Exhibit 28

to the Declaration of Cullen N. Pendleton in Support of Amgen's Opposition to Roche's Motion for Summary Judgment that Claim 7 of the '349 Patent is Invalid Under 35 USC §112 and is Not Infringed

J. Lab. Clin. Med.
December, 1982

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Original articles

A radioimmunoassay for erythropoietin: serum levels in normal human subjects and patients with hemopoietic disorders

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An RIA for Ep has been developed that is highly sensitive and specific. A homogeneous Ep preparation was labeled with ^{125}I by the chloramine-T method to a specific activity of 90 to 136 $\mu Ci/\mu g$ and immunoreactivity of 80%. Ep antiserum, which was produced to a human urinary Ep preparation (80 U/mg of protein), was adsorbed with normal human urinary and serum proteins without any loss in sensitivity of the RIA to increase the specificity of the assay. A good correlation was seen between the RIA and the exhypoxic polycythemic mouse assay (corr. coef. 0.967; slope 1.05 and "y" intercept 0.75). Ep titers in sera from 175 hematologically normal human subjects exhibited a normal frequency distribution and ranged between 5.8 and 36.6 mU/ml with a mean of 14.9 ± 4.7 (S.D.) and median of 14.3. Serum Ep titers were markedly elevated in seven patients with aplastic anemia and one patient with pure red cell aplasia (1350 to 20,640 mU/ml) and were lower than normal in two patients with polycythemia vera (8.1 and 9.4 mU/ml). The serum Ep titers in a prenephrectomy patient with chronic glomerulonephritis (32.1 mU/ml) decreased to below normal levels (9.04 mU/ml) after nephrectomy. The cord serum erythropoietin titers in 10 IDM [90.82 ± 134.1 (S.D.) mu/ml] returned to values within the normal range (13.86 ± 5.55) on day 3 after birth, suggesting the utility of the RIA in elucidating the role of hypoxia and/or insulin in increased erythropoiesis in IDM. The serum Ep titers in patients with anemias and polycythemias were compared to those of normal human subjects and agreed well with pathophysiologic mechanisms of these hemopoietic disorders, confirming the validity of the RIA. (J LAB CLIN MED 100:829, 1982.)

Abbreviations: erythropoietin (Ep), radioimmunoassay (RIA), bovine serum albumin (BSA), International Reference Preparation erythropoietin (IRP Ep), infants of diabetic mothers (IDM)

Erythropoietin is a hormone primarily produced in the kidney in response to hypoxia. It is the physiologic regulator of day-to-day production of red blood cells and also

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plays a key role in increasing red cell production in response to pathophysiologic stimuli such as hemorrhage and hemolysis. Serum Ep titers provide important clinical clues in making the differential diagnosis of some types of anemias and polycythemias.[1, 2] An increase in serum Ep titers occurs (1) in anemias due to decreased red cell production associated with pure red cell aplasia[2] or increased red cell destruction in hemolytic anemia[3] and (2) in secondary polycythemias associated with impaired oxygen delivery to the tissues (e.g., in congenital and acquired heart diseases), impaired pulmonary oxygen exchange, abnormal hemoglobins with increased oxygen affinity, constriction of the renal vasculature,[4-8] and inappropriate secretion of Ep caused by certain renal and extrarenal tumors.[9] Serum Ep titers are decreased in anemias due to increased oxygen delivery to tissues (e.g., abnormal hemoglobins with decreased oxygen affinity[10]), in hypophosphatemia,[11] and in polycythemia vera where there is an increase in autonomous production of red cells by the bone marrow.[12, 13]

In the present studies using a homogeneous Ep preparation to prepare the tracer, we report the development and evaluation of an RIA for Ep with respect to specificity, sensitivity, precision, correlation with the bioassay, and validity of the RIA and the serum levels of Ep in normal human subjects and in several types of anemias and polycythemia vera.

Methods

This research study was carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained and the Human Experimentation Committee of the Tulane University School of Medicine has approved this study.

Highly purified human urinary Ep with an estimated specific activity of 70,400 U/mg of protein, prepared by Miyake et al.[14] of the University of Chicago, was provided to us for our RIA studies by the Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Md.

Human urinary Ep preparations with a specific activity of 69 and 80 U/mg of protein were made from urine of patients with hookworm anemia and aplastic anemia, respectively. Carrier-free $Na^{125}I$ at a pH of 8 to 10 with a specific activity >350 mCi/ml was purchased from New England Nuclear Corp., Boston, Mass.; chloramine-T from Eastman Kodak Co., Rochester, N. Y.; sodium metabisulfite from Mallinkrodt, Inc., St. Louis, Mo.; and goat anti-rabbit gamma globulins and rabbit gamma globulins from Antibodies, Inc., Paris, Calif.

Radioiodination. The chloramine-T method of Greenwood and Hunter[15] was optimized for labeling of Ep with iodine 125. First, 0.72 μg of freeze-dried purified Ep (70,400 U/mg of protein) was solubilized in 10 μl of 0.25M sodium phosphate buffer at pH 7.5; then 1.5 mCi (2 μl) of $Na^{125}I$, 10 μg of chloramine T (2 μl), 24 μg of sodium metabisulfite (100 μl), and 100 μg of KI (100 μl) were added in rapid succession. All the reagents were dissolved in 50 mM sodium phosphate buffer at pH 7.5. The reaction mixture was immediately transferred to a 14 ml (1 by 17.8 cm) column of Sephadex G-50 superfine (Pharmacia Fine Chemicals, Piscataway, N. J.), which was presaturated with BSA and pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.5. The reaction tube and the stirrer were washed with 100 μg of KI (100 μl) and the wash was transferred to the column. The column was eluted with 50 mM sodium phosphate buffer, pH 7.5. Fractions (0.5 ml) were collected in tubes containing 0.1 ml of phosphate buffer with 5% BSA. The labeled Ep was recovered in the void volume (Fig. 1). The specific activity of the labeled Ep was 90 to 136 μCi/μg. The fractions in the void volume were pooled and refractionated on a 124 ml (1.6 by 62 cm) Sephadex G-150 column with 50 mM sodium phosphate buffer, pH 7.5, containing 1% BSA. The fractions under peak "C" (Fig. 2), which represented undamaged and unaggregated labeled Ep were pooled, and aliquots of 2 million cpm were freeze-dried and stored at −60° C until used in the RIA.

