EXHIBIT 20

Radioimmunoassay of erythropoietin: circulating levels in normal and polycythemic human beings

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Techniques are described in detail for the RIA of human Ep in unextracted plasma or serum. With 100 μl of sample, the assay is sensitive at an Ep concentration of approximately 4 mU/ml, and when required, the sensitivity can be increased to 0.4 mU/ml, a range considerably less than the concentration observed in normal human beings. This is approximately 100 times more sensitive than existing in vivo bioassays for this hormone. Studies concerned with the validation of the Ep RIA show a high degree of correlation with the polycythemic mouse bioassay. Dilutions of a variety of human serum samples show a parallel relationship with the standard reference preparation for Ep. Validation of the RIA is further confirmed by observations of appropriate increases or decreases of circulating Ep levels in physiological and clinical conditions known to be associated with stimulation or suppression of Ep secretion. Significantly different mean serum concentrations of 17.2 mU/ml for normal male subjects and 18.8 mU/ml for normal female subjects were observed. Mean plasma Ep concentrations in patients with polycythemia vera are significantly decreased, and those of patients with secondary polycythemia are significantly increased as compared to plasma levels in normal subjects. These results demonstrate an initial practical value of the Ep RIA in the hematology clinic, which will most certainly be expanded with its more extensive use. (J LAB CLIN MED 99:624, 1982.)

Abbreviations: erythropoietin (Ep), radioimmunoassay (RIA), human serum albumin (HSA), bovine serum albumin (BSA), phosphate buffer (PO_4), specific activity (S.A.), coefficient of variation (CV), International Reference Preparation (IRP), Lawrence Berkeley Laboratory (LBL), Brookhaven National Laboratory (BNL), trichloroacetic acid (TCA), counts per minute (cpm), ethylenediamine tetraacetic acid (EDTA)

Until recently, the quantitation of Ep in serum and plasma has been dependent on relatively insensitive bioassay systems. For the past 20 years, an assay employing the polycythemic mouse has been used by most investigators for the bioassay of Ep.[1] However, results from different laboratories are not always intercomparable because of experimental

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This work was supported in part by the National Heart, Lung and Blood Institute (HL 22489 and HL 22650) and the Division of Environmental Research for the U.S. Department of Energy under contracts W-7405-ENG-48 and DE-AC02-76CH00016.
Submitted for publication June 24, 1981; accepted Oct. 21, 1981.
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variability. Although this bioassay is capable of measuring increased circulating levels of Ep, measurement of normal levels requires concentration of large amounts of plasma.[3] Also, materials other than Ep may affect the bioassay results.[3] A variety of in vitro bioassay systems for Ep measurement have also been developed utilizing cultures of bone marrow,[4] spleen,[5] and fetal liver,[6] but many of these systems are also subject to the effect of nonspecific factors that are present in plasma, serum, or crude Ep preparations.

Several investigators[7-11] have been concerned with immunological techniques for the measurement of Ep, but it is only recently, with the isolation of human Ep in pure form,[12] that valid Ep immunoassays using the classical Berson and Yalow[13] RIA technique have been demonstrated.[14, 15] The purpose of this communication is to further validate the Ep RIA and to extend its use to a large series of normal humans and some polycythemic patients.

Methods

Experimental subjects. Normal human plasma and serum samples were collected from healthy volunteer members of the LBL and BNL staffs. All patient samples were obtained as residual samples from outpatients treated at the Donner Clinic at LBL. The protocols used in these studies were reviewed and approved by the LBL Human Use Committee and the University of California Human Use Committee. Informed consent was obtained from all normal subjects and patients.

Patients with polycythemia were in various stages of treatment: phlebotomy, with or without cytotoxic agents, for polycythemia vera, and phlebotomy alone for secondary polycythemia. Only polycythemia vera patients originally meeting the NIH Polycythemia Vera Study Group criteria[16] were accepted for this study; there were 17 such patients (11 males, 6 females), and 192 determinations were done on different plasma samples taken at different times. There were five patients with secondary polycythemia—three males with hypoxia (chronic congestive heart failure, congenital heart disease, pulmonary disease), one male with hemoglobinopathy with high oxygen affinity, and one female with erythrocytosis of undetermined etiology. A total of 39 individual plasma samples were assayed in the five patients with secondary polycythemia. In all polycythemic patients, the presence of polycythemia was initially documented by an increased red cell mass. Most of the samples for Ep measurement were obtained from either patients who had never been phlebotomized or patients in whom more than a month had elapsed since the most recent therapeutic phlebotomy; only eight of the Ep measurements were made earlier than this, at times ranging from 4 to 21 days after phlebotomy. Twenty-seven different plasma samples from three severely anemic patients—one with Fanconi's anemia, one with Diamond-Blackfan's anemia, and one with paroxysmal nocturnal hemoglobinuria—were included as part of the Ep RIA verification studies. All samples were separated within 30 min of being drawn, and the plasma or serum was frozen and kept at −20° C in polypropylene cryotubes (Vangard International, Inc., Neptune, N. J.) until set up in a RIA.

