

EXHIBIT 10

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Serum Concentrations of Erythropoietin Measured by Radioimmunoassay in Hematologic Disorders and Chronic Renal Failure

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Bioassays for human erythropoietin are cumbersome, time-consuming, and insensitive. The purification of human erythropoietin (EP) has provided small quantities of highly bioactive EP (~70,000 U/mg) required for the development of an EP radioimmunoassay (RIA). The RIA for EP described in this investigation, can detect 5 mU/ml of EP in the assay tube; the serum concentration of EP in normal individuals ranged from <18 to 81 mU/ml with a mean value of 29 mU/ml. In contrast, nine patients with severe aplastic anemia had markedly elevated serum EP concentrations with a mean value of 3,487 mU/ml, range 984-6,434 mU/ml. In this RIA, patients with Polycythemia vera had consistently undetectable EP concentrations, <18 mU/ml. Eleven patients with chronic renal failure had a higher mean serum EP concentration (40.5 mU/ml) than normal individuals, but the range (<18-115 mU/ml) overlapped that of normals. By immunologic and gel chromatographic criteria, EP measured in serum was similar to standard urinary EP. The EP immunoassay that we have developed has sufficient sensitivity and specificity not only to quantitate the elevated serum EP levels found in aplastic anemia but also to discriminate decreased from normal serum concentrations of EP in most circumstances. This simple, reliable RIA has provided the necessary framework upon which to increase our understanding of the importance of EP in hematopoiesis.

Key words: erythropoietin, radioimmunoassay

INTRODUCTION

Because of the inability to obtain large quantities of purified, bioactive erythropoietin (EP) from extracts of kidneys [1-4], where EP appears to be synthesized [5-8], difficulty has been encountered in the development of sensitive assays to measure this glycoprotein hormone, which is likely to be the major stimulator of erythropoiesis [8-10]. Until recently, only whole animal or cell-culture bioassays and a hemagglutination inhibition assay have been available for the measurement of EP [11-18]. Such assays are time-consuming, cumbersome, and insensitive. The purification of human EP from the urine of patients with

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aplastic anemia by Miyaki, Kung, and Goldwasser [19] has provided small quantities of highly bioactive EP ($\sim 70,000$ U/mg) needed for the development of EP radioimmunoassays [20-27].

In this report, we describe our EP radioimmunoassay developed utilizing highly purified iodinated EP as tracer and antisera from rabbits immunized with less pure EP (62 U/mg). In the resultant radioimmunoassay, patients with Polycythemia vera had decreased serum concentrations of EP compared to normal subjects; patients with aplastic anemia had dramatically elevated serum EP levels. Our EP radioimmunoassay appears to have sufficient sensitivity and specificity to be useful, clinically, in improving our understanding of the role of EP in disorders of erythropoiesis.

MATERIALS AND METHODS

Iodination of EP

Highly purified EP ($\sim 70,000$ U/mg), kindly provided by Dr. E. Goldwasser through the distribution program of the Blood Diseases Branch of the NHLBI, was iodinated with carrier-free ^{125}I ($500 \mu\text{Ci}$ for $1.44 \mu\text{g}$) to a specific activity of $150 \mu\text{Ci}/\mu\text{g}$ by the IODO-GEN method described by Fraker and Speck [28]. The iodinated EP was separated from unreacted ^{125}I by gel chromatography on a $1.5 \times 26\text{-cm}$ column of Sephadex G-25. The peak fraction of iodinated EP was then rechromatographed on a $1.5 \times 66\text{-cm}$ column of Sephadex G-100 in order to separate possible aggregated products of iodination from monomeric iodinated EP (Fig. 1). However, since aggregation was not significant, subsequently only Sephadex G-25 chromatography was utilized most of the time.