Immunization. Antibodies to Ep were prepared in New Zealand white rabbits. Antiserum 4126-P-1 was prepared by immunization with a total of 256 U of Ep (69 U/mg of protein) over a period of 4 weeks. Thirty-two units of Ep in complete Freund's adjuvant were administered twice each week intradermally and the first blood sample was drawn 7 days after the last injection. This rabbit was rested for a period of 12 months and then boosted with 40 U of highly purified Ep (70,400 U/mg of protein).

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Table I. Labeling of purified Ep with iodine 125 by the chloramine-T method

Ep (μg)	*$Na^{125}I$ (mCi)*	*Ch-T (μg)*	*Reaction time (sec)*	*cpm incorp. in protein (millions)*	*Specific activity (μCi/μg of prot.)*	*% bound at 1:500 antiserum* dilution*
0.72	0.3	50	10	38	31	62.25
0.72	0.3	50	30	103	85	20.92
0.72	1.2	50	10	139	115	36.1
0.72	1.5	10	Negligible	128	106	73.64

Ch-T = chloramine-T
Optimization of conditions for the labeling of purified Ep (70,400 U/mg of protein) with iodine 125 by the chloramine-T method. Bottom row summarizes the reaction conditions most suitable and results obtained under these conditions. Chloramine-T concentrations of less than 10 μg were not evaluated.
*Antiserum 4126-P-1.

Antiserum 411 was prepared as follows: 0.4 mg of protein per milliliter of the immunogen (human urinary Ep preparation with a specific activity of 80 U/mg of protein) was prepared by emulsifying 3.86 mg of solid Ep (1.4 mg of protein) dissolved in 1.75 ml of isotonic saline with 1.75 ml of complete Freund's adjuvant. Three rabbits were injected subcutaneously on the back with 1 ml of total immunogen per rabbit. Between 50 and 100 injections were made per animal, with the injection sites approximately 1.5 cm apart. After 6 weeks, the rabbits were boosted with 1 ml of immunogen (0.4 mg/ml) per rabbit, subcutaneously. On the seventh day an additional 1 ml of the immunogen in saline (0.4 mg/ml) was injected intravenously into each rabbit and the first blood sample was drawn 3 days later.

Adsorption of antiserum. To prepare adsorbed antiserum, 63 mg of normal human urinary proteins and 155 mg of normal human serum proteins were coupled, respectively, to 3.62 and 8.86 gm of cyanogen bromide–activated Sepharose 4-B (Pharmacia) in 0.1M bicarbonate buffer at pH 8.3 following the procedure recommended by Pharmacia.[15a] The two coupled gels were incubated together with 1 ml of antiserum at 4° C overnight. The gel was kept in suspension with the use of a Dubnoff shaker. The gel was then packed in a column, allowed to drain to the top of the gel bed, and washed with 50 mM sodium phosphate buffer at pH 7.5 until all the unadsorbed protein was washed from the column. The fractions under the protein peak were pooled and concentrated with an Amicon stirred cell (Amicon Corp., Lexington, Mass.) and a YM-10 membrane to the desired volume.

Bioassay. The exhypoxic polycythemic mouse assay as modified by our laboratory from the technique developed by Cotes and Bangham[16] was used to measure Ep in vivo. CD-1 strain mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were placed in a hypobaric chamber at 0.42 atm for 22 hr per day for 2 weeks. On the sixth and seventh days out of the tank, the mice were injected subcutaneously with two equally divided doses of the Ep sample; 0.4 μCi of ^{59}Fe ferrous citrate was injected intravenously 24 hr later. The mice were exsanguinated 48 hr later and ^{59}Fe incorporation into circulating red blood cells determined. The log dose-response curve was linear between 50 and 1000 mU of Ep per mouse. The slope of the regression line was 13.19, with an S.D. of ±1.43, and the index of precision was 0.11.

Radioimmunoassay. One hundred microliters of the IRP Ep standard solution (obtained from the Bureau of Biological Standards, National Institute of Medical Research, London, England) or 100 to 200 μl of serum sample were incubated with 100 μl of Ep antiserum, 100 μl of tracer, and the volume of diluent sufficient to make a final volume of 500 μl. All tubes were gently vortexed and allowed to remain at 4° C for 4 days. Fifty microliters of a 1:3 dilution of goat anti-rabbit gamma globulins were then added to each tube. The tubes were gently vortexed and allowed to remain at room temperature for 1 hr. Fifty microliters of a 1:30 dilution of rabbit gamma globulins were then added to increase the bulk of the precipitating antigen-antibody complex. The tubes were gently vortexed and incubated at 4° C for at least an additional 16 hr. The tubes were then counted for total radioactivity and centrifuged at 5000 rpm for 30 min, the supernatant was aspirated, and the tubes with the pellet were counted for bound radioactivity. Zero antigen binding was determined by substituting for standard or sample with 5% BSA. Nonspecific binding was determined by substituting for standard or sample with 5% BSA and antiserum with the diluent. The diluent consisted of 50 mM sodium phosphate buffer, pH 7.5, containing 1% BSA. Dilutions of tracer and antiserum were made in diluent, whereas dilutions of standard and samples were made in 50 mM sodium phosphate buffer,