Ep iodination. The Ep used for iodination was obtained in pure form.[12] Some was kindly supplied by Dr. Eugene Goldwasser,* coded HT 6-30-76 Fr2, and consisted of approximately 1 μg of lyophilized Ep in the bottom of a polyethylene microfuge tube. The rest was obtained from a distribution program through the National Heart, Lung and Blood Institute,† coded M-7-72-2 (also originally from Dr. Goldwasser), and consisted of approximately 0.7 μg of lyophilized Ep in glass capillary tubing 1 to 2 mm in diameter and approximately 4 cm long. The Ep was obtained by extraction of urine from severely anemic humans and purified to an S.A. of approximately 70,400 U/mg of protein.[12] The iodinations were carried out with either a modification of the lactoperoxidase method[17] or a method by Fraker and Speck[18] that utilized 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (IODO-GEN; Pierce Chemical Co., Rockford, Ill.). Details of the application of these two iodination methods to Ep have been presented earlier.[14] Ep iodinated with either of these methods was equally immunologically reactive.

Ep antibody. Details of Ep antibody production have been presented elsewhere.[9] The anti-Ep used here was obtained from a rabbit that received a booster immunization of a partially purified

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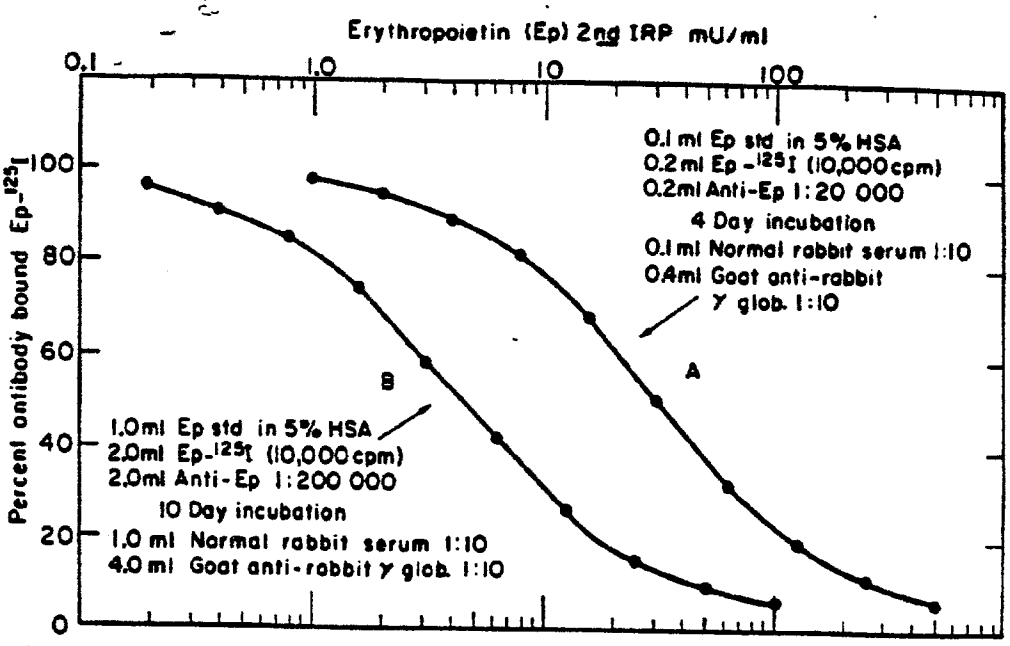

Fig. 1. Typical curves for the Ep RIA. The protocol for *curve A* was used for most assays and the protocol for *curve B* was reserved for samples with very low Ep concentrations.

human Ep preparation* with an S.A. of 8000 U/mg.[19] The rabbit had been previously immunized with a crude extract of human urinary Ep prepared by pressure filtration through a collodion membrane.[20] This antiserum has the ability to neutralize more than 300 U of human Ep per milliliter.[9] Although neutralization studies against other species' Ep's have not been carried out with this antiserum, it does cross-react with a variety of animal Ep's in the RIA system. In addition to human Ep, cross-reactions with mouse, rat, rabbit, sheep, dog, baboon, and monkey Ep's have been observed.[14] This antiserum was used, purposefully unabsorbed, in the RIA at a final incubation dilution of 1:50,000.

