EXHIBIT 21

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Erythropoietin Radioimmunoassay in Evaluating Patients with Polycythemia

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We measured serum concentrations of erythropoietin in 59 patients with polycythemia using a sensitive and specific radioimmunoassay. The mean concentration was 17.5 ± 8.4 mU/mL (± SD) in 26 patients with polycythemia vera and 14.9 ± 4.2 mU/mL in 26 normal persons. In contrast, the average concentration was 94.3 ± 101.2 mU/mL in 33 patients with secondary polycythemia, representing a highly significant elevation ($p < 0.0001$) compared to both normal and polycythemia vera groups. The average hematocrit value did not differ between the polycythemia vera and the secondary polycythemia patients, and both groups had higher values (median, 55%) than the normal donors (median, 41%). Erythropoietin concentrations ascertained by radioimmunoassay helped discriminate between polycythemia vera and secondary polycythemia. Ninety-two percent of polycythemia vera patients had concentrations less than 30 mU/mL (the concentration used as a cut off point), and 94% of secondary polycythemia patients had concentrations greater than 30 mU/mL. This represents an overall correct classification of 93% of the patients. Serum erythropoietin levels as ascertained by radioimmunoassay can distinguish between most polycythemia vera and secondary polycythemia patients and should prove useful in the differential diagnosis of polycythemia.

PERSONS with an elevated circulating erythrocyte volume may be classified as those with polycythemia vera or as those with polycythemia secondary to hypoxia or inappropriate erythropoietin secretion. Polycythemia vera is a clonal hematopoietic stem cell disorder (1), whereas secondary polycythemia may be a result of such conditions as severe, chronic obstructive pulmonary disease, cyanotic congenital heart disease, high oxygen affinity hemoglobinopathy, and erythropoietin-secreting tumor (2, 3). Patients with secondary polycythemia often have elevated urine or plasma erythropoietin concentrations, or both (3, 4). Patients with polycythemia vera have an inappropriately increased erythropoiesis without an increased erythropoietin level (4-6). The distinction between polycythemia vera and secondary polycythemia is often not difficult. There are, however, situations in which diagnosis is not clear, and reliable erythropoietin levels would be diagnostically helpful. Many in-vivo and in-vitro assays for erythropoietin have not been satisfactory for clinical purposes due to lack of reliability, sensitivity, specificity, or convenience (7-16). The recent purification of erythropoietin (17) has permitted the development of a specific and sensitive radioimmunoassay for erythropoietin (18, 19). We report herein that erythropoietin concentrations measured by radioimmunoassay can be used to discriminate between patients with polycythemia vera and patients with secondary polycythemia.

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Materials and Methods

Blood was obtained with informed consent from normal volunteers and from patients with polycythemia. The blood was allowed to clot at room temperature, and the serum was isolated and tested for erythropoietin concentration by radioimmunoassay, as previously described (18). All patient samples and erythropoietin standards were assayed in quadruplicate and at two or more different concentrations of serum. All samples were coded and assayed without knowledge of the diagnosis of the patient.

Twenty-six patients with polycythemia vera and 33 with secondary polycythemia were investigated. The patients with secondary polycythemia consisted of 13 patients with cyanotic congenital heart disease; seven with lung-associated disease including four patients with severe chronic obstructive pulmonary disease, one with Pickwickian syndrome, and two heavy cigarette smokers with elevated carboxyhemoglobin levels; six patients with kidney disease including four with a cystic renal mass and two with renal artery stenosis; three patients with cancer including two with renal cell carcinoma and one with a cerebellar tumor; and four with no known diagnosis.

At the time of study, all patients had hematocrit values of 51% or greater, were not receiving chemotherapy, had had no phlebotomies within 5 weeks, and had not been treated with ^{32}P within the past 1½ years. All patients with polycythemia vera fulfilled the criteria for disease as developed by the Polycythemia Vera Study Group (2). At diagnosis, the total erythrocyte volume was elevated (men ≥ 36 mL/kg and women ≥ 32 mL/kg of body weight) in patients with polycythemia vera and secondary polycythemia.

Twenty-six normal control subjects were included in the analysis for comparison. The healthy volunteers ranged between 19 and 45 years, and there were 10 men and 16 women.

Results

The data obtained using the radioimmunoassay for measuring serum erythropoietin concentration in patients with polycythemia vera, those with secondary polycythemia, and normal persons are presented in Table 1 and Figure 1, *left*. The mean erythropoietin concentration was 17.5 ± 8.4 mU/mL (± SD) in polycythemia vera patients, 14.9 ± 4.2 mU/mL in normal control subjects, and 94.3 ± 101.2 mU/mL in patients with secondary polycythemia (Table 1). The patients with cyanotic heart disease had a mean erythropoietin value of 151 mU/mL, which was the highest average titer in the secondary polycythemia group. The large standard deviation in the secondary polycythemia group was due to several patients with very high erythropoietin concentrations (300, 356, and 420 mU/mL). The median concentration was 16 mU/mL for polycythemia vera patients, 14 mU/mL for normal persons, and 53 mU/mL for patients with secondary polycythemia (Figure 1, *left*). The erythropoietin concentration differed significantly between the secondary polycythemia patient group and both the normal and polycythemia vera groups ($p < 0.0001$, Wilcoxon rank-sum test). No significant difference in serum erythropoietin concentration was found between the

normal and the polycythemia vera groups.