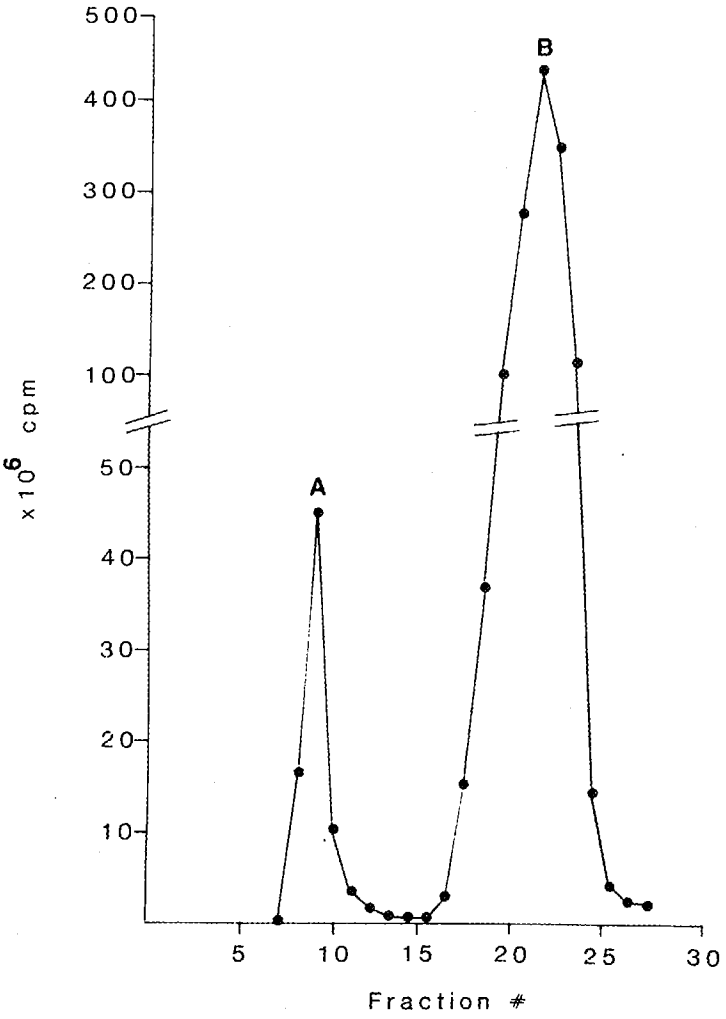

Fig. 1. Fractionation of the iodination reaction mixture using a Sephadex G-50 superfine column (1 by 14 cm). Fraction volume 0.5 ml. The first peak, *A*, in the void volume is the iodinated Ep clearly separated from the unreacted iodide (peak *B*).

pH 7.5, containing 5% BSA. The RIA data are expressed as percent of initial binding in the absence of unlabeled Ep (% B/Bo).

Results

Iodination. The optimization of the conditions for iodination of purified Ep (70,400 U/mg of protein) by the chloramine-T method are summarized in Table I. In order to minimize loss of immunoreactivity, it was important to reduce the reaction time and the concentration of chloramine-T to a minimum. With a negligible reaction time (~2 sec) and a chloramine-T concentration of 10 μg, it was necessary to load the reaction mixture with $Na^{125}I$ (1.5 mCi) to obtain the desired high specific activity.

The labeled Ep appeared as a sharp peak in the void volume and was clearly separated from the unreacted iodide by the fractionation of the iodination reaction mixture on

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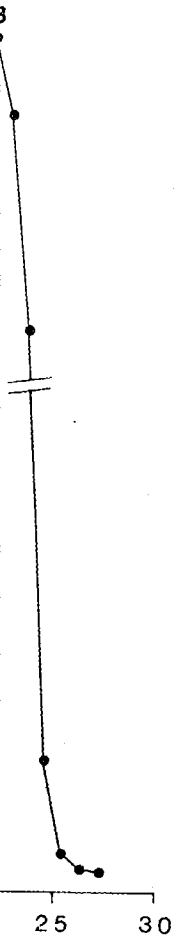

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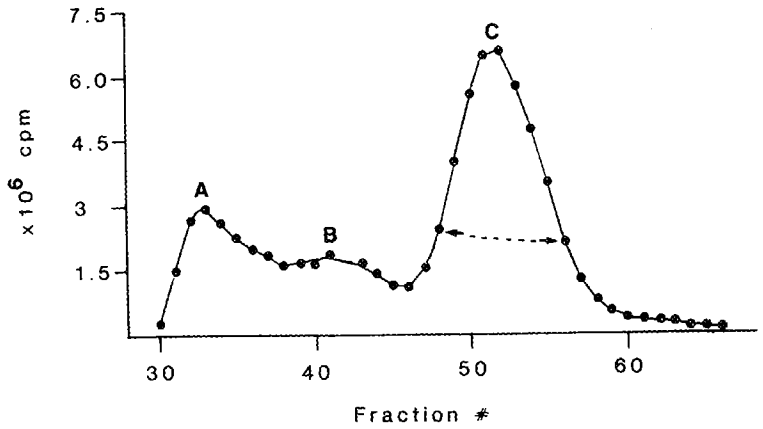

Fig. 2. Purification of labeled Ep (pooled fractions under peak A, Sephadex G-50, shown in Fig. 1) on a Sephadex G-150 column (1.6 by 62 cm). Fraction volume 1.5 ml. Fractions 48 through 56 under peak C representing unaggregated undamaged Ep were pooled and used as a tracer in the RIA.