Ep RIA. Samples of 100 μl of undiluted or diluted plasma or serum were pipetted into disposable polystyrene tubes (12 by 75 mm). Similarly, 100 μl of halving dilutions of Ep standard (second IRP), from 500 mU/ml down to 1 mU/ml, were pipetted into such tubes. Phosphate buffer, 0.05M, pH 7.5, with 5% HSA (Cutter Laboratories, Inc., Berkeley, Calif.) added, was used as the diluent for the Ep standard and for any dilution of plasma or serum required. The high HSA content was an attempt to maintain similar protein concentrations in all tubes, both standards and unknowns. Two hundred microliters of diluted labeled Ep and 200 μl of diluted anti-Ep antiserum were added simultaneously with an automatic pipette (Micromedic Systems, Horsham, Pa.). The labeled Ep was diluted so that the 200 μl contained approximately 10,000 cpm, and the anti-Ep was a dilution of 1:20,000 (final incubation dilution of 1:50,000). The diluent for both of these reactants consisted of 0.05M PO_4, pH 7.5, with 0.5% BSA added. The tubes were then vortexed and kept at 4° C for an incubation period of 4 days.

Separation of the gamma globulin–bound Ep was accomplished with a goat anti-rabbit gamma globulin antiserum as described previously.[21] The tubes were centrifuged in a refrigerated centrifuge (J-6B; Beckman Instruments, Inc., Palo Alto, Calif.) for 30 min at 2000 rpm (900 × g). The tubes were decanted and then counted by using a well-type gamma counter (MS-588; Micromedic). The data were collected on paper tape and an electronic recording cassette using an electronic data terminal (Silent 700 ASR; Texas Instruments, Inc., Houston, Texas). Results were analyzed with the sigmoid computer program of Rodbard and Hutt[22] and the LBL CDC 7600 computer, which derives the standard curve relating percent bound to log mUml/Ep and determines values for unknowns in terms of milliunits per milliliter.

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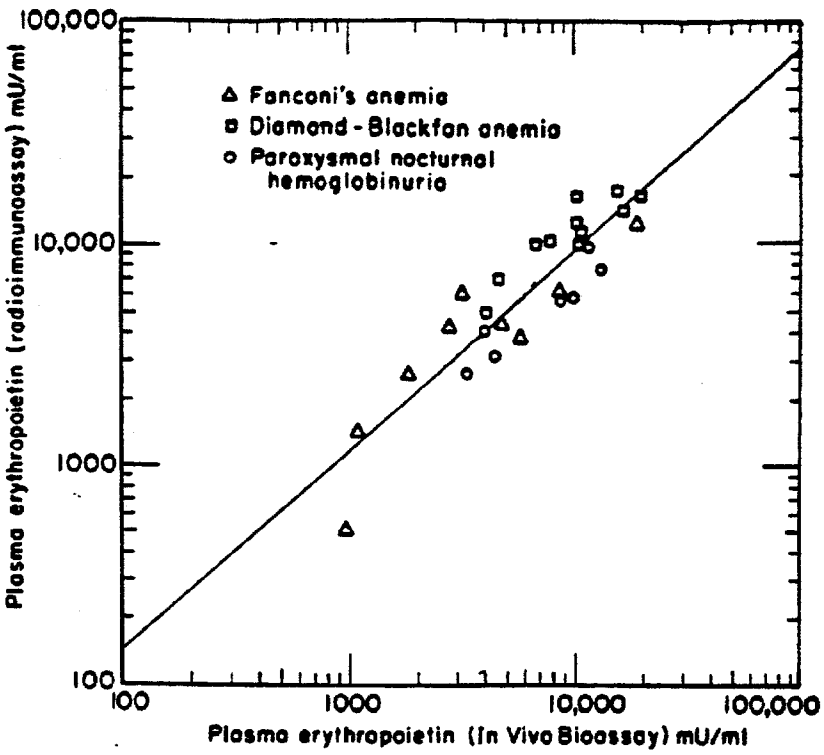

Fig. 2. Correlation of Ep RIA results with polycythemic mouse bioassay results, using 27 different plasma samples from three anemic patients. The correlation coefficient was 0.898. No significant difference between the two assays was found, with $p > 0.6$ by Student's paired t test.