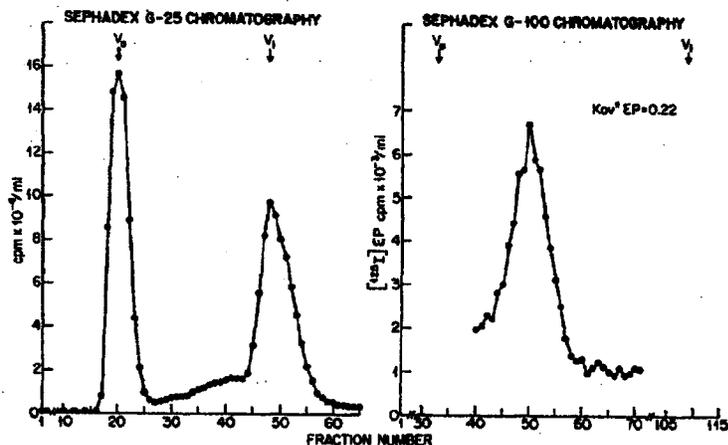


Fig. 1. Gel chromatography of iodinated EP on a $1.5 \times 26\text{-cm}$ column of Sephadex G-25 (left panel). Each 1-ml fraction collected was counted, using an altered geometry method in which the counting efficiency was purposefully decreased to 0.12% because of the large number of cpm. Iodinated EP eluted in the V_0 of the column. The peak fraction was then rechromatographed on a $1.5 \times 66\text{-cm}$ column of Sephadex G-100. These 1-ml fractions were collected and counted using normal spectrophotometer geometry with a counting efficiency of 65%. The partition coefficient (K_{av}) of iodinated EP was 0.22.

Technique: Erythropoietin Values by Radioimmunoassay 87**Antisera to EP**

Antibodies to a less purified preparation of human urinary EP (62 U/mg by bioassay), kindly provided by Dr. Peter Dukas, Los Angeles, CA, were induced in rabbits by multiple subcutaneous injections of the EP (405 μ g initial immunizing dose followed by 3 booster injections at 6-week intervals) dissolved in 0.05 M sodium phosphate, pH 7.4, and complete Freund's adjuvant (50% vol/vol) [29]. The antisera developed were initially evaluated in the hypertransfused, polycythemic mouse bioassay for their ability to neutralize human urinary EP [30]. One antiserum was selected for use in the radioimmunoassay, since 1 ml neutralized > 6 U of EP. This rabbit had a hematocrit of 38%, whereas a control rabbit had a hematocrit of 42%. The antiserum used at a final concentration of 1:1,000 in the radioimmunoassay yielded a binding of 12% of the iodinated EP.

Radioimmunoassay of EP

An aliquot of human urinary EP (62 U/mg) was used as the standard for the immunoassay. Standards (100 μ l) and unknown sera (5–300 μ l) were assayed in duplicate 1-ml incubates containing labeled EP, antibody, ovalbumin (0.1% vol/vol, Sigma) and nonimmune rabbit serum (0.5% vol/vol, Pel-Freez) diluted in 0.05 M sodium phosphate, pH 7.4. Samples were preincubated for 2 days at 4°C prior to the addition of labeled EP and then incubated for 2 more days at 4°C after the addition of tracer. Goat anti-rabbit gamma globulin (an amount previously shown to fully precipitate the gamma globulins in 5 μ l of rabbit nonimmune serum plus 1 μ l of anti-EP serum) was then added to precipitate the bound radioactivity; after an additional 20-hr incubation at 4°C, the samples were centrifuged, the supernatant decanted, and the drained precipitates counted in an automatic well-type gamma spectrometer. In control tubes without antibody, 1–2% of the total radioactivity was precipitated; this blank was subtracted from all measurements. The immunoassay data were expressed as percent of initial binding in the absence of unlabeled EP (% B/B₀). The specificity of the EP radioimmunoassay was evaluated by determining the cross-reactivity of human thyroid-stimulating hormone (Medical Research Standard B) which is another glycoprotein hormone, human serum albumin (Pentex), and human gamma globulins (Pentex). Since human gamma globulins demonstrated a small cross-reactivity in the EP immunoassay, standards were subsequently incubated with the average amount of gamma globulin found in the volume of unknown serum measured. In this fashion, the effect of nonspecific displacement of antibody from tracer by gamma globulins was negated.

Experimental Subjects

Serum samples were obtained from 19 normal individuals, nine patients with severe aplastic anemia, three patients with untreated Polycythemia vera, and 11 patients with chronic renal failure on a hemodialysis program. In addition, three anemic cancer patients and one patient with erythroid aplasia requiring transfusion of red blood cells had serum EP values measured immediately before and within four hours after transfusion. All samples were measured in duplicate at three or more different volumes of serum.

Immunological Identity of Serum EP With Urinary Standard EP

Various amounts of serum (5–75 μ l) from the patients with aplastic anemia were used to generate lines in the immunoassay in order to evaluate parallelism with the standard curve.

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Gel Chromatography of Serum From a Patient With Aplastic Anemia

A 1-ml aliquot of serum from a patient with aplastic anemia was fractionated on a 1.5 X 82-cm column of Sephadex G-100. The column was equilibrated at 4°C and eluted with a solution of 0.01 M sodium phosphate, 0.15 M sodium chloride, and 0.003 M sodium azide, pH 7.4, with a hydrostatic pressure of 20 cm and a flow rate of 9 ml/hr. The serum was cochromatographed with ¹²⁵I bovine thyroglobulin to determine the void volume of the column (V₀), ¹²⁵I, to determine the inner bed volume of the column (V_i), and [¹²⁵I]-EP. One-milliliter fractions were collected, counted, and assayed in the EP radioimmunoassay. Absorbance at 280 nm was also measured in each fraction.