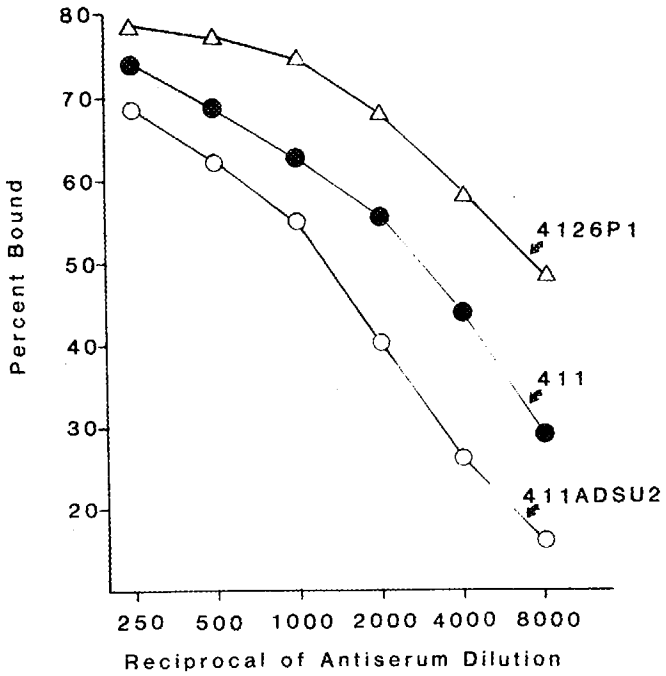

Fig. 3. Antiserum dilution curves for antisera 4126P1, 411, and 411ADSU2. Antiserum 411ADSU2, which is antiserum 411 adsorbed with normal urinary and serum proteins, was used in the RIA at a 1:8000 final dilution.

Sephadex G-50 superfine (Fig. 1). The specific activity of the labeled Ep (peak A, Fig. 1) under these iodination conditions ranged from 90 to 136 μCi/μg, corresponding to the incorporation of 1.5 to 2.2 iodine atoms per molecule of Ep.

The fractions under the protein peak (peak A, Fig. 1) were pooled and refractionated on a 124 ml (1.6 by 62 cm) Sephadex G-150 column (Fig. 2). Fractions under peak C,

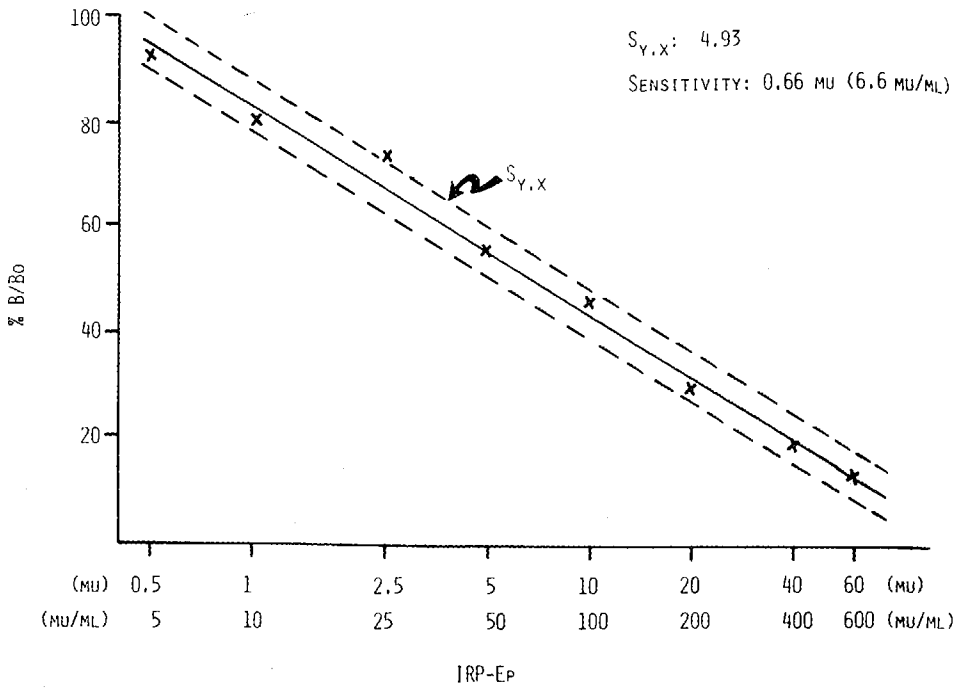

Fig. 4. IRP Ep standard dose-response regression line. The standard dose-response curve was linear from 5 to 600 mU/ml IRP-Ep. The standard error of estimate of γ on χ ($S_y \cdot \chi$) was 4.93. The sensitivity of the assay was 0.66 mU or 6.6 mU/ml.

which represented undamaged, unaggregated labeled Ep were pooled, and freeze-dried in aliquots, and stored at −60° C.

Selection of the antiserum. Antiserum dilution curves for antisera 4126-P-1 and 411 are shown in Fig. 3. Antiserum 4126-P-1 had a higher Ep-antibody titer than 411. However, it also exhibited slightly less affinity for Ep than antiserum 411, as evidenced by the sensitivity of the standard dose-response curve. Moreover, both antisera displayed slight cross-reactivity with some serum proteins, as indicated by the nonparallelism of the normal serum concentration curve or aplastic serum dilution curve with the standard dose-response curve. Therefore antiserum 411 was adsorbed (411ADSU-2) with normal human urine and serum proteins as described in the Methods. The completion of adsorption was confirmed by Ouchterlony double gel diffusion and by immunoelectrophoresis.