This protocol is used for most Ep RIAs and the resulting curve is presented (*curve A*) in Fig. 1. Standard curves are dispersed throughout an assay, so that such a complete curve is set up along with approximately every 200 RIA tubes. Along with each curve, three control tubes are set up. In one, the total amount of Ep-^{125}I that is precipitable by TCA is measured. This is accomplished by the addition of 1 ml of 10% TCA. The amount of radioiodine that is precipitated by TCA is routinely 80% of the total radioactivity added. Another tube contains all the reactants but does not contain any unlabeled Ep. It is on the basis of this tube that the total amount of antibody precipitable Ep-^{125}I is determined. The anti-Ep binding has ranged from 33% to 39% of the TCA-precipitable Ep-^{125}I. The third tube has all reactants except anti-Ep. This serves as a background tube, and the amount of Ep-^{125}I trapped in the pellet in this tube is subtracted from the cpm of all tubes. Also, along with each standard curve, assays of aliquots of pools of three different serum samples are measured. These serve as internal standards for intraassay and interassay comparison. One is a normal human serum pool, another is a pool of serum from anephric patients, and the third is a pool of serum from patients with iron-deficiency anemia. The mean value for the normal human serum pool is 20.03 mU/ml, with an intraassay CV of 8.37% and an interassay CV of 9.70%.[22] The anephric serum had a mean of 14.39 mU/ml, with an intraassay CV of 9.94% and an interassay CV of 13.20%, and the mean for the iron deficiency pool was 97.08 mU/ml, with an intraassay CV of 6.38% and an interassay CV of 8.83%.

Curve A in Fig. 1 shows that within the anti-Ep binding limits of 90% to 10% a range of Ep concentrations of approximately 4 to 250 mU/ml can be measured, with 50% binding at 32.0 mU/ml. All unknown samples are done in duplicate, and values in the range of 100 mU/ml or higher are repeated in a series of halving dilutions, with values assigned from determinations that fall closer to the middle of the RIA range. Since 100 µl of each standard dilution have been used in the development of this curve, it should be pointed out that the range of the Ep RIA in terms of absolute units is 0.4 to 25 mU.

The possibility of extending the range of measurable Ep concentration to a more sensitive range was considered. Since one of the limiting factors in the Ep RIA is the amount of Ep used in the labeled form, the sensitivity could be extended to a lower range by increasing the amount of unlabeled Ep relative to labeled Ep. This was accomplished by increasing the volume of unknown to 1 ml and keeping the amount of labeled Ep to that giving a counting rate of 10,000 cpm. Since the original protocol appeared to work well, the same buffers and relative volume relationships were maintained. However, the anti-Ep concentration was reduced and the incubation period increased. Such an approach has been used to extend the range of a human growth hormone RIA.[31]

Thus 1 ml of diluted or undiluted plasma or serum was pipetted into disposable polystyrene tubes (16 by 119 mm). For the Ep standard curve, 1 ml of halving dilutions of Ep (second IRP) from a concentration of 100 mU/ml down to 0.2 mU/ml were made and 1 ml was pipetted into each tube. All dilutions of standard Ep and plasma or serum were made with the phosphate buffer with 5% HSA added. Two milliliters of labeled Ep (approximately 10,000 cpm) and 2 ml of anti-Ep at a dilution of 1:200,000 made up the remainder of the incubation medium. The incubation period was extended to 10 days at 4° C, after which the anti-Ep was precipitated with goat anti-rabbit gamma globulin serum as before.

Curve B in Fig. 1 was obtained with this protocol. Now, within the anti-Ep binding limits of 90% to 10%, a range of Ep concentrations of approximately 0.4 to 50 mU/ml can be measured, with a 50% binding at 4.5 mU/ml. Such a curve is more sensitive by a factor of approximately 7; however, the absolute Ep range of the assay is similar to that of the previous assay. The antibody binding with this protocol was approximately 20% as compared to 33% to 39% for the usual assay. Also, it is more cumbersome in incubation time and volume of second antibody and is not as adaptable to our automation system. Thus its use has been reserved for very low Ep concentrations and for dilution studies as shown below.

Ep bioassay. Ep was bioassayed in female LAF_1 mice (Jackson Laboratories, Bar Harbor, Me.) made polycythemic by exposure to increasing levels of carbon monoxide.[34] Such animals exposed for 8 hr/day, 5 days a week reach hematocrits of over 70% in about 3 weeks. After exposure, the polycythemic mice are then rested for 7 days prior to being used as assay animals. Ep preparations were injected subcutaneously, and 48 hr later 0.5 μCi of ^{59}Fe (New England Nuclear, Cambridge, Mass.) was injected intraperitoneally. Seventy-two hours after the radioiron injection, the mice were anesthetized with ether and bled by cardiac puncture. Results on mice with hematocrits less than 55% at autopsy were discarded. The ^{59}Fe in an aliquot of blood was counted in a well-type gamma counter (Nuclear Chicago, Des Plaines, Ill.) and the percent incorporation into the total red cell volume was calculated on the basis of a blood volume of 7% body weight. This was compared to a curve generated with standard Ep (second IRP) covering a range from 0.1 to 0.8 U. All Ep injections had a volume of 1 ml and consisted of dilutions of serum or standard Ep made up with 5% HSA. Values for serum Ep were accepted for at least two dilutions falling within the range of Ep used to develop the standard curve. The limit of detection for Ep is about 50 mU with this bioassay.