All the polycythemia patients had a higher hematocrit value (median, 55%) than all the normal persons (median, 40%). There was no significant difference in mean hematocrit value between polycythemia vera and secondary polycythemia patients and no significant correlations between the erythropoietin concentration and the hematocrit value in any of the groups.

We evaluated the serum erythropoietin concentration of one person in the polycythemia vera group 14 times over 8 months. His mean concentrations did not vary much over time, and the variability (SD = 2.9) was much less than that seen in the control (SD = 6.0) and polycythemia vera (SD = 8.0) groups. This suggested that the variability of the radioimmunoassay was small relative to the variability among people in each of the groups. A patient with an erythropoietin-secreting renal adenocarcinoma who had a serum erythropoietin concentration of 35 mU/mL and a hematocrit value of 58% on two separate days was of particular interest. Seven days after removal of the tumor, the serum erythropoietin concentration fell to 13 mU/mL and the hematocrit value decreased to 41%.

We investigated the usefulness of the erythropoietin level ascertained by radioimmunoassay in differentiating between polycythemia vera and secondary polycythemia patients. Various erythropoietin concentrations were arbitrarily chosen as cutoff points, and the percentage of correct diagnoses were calculated (Figure 1, *right*). The percentage of polycythemia vera patients correctly diagnosed is plotted on the ordinate, and the percentage of incorrectly diagnosed secondary polycythemia patients is plotted on the abscissa. If the erythropoietin concentration as assayed by the radioimmunoassay was a perfect discriminant, there would be 100% true-positive and 0% false-positive patient diagnoses. We found the best erythropoietin cutoff point to be 30 mU/mL: At this concentration, 92% of polycythemia vera patients had erythropoietin levels under 30 mU/mL and were correctly classified, whereas 6% of the secondary polycythemia patients had levels under 30 mU/mL and were incorrectly diagnosed. This represents an overall correct classification of 93%. An erythropoietin of 50 mU/mL gave a 100% correct classification of polycythemia vera but increased the number of incorrectly classified secondary polycythemia patients to 39%.

Discussion

A number of assay methods have been used to measure erythropoietin levels in humans. Each, however, has limitations making it unsuitable for general clinical application. In whole-animal assays, the lower limits of erythropoietin detection are 50 mU using the plethoric mouse assay and 1000 mU using the fasted-rat method (7, 8). These methods are not sensitive enough to detect erythropoietin titers in the normal range, lack precision, are