RIA of human sera. The standard dose-response regression line (Fig. 4) using IRP Ep as a standard was linear between 0.5 and 60 mU (5 to 600 mU/ml). The standard error of the estimate of γ on χ ($S_y \cdot \chi$) was 4.93%. The dose-response regression lines with either IRP Ep (2 U/mg of solid) or highly purified Ep (70,400 U/mg of protein) as standards were superimposed (Fig. 5). The antiserum dilution curves obtained with labeled Ep mixed with unlabeled Ep in a ratio of 1:9 dissolved in either 5% BSA or normal human serum were superimposed (Fig. 6). The standard dose-response regression line with IRP as the standard was parallel to the dilution curve obtained with an aplastic anemia urine concentrate and a concentration curve of a normal human serum (Fig. 7). Several human serum proteins were tested for cross-reactivity with Ep antiserum. Neither albumin, α-

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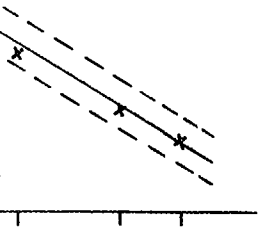

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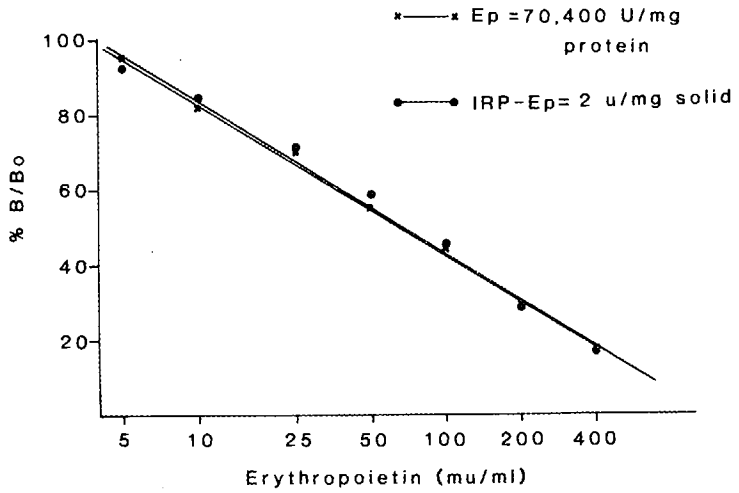

Fig. 5. RIA dose-response regression lines, with highly purified Ep and IRP Ep used as standards.

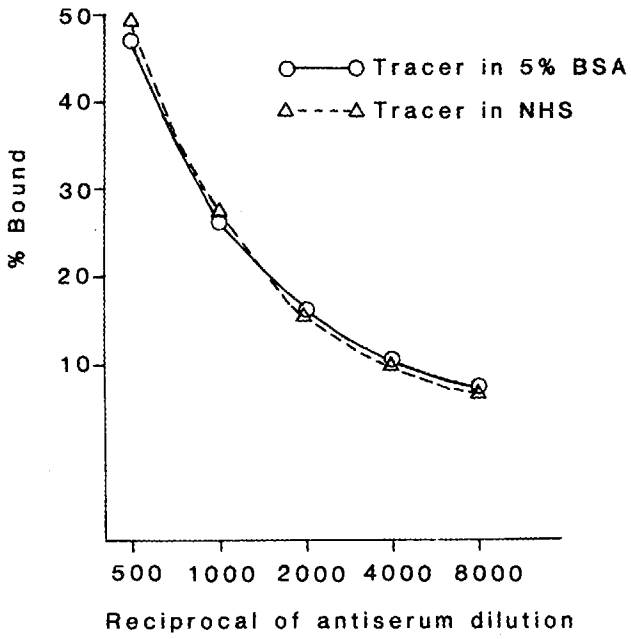

Fig. 6. Antiserum dilution curves. Tracers were labeled Ep (45 pg) diluted with unlabeled Ep (405 pg) that was dissolved either in 5% BSA or in normal human serum (*NHS*).

1-antitrypsin, or orosomucoid at concentrations present in normal serum showed any cross reactivity with Ep for binding with the antiserum 411ADSU-2. Two serum samples, one with a low Ep titer and one with a high Ep titer, were assayed 10 to 12 different times in the same assay to determine intra-assay variation (Table II). The same two samples were assayed in five different assays over a period of 14 days to study interassay variation (Table III). Both intra-assay and interassay comparisons showed a higher than desired coefficient

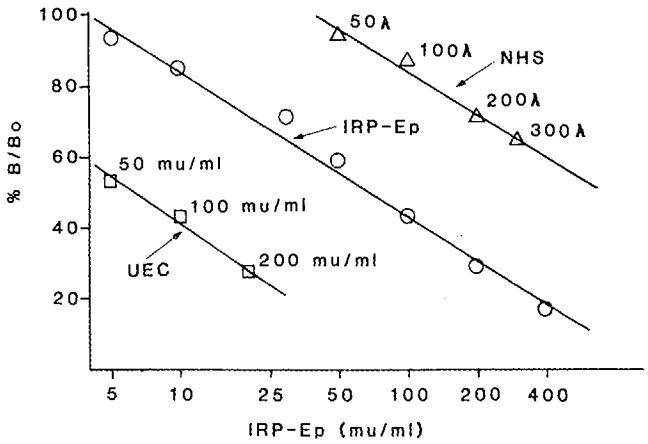

Fig. 7. Parallelism of concentration curve for normal human serum (*NHS*) and dilution curve for a urinary Ep concentrate from an aplastic anemia patient (*UEC*) with the IRP Ep standard dose-response curve. λ, Microliter.

Table II. Intra-assay variance

Sample	*No. of single determinations*	*Mean Ep (mU/ml)*	*S.D.*	*S.E.M.*	*CV (%)*
Low Ep	12	19	2.96	0.85	15.57
High Ep	10	311	31	9.82	9.97

CV = coefficient of variation.

Table III. Interassay variance

Sample	*No. of runs*	*Mean Ep (mU/ml)*	*S.D.*	*S.E.M.*	*CV (%)*
Low Ep	5	16.61	3.72	1.66	22.39
High Ep	5	327	41	18.31	12.52

CV = coefficient of variation.