Results

Comparisons of Ep RIA values with Ep results obtained with the polycythemic mouse bioassay were made with plasma from the three anemic patients studied. These results are presented in Fig. 2 and indicate a high degree of correlation between these two methods, with a correlation coefficient of 0.898. The plasma Ep concentration ranged from 500 to 16,700 mU/ml with the RIA and from 950 to 20,000 mU/ml with the in vivo bioassay. Dilutions of these plasmas, to bring them within the Ep range of the respective assay systems, were made with 5% HSA. Also, the same Ep (second IRP) was used as the reference material for both assays.

When the more sensitive Ep RIA protocol (*curve B*, Fig. 1) was used, selected human sera were serially diluted, and the RIA results were examined for parallelism with the curve obtained with the second IRP of human Ep. These results are presented in Fig. 3. The three internal standards of normal, anephric, and iron-deficiency sera were tested in this way, as was a sample of polycythemia vera serum. The results obtained by serial dilution of these sera are reasonably parallel to those obtained with the second IRP Ep.

Plasma and serum Ep measurements were done on a total of 445 healthy normal

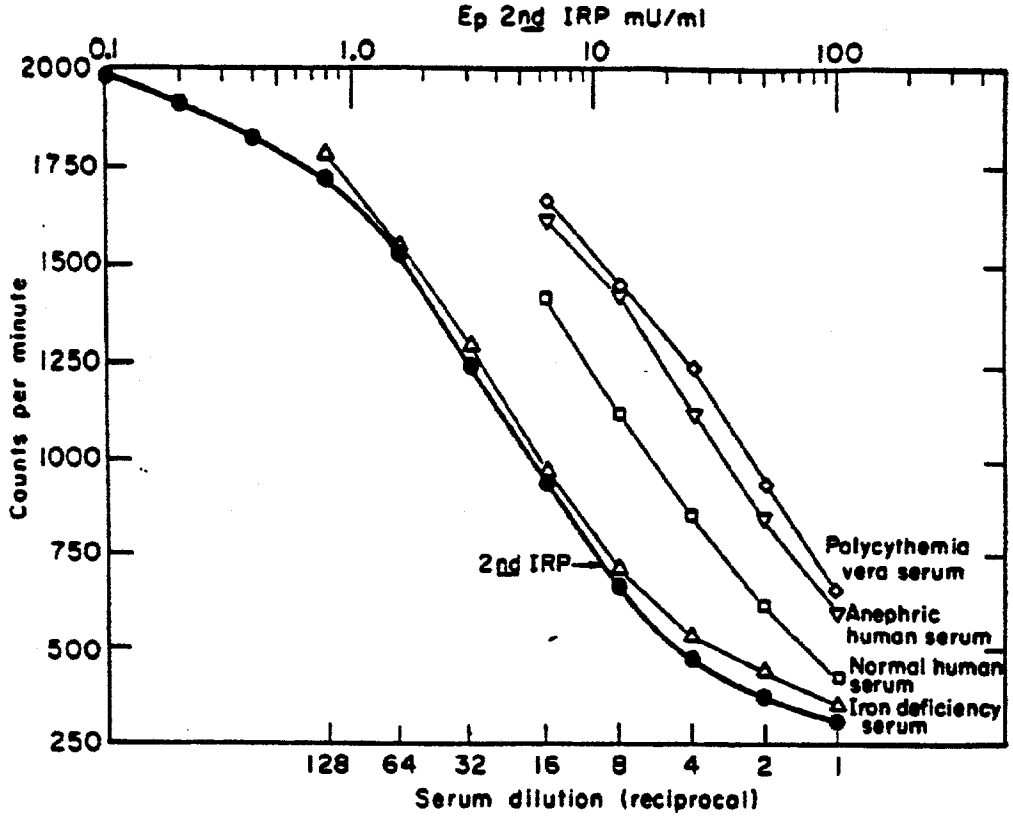

Fig. 3. Serial dilution of various human sera demonstrating a parallel relationship with the standard reference preparation of human Ep.