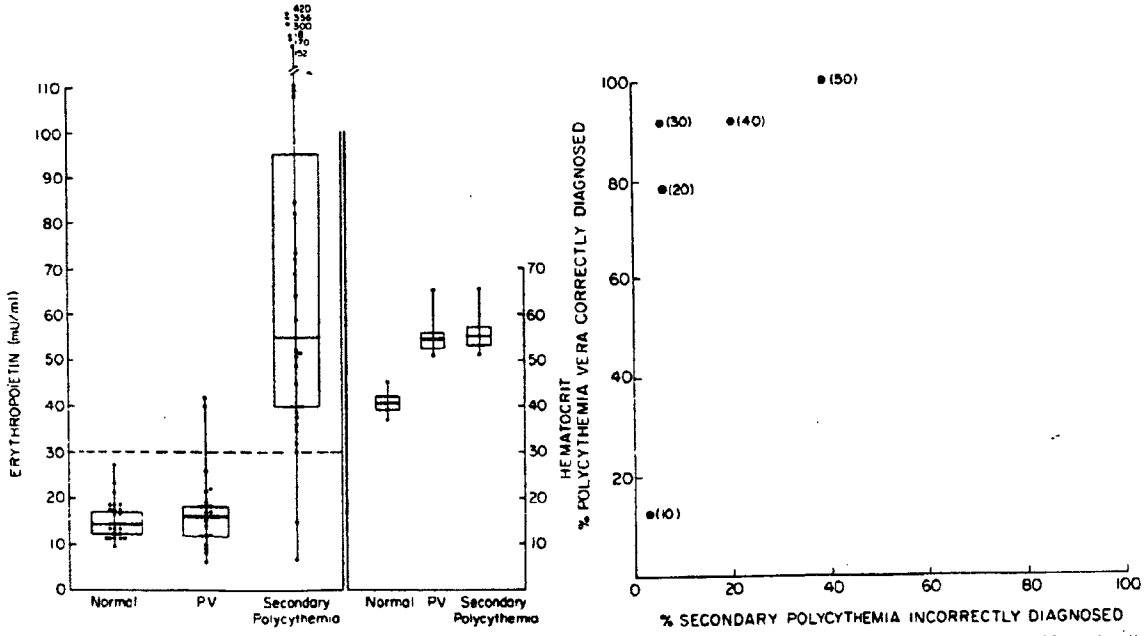

Figure 1 Left. The median (*heavy bars*), the 25th to 75th percentile (*light bars*), and range of erythropoietin concentrations and hematocrit values of 26 normal persons, 26 patients with polycythemia vera, and 33 patients with secondary polycythemia (represented by circles). **Right.** Erythropoietin discriminate curve based on erythropoietin concentration cutoff points of 10, 20, 30, 40, and 50 mU/mL (cutoff point is in parentheses). All patients with erythropoietin levels less than the cutoff point are placed in the polycythemia vera classification. The ordinate represents the percent of polycythemia patients correctly diagnosed, and the abscissa represents the percent of secondary polycythemia patients incorrectly diagnosed. The analysis is based on all the patients represented in Figure 1 *left* (polycythemia vera, 26 patients; secondary polycythemia, 33 patients).

Table 1. Composite Erythropoietin Data

Patient Diagnosis	Patients	Hematocrit	Erythropoietin (Mean ± SD)
	n	%	*mU/mL*
Polycythemia vera	26	54	17 ± 8
Secondary polycythemia	33	56	94 ± 101
Lung-associated disease	7	55	80 ± 99
Heart disease	13	58	151 ± 126
Kidney disease	6	55	55 ± 19
Cancer	3	56	39 ± 5
Unknown	4	53	35 ± 19
Normal donors	26	41	15 ± 4

labor intensive, and require a large number of animals, therefore being expensive. Sensitive tissue culture assays can detect 2 to 5 mU of erythropoietin (9-12), but they are often affected by nonspecific inhibitors. A hemagglutination inhibition assay has been developed (20) and commercially marketed, but the assay appears to lack specificity (21). There have been radioimmunoassays developed that used erythropoietin preparations and antisera that were not pure and hence did not yield reliable results (14, 16). Human urinary erythropoietin has been purified to apparent homogeneity (17), thereby permitting the development of an erythropoietin radioimmunoassay that is both specific and sensitive enough to detect 2 to 3 mU of human erythropoietin (18, 19). We used this radioimmunoassay to study erythropoietin concentrations in polycythemia patients and found the concentrations to be similar in patients with polycythemia vera (mean, 17.5; median, 16 mU/mL) and in normal persons (mean, 14.9; median, 14 mU/mL). Both groups had significantly ($p < 0.0001$) lower mean and median erythropoietin concentrations compared to those in the secondary polycythemia group (mean, 94.3; median 53 mU/mL). Values for the normal and the polycythemia vera group clustered about the mean erythropoietin concentration (coefficient of variation, 28% for the normal group and 48% for the polycythemia vera group), and the values for the secondary polycythemia group varied widely in their elevated erythropoietin levels (coefficient of variation, 107%).

Among the 33 patients with secondary polycythemia, 31 had erythropoietin titers greater than 30 mU/mL. These elevated concentrations are consistent with our understanding that secondary polycythemia occurs because of compensation for tissue hypoxia or because of a nonphysiologic increase in erythropoietin secretion, seen most commonly with certain tumors or benign disorders of the kidney (3, 5, 22).

Among the 26 patients with polycythemia vera, 24 had serum erythropoietin concentrations less than 30 mU/mL. Adamson and coworkers (5, 6) found that urinary excretion of erythropoietin from patients with polycythemia vera was undetectable using the polycythemia mouse assay. After reduction of the hematocrit by bleeding, erythropoietin excretion rose to levels equal to those observed in normal persons with similar hematocrit values. Erslev and coworkers (4) measured erythropoietin concentration in plasma of polycythemic patients. The plasma was concentrated 40-to-100 times, and erythropoietin was bioassayed in the polycythemic mouse assay. The mean level in the normal subject was 7.8 mU/mL. All the patients with polycythemia vera and 24% of those with secondary polycythemia had undetectable plasma erythropoietin levels. We were not able to distinguish between normal subjects and polycythemia vera patients by the erythropoietin concentrations. This may reflect potential differences in measurement of immunoreactive versus bioactive hormone. A previous study, however, showed that comparable erythropoietin levels were obtained from the sera of anemic patients that were assayed by both radioimmunoassay and the conventional plethoric mouse bioassay (18). There was, however, a significant discrepancy between the radioimmunoassay and bioassay values of serum from a uremic patient, suggesting the existence of an immunoreactive but biologically inactive component.

In this study we show that the serum erythropoietin concentration measured by the erythropoietin radioimmunoassay can be used to differentiate between polycythemia vera and secondary polycythemia. Using an erythropoietin concentration of 30 mU/mL, 92% of the polycythemia vera patients were correctly classified and 6% of the secondary polycythemia patients were incorrectly classified. Erythropoietin titers measured by radioimmunoassay should prove useful in the differential diagnosis of polycythemia. The assay may also be helpful in detecting early the recurrence of erythropoietin-secreting tumors and renal transplant rejection.

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