Table IV. Recovery study

Sample	*Human serum endogeneous Ep (mU/ml)*	*Exogeneous Ep added (mU/ml)*	*Total Ep/sample (mU/ml)*	*Total Ep recovered (mU/ml)*	*Ep recovery*	
					%	*Mean ± S.D.*
1	5	50	27.5	23.99	87.23	
2	5	100	52.5	49.36	94.02	
3	5	200	102.5	99.29	96.86	92.76 ± 4.49
4	5	400	202.5	196.1	96.83	
5	5	500	252.5	224.4	88.87	

Equal volumes of exogenous Ep (assayed previously with the polycythemic mouse assay) and normal human serum (assayed previously by RIA) were mixed and assayed by RIA. The percent recovery was calculated as the percent of the total Ep (original + added) in each sample that was measurable by RIA.

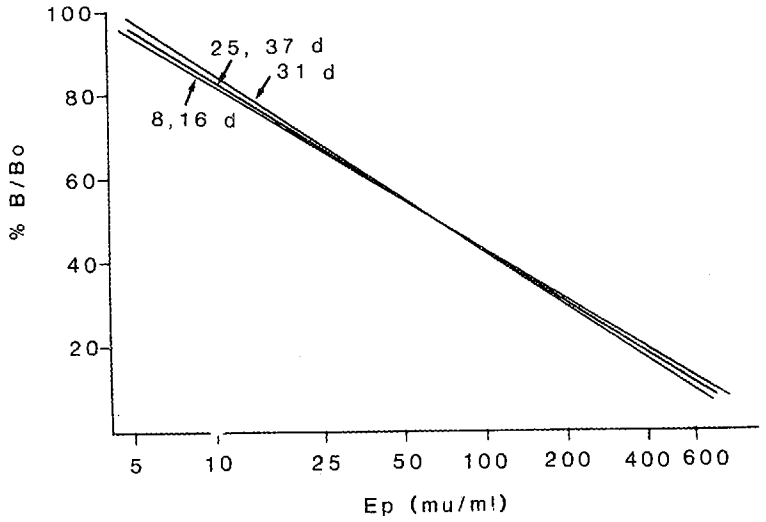

Fig. 8. Stability studies on the ^{125}I-labeled Ep. IRP Ep standard dose-response curves with 8-, 16-, 25-, 31-, and 37-day-old tracer.

Table V. Correlation of RIA and exhypoxic polycythemic mouse bioassay

Serum sample	*Diagnosis*	*Ep titer (U/ml)*	
		RIA	*Bioassay*
1	Aplastic anemia	15.2	20.7
2	Aplastic anemia	20.6	21.0
3	Aplastic anemia	3.4	4.3
4	Aplastic anemia	1.4	2.3
5	Aplastic anemia	3.8	5.3
6	Aplastic anemia	2.2	2.7
7	Aplastic anemia	11.6	10.2
8	Red cell aplasia	6.6	7.7

of variation, particularly for the determinations of low Ep titers. The accuracy of the RIA was determined by adding known concentrations of exogenous Ep to known concentrations of endogenous Ep and by correlating the total Ep that was present in the sample with the total Ep determined by the RIA. The recovery of Ep varied from 87.23% to 96.86%, with a mean percent recovery of 92.76 ± 4.49 (S.D.) (Table IV).

To determine the period over which a single labeled Ep preparation could be used in the RIA without further purification, stability of the labeled Ep was evaluated by comparing the standard dose-response curves obtained over a period of 57 days. Although some free iodide was liberated during storage, the standard dose-response curves were superimposed for up to 37 days after labeling (Fig. 8). Removal of the iodide liberated during storage was not necessary. There was no significant change in nonspecific binding, and the maximum binding in the absence of unlabeled antigen decreased very slowly over the period of 37 days. The results obtained from the assay set up on the forty-fourth day after

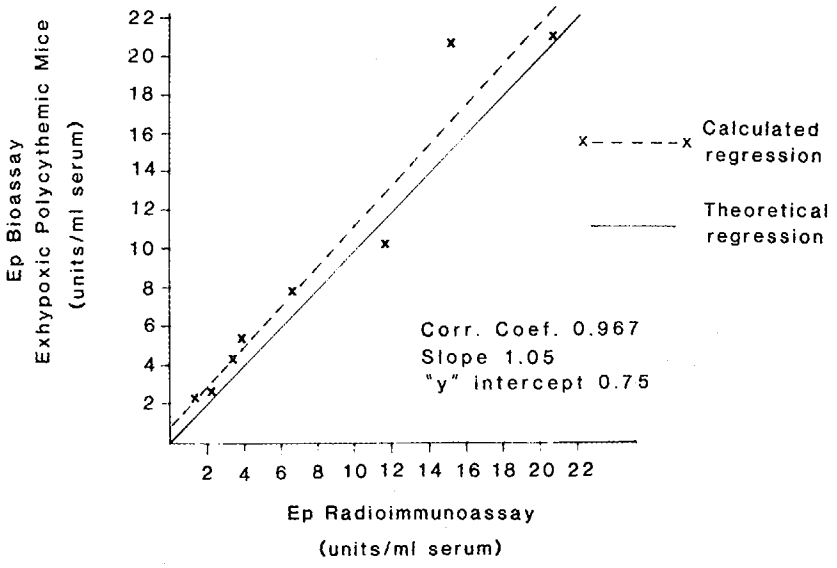

Fig. 9. Correlation of results of the polycythemic mouse assay and the RIA for Ep. Sera from seven patients with aplastic anemia and one patient with pure red cell aplasia were assayed for Ep by the exhypoxic polycythemic mouse assay and the RIA.