subjects with an age range of 18 to 64 years, 293 males and 152 females. Some were unfasted volunteers from the LBL staff (136), and some were volunteers from the BNL staff (309) after an overnight fast. The LBL subjects were sampled twice, between 7:30 and 9:30 A.M. and 1:30 to 3:30 P.M. The BNL subjects were all sampled between 9:00 and 11:00 A.M. All samples were collected as serum except for an additional sample of EDTA plasma at the morning sampling of the LBL subjects; at this time, hemoglobin concentrations and hematocrit determinations were made. The serum Ep concentrations are presented in Table I. There was no significant difference in the male and female values of both the LBL and BNL groups. In fact, there was remarkable agreement between the LBL and BNL groups, in spite of the fasting and nonfasting states. There was no difference between morning and afternoon sampling. However, when the results of the entire serum sampling were pooled, the mean male Ep concentration of 17.2 mU/ml was significantly different from the mean female concentration of 18.8 mU/ml, with a p value of <0.005. Results on the plasma samples taken with EDTA, at the same time as the morning LBL serum samples, showed a significant reduction from their respective serum values. The mean for the EDTA male normal plasma was 15.2 mU/ml with an S.D. of 5.5, and the female normal mean was 15.8 mU/ml with an S.D. of 4.6. These normal plasma values are presented in Table II and significantly differ from their respective serum values as presented in Table I, with a p value of <0.005.

Also in Table II are presented results for Ep concentrations on plasma samples ob-

Table I. Serum Ep concentrations in normal human beings

	Hemoglobin concentration (gm/dl)	*Hematocrit (%)*	*Erythropoietin concentration (mU/ml)*
Male			
LBL* (81) 7:30-9:30 A.M.	15.5 ± 1.0	44.5 ± 2.8	17.6 ± 5.5
LBL (81) 1:30-3:30 P.M.			17.5 ± 5.0
BNL* (212) 9:00-11:00 A.M.	15.4 ± 1.0	44.4 ± 2.8	17.0 ± 5.7
Entire male sampling (364)			17.2 ± 5.5
Female			
LBL (55) 7:30-9:30 A.M.	13.3 ± 1.0	38.7 ± 2.5	19.0 ± 5.8
LBL (55) 1:30-3:30 P.M.			18.7 ± 4.7
BNL (97) 9:00-11:00 A.M.	13.5 ± 0.8	39.5 ± 2.5	18.7 ± 7.0
Entire female sampling (199)			18.8 ± 6.2

Values are mean ± S.D.; number of Ep measurements in parentheses.
*The LBL samples were from nonfasted individuals, whereas the BNL samples were obtained after an overnight fast.

Table II. Plasma Ep concentrations in normal and polycythemic human beings*

	Hemoglobin concentration (gm/dl)	*Erythropoietin concentration (mU/ml)*
Male†		
Normal (81)	15.5 ± 1.0	15.2 ± 5.5
Polycythemia vera (113)	15.4 ± 1.1	9.2 ± 5.0
Secondary polycythemia (29)	15.9 ± 0.9	153.7 ± 106.7
Female†		
Normal (53)	13.3 ± 1.0	15.8 ± 4.6
Polycythemia vera (79)	15.6 ± 1.3	8.7 ± 5.6
Secondary polycythemia (10)	15.3 ± 0.7	75.7 ± 29.8

Values are mean ± S.D.; number of erythropoietin measurements in parentheses.
*All plasma samples were collected with EDTA as the anticoagulant.
†Within each sex, all Ep means are significantly different from each other, with $p < 0.001$.

tained from patients with polycythemia in various stages of treatment from the Donner Clinic. All plasma results reported here were on plasma samples collected with EDTA as the anticoagulant. The mean hemoglobin concentration for the treated primary and secondary polycythemic males was 15.4 and 15.9 gm/dl, respectively, as compared to 15.5 for the normal males. The mean Ep concentrations were 9.3 mU/ml for 113 polycythemia vera plasma samples and 153.7 mU/ml for 29 secondary polycythemia plasma samples. The mean Ep concentrations for the primary and secondary polycythemic plasma samples were significantly different from each other, as well as from the mean normal plasma value, with p values of <0.001. Although the individual secondary polycythemia values were definitely increased over the normal and polycythemia vera values, individual polycythemia vera values were not distinguishable from normal values. This is further depicted in Fig. 4, where no overlap is seen between the Ep values on plasma samples from male patients with secondary polycythemia and those with polycythemia vera. Results on plasma from the Diamond-Blackfan anemia and a polycythemia with myelofibrosis are also presented in Fig. 4 to emphasize the inverse hemoglobin–Ep concentration relationship.

