia vera. Enhanced erythropoietin-stimulated erythropoiesis may be due to increased sensitivity of target cells to low concentrations of erythropoietin, as postulated in polycythemia vera. Alternatively, an increased number of target cells per nucleated cells plated, as compared with normal, may explain this observation.

This study of an unusual form of pure erythrocytosis clearly demonstrates the fallibility of current methods for categorizing individuals with erythrocytosis. These individuals must be carefully investigated in order to determine the relation-
ships of their diseases to the myelodysplastic disorders so that inappropriate chemotherapy or radiotherapy will not be given.

ACKNOWLEDGMENT

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