Table VI. Serum Ep titers in normal human subjects

Sex	*N*	*Ep range (mU/ml)*	*Mean*	*S.D.*	*S.E.M.*	*Median*
M + F	175	5.8-36.6	14.9	4.7	0.35	14.3
M	80	7.7-32.2	14.9	4.3	0.47	14.9
F	95	5.8-36.6	15.0	5.0	0.51	14.0

Table VII. Serum Ep titers in patients with anemia associated with aplastic marrow, pure red cell aplasia, chronic glomerulonephritis, and polycythemia vera

Diagnosis	*N*	*Ep (mU/ml)*
Aplastic	7	1350-20,640
Pure red cell aplasia	1	6560
Chronic glomerulonephritis	1	
Prenephrectomy		32.09
Postnephrectomy		9.04
Polycythemia vera	2	8.1; 9.4
Normal	175	14.9 ± 4.7*

*Mean ± S.D.

labeling were slightly erratic. The tracer did not bind to the antisera when the assay was set up 57 days after labeling.

In order to correlate the RIA with the in vivo bioassay (exhypoxic polycythemic mouse assay), eight sera with high Ep titers (seven patients with aplastic anemia and one patient with pure red cell aplasia) and all with hemoglobin values of <6 gm/100 ml were assayed

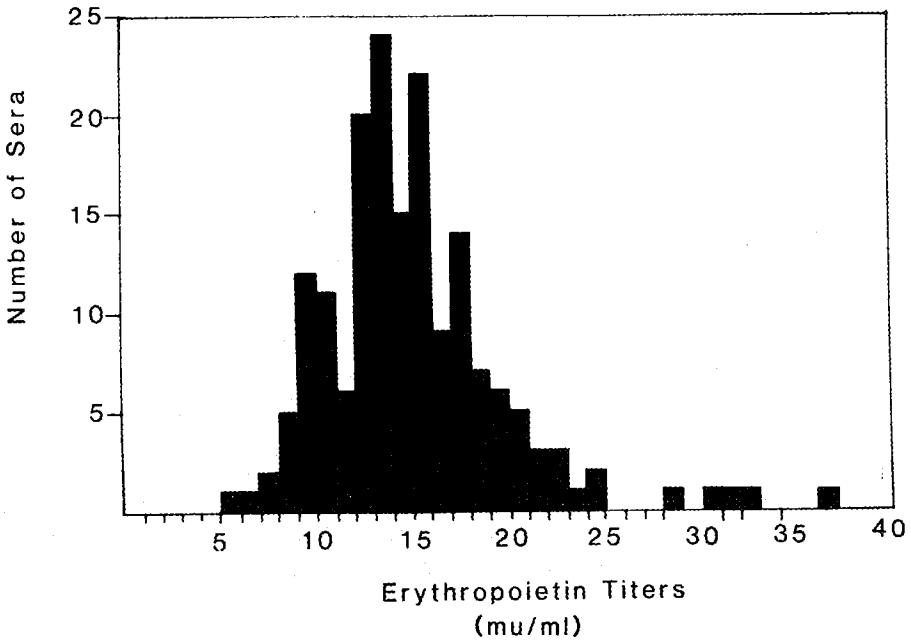

Fig. 10. Frequency distribution of serum Ep titers in 175 hematologically normal human subjects (male, hemoglobin 13.5 to 17.0 gm/100 ml; female, hemoglobin 12.5 to 17 gm/100 ml).

Table VIII. Cord serum and day 3 serum Ep titers in IDM

	Ep titer (mU/ml)	
	*Cord serum**	*Day 3 serum†*
1	27.84	6.54
2	12.74	8.43
3	40.31	9.09
4	318	20.62
5	14.75	13.31
6	27.86	19.12
7	31.06	18.03
8	25.42	9.48
9	369.3	21.31
10	40.74	12.7

*Mean 90.82 ± 134.1 (S.D.).
†Mean 13.86 ± 5.55 (S.D.).

both by RIA and with the bioassay. The correlation coefficient was 0.967 with a slope of 1.05 and a y intercept of 0.75 (Table V, Fig. 9).

The serum Ep titers in 175 hematologically normal human subjects (hemoglobin range 13.5 to 17 gm/100 ml for males and 12.5 to 17 for females) showed a normal frequency distribution (Fig. 10). The Ep titers ranged from 5.6 to 36.6 mU/ml, with a mean of 14.9 ± 4.7 (S.D.) and a median of 14.3 (Table VI). There was no significant difference between the serum Ep titers in male and female subjects. In order to study the validity of the RIA in the differential diagnosis of anemias and polycythemias, sera from

seven patients with aplastic anemia, one patient with pure red cell aplasia, one patient with chronic glomerulonephritis before (hematocrit 18%) and after nephrectomy, and two patients with polycythemia vera were assayed for Ep by using our RIA. As expected, the two patients with polycythemia vera had Ep titers lower than the mean serum Ep titers in normal human subjects, whereas patients with aplastic anemia and pure red cell aplasia had markedly elevated serum Ep titers. One patient with chronic glomerulonephritis had higher than normal levels of Ep before nephrectomy and lower than the mean normal titer after nephrectomy (Table VII).

The validity of the RIA was further confirmed by the ability of this assay to discriminate between cord serum and day 3 serum Ep titers of IDM (Table VIII). The mean cord serum Ep titer was 90.82 mU/ml and the mean day 3 serum Ep titer was 13.86 mU/ml.

Discussion

A number of assay methods have been used to measure Ep levels in human serum. These methods have included in vivo bioassays such as the exhypoxic polycythemic mouse assay,[16] the fasted rat assay,[17] or the hypertransfused mouse assay[18]; in vitro bioassay methods such as rabbit or mouse bone marrow cultures[19–23] or fetal mouse liver cells[24–28]; and several immunoassays such as hemagglutination inhibition,[29] RIA,[30–33] and immunoradiometric assay.[34] In vivo bioassays are too cumbersome, expensive, and time-consuming and have limited sensitivity (e.g., 50 to 100 mU for the exhypoxic polycythemic mouse and hypertransfused mouse assays; 1000 mU for the fasted rat assay). In vitro bioassays are neither specific nor sensitive and are affected by nonspecific inhibitors in the serum samples. The hemagglutination inhibition assay currently marketed is also nonspecific.[35, 36] Prior to the availability of highly purified Ep, studies carried out in our laboratory[37, 38] and that of Garcia[39] reported the use of partially purified Ep as a tracer for use in RIA by immunopurification of the labeled Ep, fully recognizing the possible problem of nonspecificity with these impure Ep preparations. After the purification of Ep to homogeneity by Miyake et al.[14] and its availability for RIA studies, several investigators[30–33] have reported the iodination of this purified Ep and its use as a tracer in an RIA for Ep. Sherwood and Goldwasser[31] and Zaroulis et al.[32] used the iodogen method, whereas Garcia et al.[30] used an enzymatic iodination method with lactoperoxidase for iodination of Ep. We optimized the chloramine-T method for iodination, since the amount of protein to be labeled is not the limiting factor with this method and also it is the least expensive and most simple.