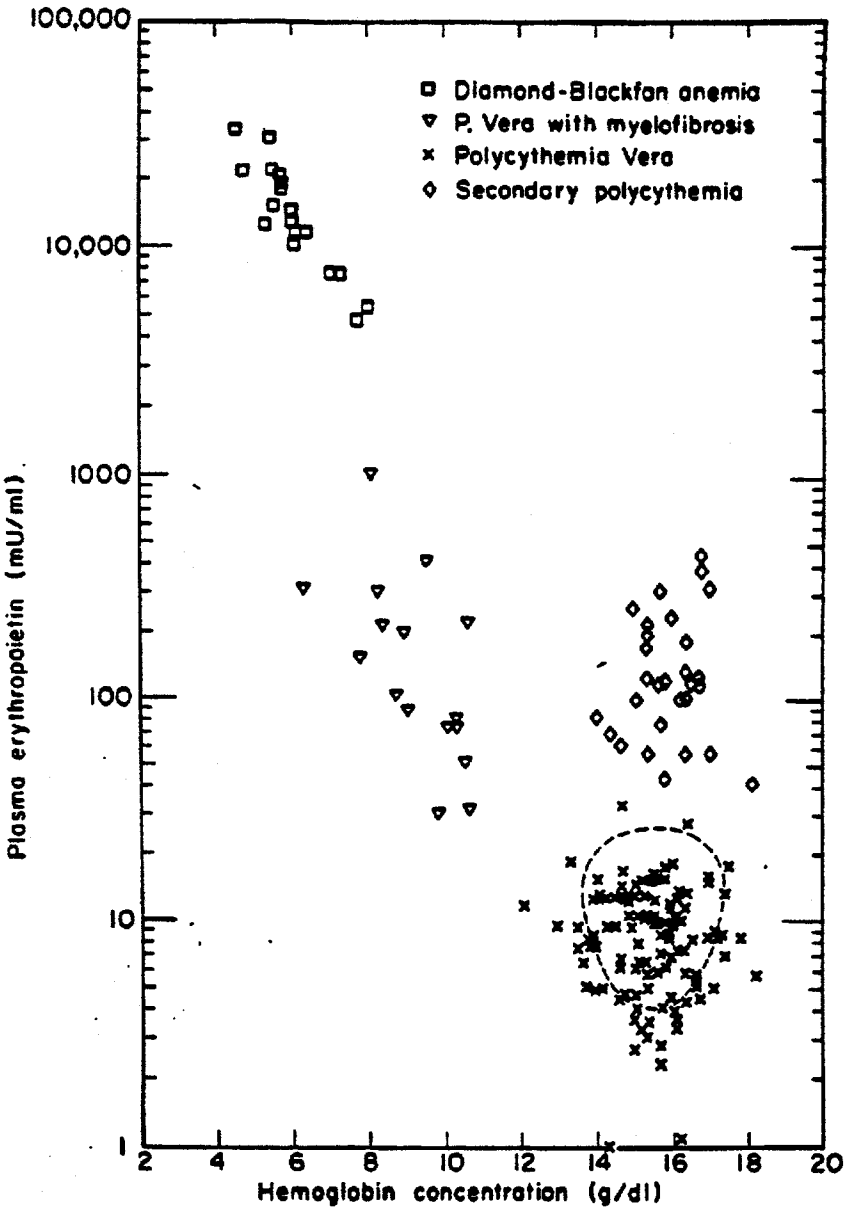

Fig. 4. Plasma Ep concentration as a function of hemoglobin concentration. Each point represents a different plasma sample. *Solid circle*, Mean for normal male plasma; *dashed line*, 2 S.D. of the mean.

The mean hemoglobin concentration for the treated polycythemia vera and secondary polycythemia females was 15.6 and 15.3 gm/dl, respectively, as compared to 13.3 for the normal females. The mean Ep concentrations were 8.7 mU/ml for 70 female polycythemia vera plasma samples and 75.7 mU/ml for 10 secondary polycythemia plasma samples. These values were significantly different from each other and from the normal female plasma mean, with p values of <0.001. Also, as seen in Fig. 5, there was no overlap between the secondary polycythemia Ep values and the values obtained from plasma of patients with polycythemia vera.