RESULTS

The radioimmunoassay was able to detect 5 mU/ml of EP in the assay tube using an anti-EP serum at a final concentration of 1:1,000 (Fig. 2). Since there was a small cross reactivity of human gamma globulins in the immunoassay, separate standard curves were used containing 1.75, 3.5, and 7.0 mg/ml gamma globulins for the determination of EP in 100, 200, and 300 μl of unknown serum, respectively. Thus, we did not overestimate serum EP concentrations by including non-specific displacement of antibody from tracer by gamma globulins.

The serum concentration of EP in 19 normal individuals ranged from <18 to 81 mU/ml with a mean value of 29 mU/ml (Table I). In contrast, nine patients with severe aplastic anemia had markedly elevated serum concentrations of EP with a mean value of 3,487 mU/ml (range 984–6,434 mU/ml). In our immunoassay, three patients with Polycythemia vera had decreased levels of EP (<18 mU/ml). Only 20% of our normal subjects had undetectable serum EP concentrations. On the other hand, although 11 patients with chronic renal failure had a higher mean serum EP concentration (40.5 mU/ml) than normal subjects, the range (<18–115 mU/ml) overlapped that of the normal subjects. Moreover, the serum creatinine concentrations in these 11 patients ranged from 5–17 mg/dl immediately prior to hemodialysis, which was the time at which serum EP levels were measured. The degree of anemia in the patients with chronic renal failure was also highly variable.

The acute effect of transfusion on serum EP concentration was evaluated in three anemic cancer patients and one patient with erythroid aplasia. Although the hematocrit increased in only two of the cancer patients after transfusion, the EP level decreased somewhat in all three cancer patients; furthermore, the EP value decreased dramatically in the patient with erythroid aplasia (Table II).

Moreover, various amounts of serum from the patients with aplastic anemia (5–75 μl) used to generate lines in the immunoassay showed parallelism with that of standard urinary EP, suggesting immunological similarity between serum and urinary EP (Fig. 3). In addition, gel chromatography on Sephadex G-100 of serum from a patient with aplastic anemia yielded similar elution volumes for immunoactive serum EP and iodinated urinary EP (Fig. 4).

DISCUSSION

The availability of small quantities of highly purified human urinary EP (70,000 U/mg by bioassay) for iodination [19] has allowed for the development of EP radioimmunoassays by several groups of investigators [20–27]. The EP immunoassay that we have de-

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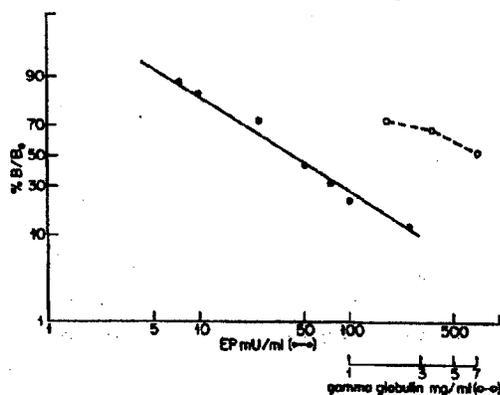


Fig. 2. Radioimmunoassay standard curve for EP using anti-EP at a final concentration of 1:1,000. Average assay sensitivity was 5 mU/ml EP. Human thyroid-stimulating hormone and albumin showed no cross-reactivity. The small cross-reactivity of human gamma globulins is depicted, 7×10^{-3} % (wt/wt).

TABLE I. EP Concentrations in Normal Individuals, Patients With Hematologic Disorders and Patients With Chronic Renal Failure

Subjects	No.	Age	Sex	EP (mU/ml)	
				Mean \pm SD	Range
Normal	19	22-52	11 M, 8 F	29 \pm 17	<18-81
Aplastic anemia	9	1-36	6 M, 3 F	3,487 \pm 2,243	984-6,434
P. vera	3	26-63	3 M	<18	<18
Chronic renal failure	11	25-80	4 M, 7 F	40.5 \pm 30	<18-115

TABLE II. EP Response to Transfusion in Anemic Cancer Patients and One Patient With Erythroid Aplasia

Subject	Age	Sex	Hematocrit (%)		EP (mU/ml)	
			Pre	Post	Pre	Post
1	63	M	32	36	45	<18
2	65	F	30	30	48	23
3	76	M	22	30	43	39
4*	28	F	29	35	1,500	150

*Patient with erythroid aplasia.

scribed in this report has sufficient sensitivity and specificity not only to quantitate the elevated serum EP levels found in aplastic anemia but also to discriminate decreased from normal concentrations of EP in most circumstances. We have found that 80% of our normal subjects had detectable serum EP concentrations (mean = 29 mU/ml), whereas the three patients studied with Polycythemia vera had undetectable levels (<18 mU/ml) [31]. All patients with Polycythemia vera, studied in the present investigation, were untreated at the time when their serum concentrations of EP were determined by radioimmunoassay.