Two Ep antisera used in the present RIA studies were produced in rabbits by different protocols, and antiserum 4126-P-1 was obtained by booster immunization with highly purified Ep (70,400 U/mg of protein). We found it necessary to adsorb the antisera with human serum and urinary proteins in order to increase the specificity of our RIA for Ep. The sensitivity of the RIA was not decreased by adsorption of the antisera. The sensitivity of our RIA was 0.66 mU or 6.6 mU/ml. The specificity of the RIA for Ep was confirmed by the following observations. The dose-response curves using as standards either highly purified Ep (70,400 U/mg of protein) or IRP Ep (2 U/mg of solid) were superimposed; the antiserum dilution curves using as tracers 1 part of labeled Ep diluted with 9 parts of unlabeled Ep dissolved in either 5% BSA or normal human serum were superimposed; increasing concentrations of normal human serum and dilutions of aplastic anemia urine concentrates gave curves parallel to the standard dose-response curve; and finally human serum albumin, α-1-antitrypsin, and orosomucoid showed no cross-reactivity at concentrations equal to that present in normal human serum.

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Ep levels in human serum.
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Intra-assay and interassay variation studies showed a higher than desired coefficient of variation, particularly for Ep titers in the lower range of the dose-response curve. The reason for this low precision is that the use of antiserum at the maximum permissible dilution gives results in the assay range of 17% to 3% bound. The use of the antiserum at a maximum permissible dilution (1:8000 final dilution) at the sacrifice of some precision was necessary to increase the sensitivity of the assay and to minimize cross-reactivity. The recovery of added Ep was almost quantitative, indicating a high degree of accuracy of the RIA. The labeled Ep was stable when stored freeze-dried at −60° C and was suitable for use in the RIA for up to 37 days after labeling. There was also an excellent correlation between the RIA and the bioassay (exhypoxic polycythemic mouse assay), which has heretofore never been reported by any other investigator.

Ep titers in normal human serum have been a controversial subject for several years, using various in vivo and in vitro assays. The in vivo bioassay (polycythemic mouse) is the most reliable assay and the standard assay accepted internationally. It is not sensitive enough to measure normal human serum Ep titers. However, normal serum Ep titers as high as 2120 ± 840 mU/ml have been reported by in vitro bioassays,[28] and titers between 100 and 300 mU/ml are not uncommon by in vitro bioassays.[24, 25] Considering several in vitro assays, titers of 30 ± 10 mU/ml[34] and 40 mU/ml[40] have been suggested for Ep in normal human serum. With serum concentrates and an in vivo bioassay, normal serum Ep titers have been reported to be <5 to 18 mU/ml[6] and 3.9 to 15 mU/ml.[41] However, the assumption of a constant loss of Ep during the concentration method used may not be valid.[42] Normal serum Ep titers measured by the hemagglutination inhibition assay was reported to be 37 ± 22 mU/ml.[29] More recently, normal serum Ep titers of 21 and 23 mU/ml, respectively, for a pool of normal female and normal male sera[30]; 14.9 ± 4.2 mU/ml (n = 26)[33] and <18 to 81 mU/ml, with a mean of 29 mU/ml (n = 19)[32] have been reported by RIA using highly purified labeled Ep (70,400 U/mg of protein) as a tracer. Our present study utilizing serum samples from 175 hematologically normal human subjects showed a normal frequency distribution for the serum Ep titers that ranged from 5.8 to 36.6 mU/ml with a mean of 14.9 ± 4.7 (S.D.) and a median of 14.3. There was no significant difference between normal male and normal female serum Ep titers. Two patients with polycythemia vera had slightly lower serum Ep titers (8.1 and 9.4 mU/ml). Serum Ep titer in one patient with chronic glomerulonephritis before nephrectomy was slightly higher than normal (32.09 mU/ml) and decreased after nephrectomy to 9.04 mU/ml. As expected, markedly elevated serum Ep titers were observed in seven patients with aplastic anemia (1.3 to 20.6 U/ml) and one patient with pure red cell aplasia (6.56 U/ml).

Recently, Widness et al.[43] have reported elevated umbilical cord plasma Ep levels in 22 of 61 IDM and have postulated that increased fetal erythropoiesis in IDM may be due either to a direct action of insulin or to hypoxia secondary to hyperinsulinemia. In a collaborative preliminary study with Dr. Keith Peevy of the University of South Alabama, we observed that two of 10 IDM had markedly elevated cord serum Ep titers and that serum Ep titers of all IDM were in a normal range within 3 days after birth.

In summary, we have developed a specific and sensitive RIA for Ep. The sensitivity of our RIA is 0.66 mU or 6.6 mU/ml and is sufficient to detect normal serum Ep levels. The mean normal serum Ep titer with our RIA was found to be 14.9 ± 4.7 mU/ml (n = 175). The validity of our RIA for Ep was further confirmed by the observations that the serum Ep titers were in a low normal range in patients with polycythemia vera and in a surgically anephric patient and were markedly elevated in patients with aplastic anemia and pure red cell aplasia.

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