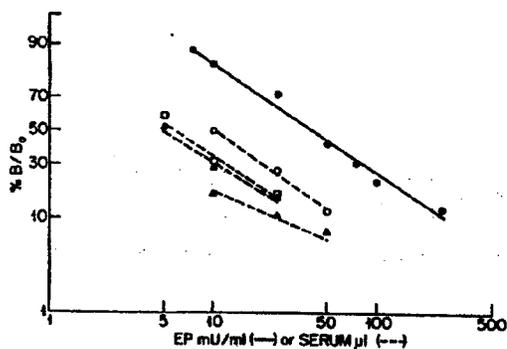


Fig. 3. Dilutions of serum from four representative patients with aplastic anemia (△, ▲, □, and ○) generated lines parallel to that of standard urinary EP (●). The slopes of the lines generated by the EP immunoactivity in these four patients (-0.04 to -0.10) were statistically the same as that of standard EP (-0.04).

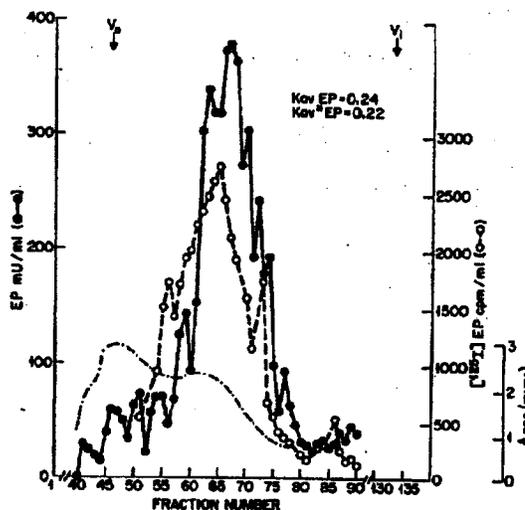


Fig. 4. Gel chromatography on a Sephadex G-100 column of 1 ml of serum from a patient with aplastic anemia. [¹²⁵I]EP was cochromatographed with the serum, and the fractions collected were counted. EP was measured in 250 μl of each fraction by radioimmunoassay. The Kav of immunoactive EP was 0.24 and that of labeled EP (¹²⁵I EP) was very similar (0.22). A₂₈₀ shows protein peaks for globulins and albumin eluting prior to the EP peak.

The patients with Polycythemia vera would have been expected to have undetectable values of serum EP because this disease appears to be an uncontrolled proliferation of a malignant erythroid clone.

Furthermore, our EP radioimmunoassay demonstrates that a reliable assay can be developed with antisera of low titer and high affinity for EP, produced using impure EP as the

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immunogen, provided that pure EP is utilized as tracer. Our normal EP concentrations were similar to those reported by Sherwood and Goldwasser [26] and Garcia, Sherwood, and Goldwasser [25]. Nevertheless, the future availability of larger quantities of highly purified EP should permit the induction of antibodies of both higher titer and affinity.

The rapid changes in serum immunoactive EP levels we have observed following transfusion of red blood cells to anemic patients suggested that the EP we have measured by radioimmunoassay was biologically active. The wide range of serum EP levels in patients with chronic renal failure can be explained in several fashions. The patients had varying degrees of anemia, as well as differing residual renal function; both of these factors could account for very diverse serum EP levels. Synthesis of EP might either be increased by the anemia or decreased due to impaired kidney function. In addition, the metabolic clearance rate of EP would most likely be reduced due to kidney failure. Sherwood, Emmanouel, and Goldwasser [32] have recently described various sizes by gel chromatography of circulating immunoactive EP in patients with renal failure. These forms would also be likely to be cleared from the circulation at different rates and probably have differing bioactivity.

Nevertheless, the circulating EP in patients with aplastic anemia was similar by immunologic and gel chromatographic criteria to standard urinary EP (Figs. 3, 4). Thus, as anticipated, the patients with severe aplastic anemia had markedly elevated serum concentrations of EP, similar to urinary standard EP; because of their marrow failure, their concentrations remained dramatically higher than normal.

The development of a simple, reliable radioimmunoassay has provided the framework upon which to expand our understanding of the physiologic importance of EP in hematopoiesis.

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