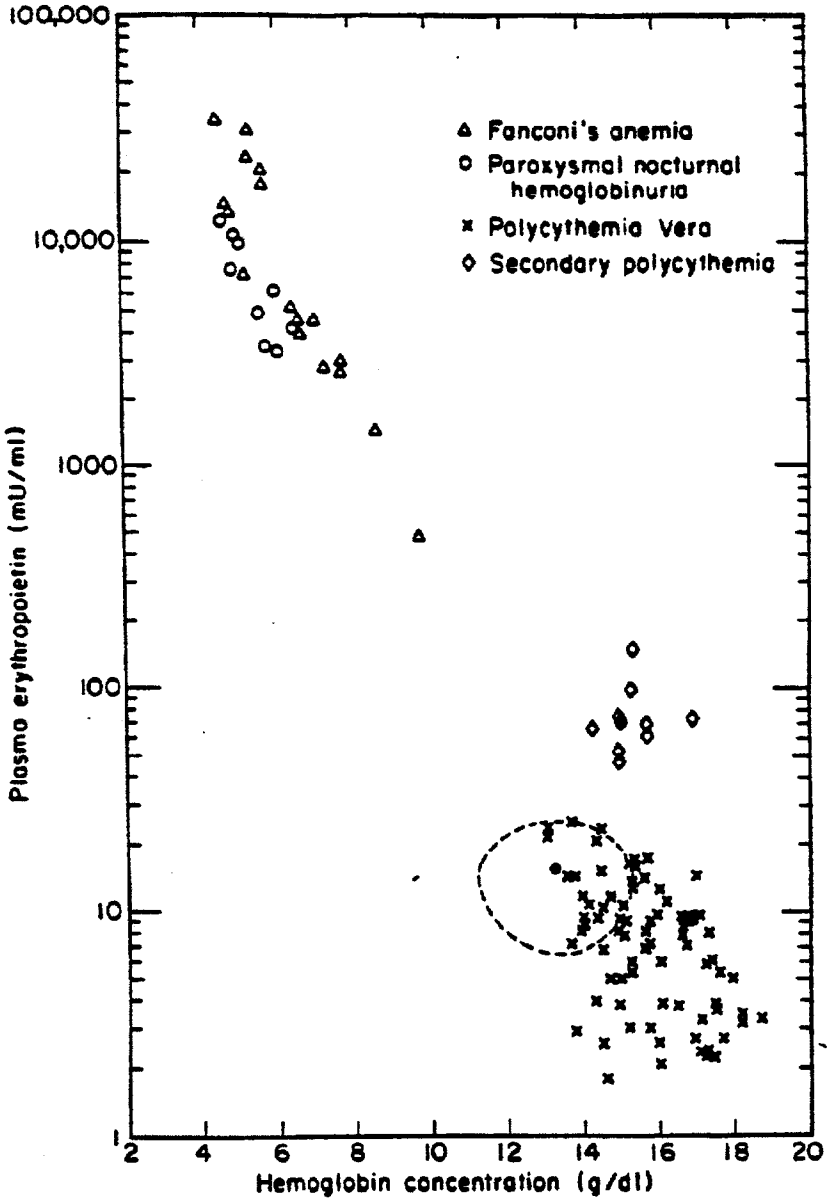

Fig. 5. Plasma Ep concentration as a function of hemoglobin concentration. Each point represents a different plasma sample. *Solid circle*, Mean for normal female plasma; *dashed line*, 2 S.D. of the mean.

Discussion

The RIA for Ep presented is sensitive to an absolute amount of Ep equivalent to 0.4 mU, which allows for the measurement of not only normal circulating Ep levels but also Ep levels that are considerably depressed below normal. The RIA shows a high degree of correlation with the polycythemic mouse bioassay, and halving dilutions of human anephric, polycythemic, and normal sera show a reasonably parallel relationship to the Ep reference standard, thus supporting the hypothesis that the small amount of immunoreactive material present in these sera is, in fact, Ep.

In addition, detection of appropriate increases or decreases in circulating Ep concentrations in response to physiological and clinical conditions known to be associated with stimulation or suppression of Ep secretion further validates the Ep RIA. In human subjects, blood loss, blood transfusion,[14] anemia, and secondary polycythemia have shown appropriate changes in Ep. With the anti-Ep antiserum used here, a variety of animal Ep's are also reactive in competing with the labeled human Ep for the anti-Ep. Appropriate changes in serum Ep measured with the RIA have been shown to occur in the hypoxic rat and baboon.[14] Acute bleeding of sheep resulted in a prompt rise in radioimmunoassayable Ep 24 hr later.[25] Also, hypertransfusion of normal mice results in a depression of plasma Ep measurable by the RIA.[26] Thus results obtained with the Ep RIA are consistent with the known physiological and clinical information concerning Ep. Perhaps the only discrepancy is that regarding the inability to distinguish between intact and asialoerythropoietin.[14, 15] This, however, has also been a problem with certain in vitro bioassay methods for Ep.[4]

In the present Ep RIA, means for normal male serum of 17.2 mU/ml and normal female serum of 18.8 were obtained. These values are approximately four times the values seen in a previous Ep RIA attempt,[27] which utilized a partially purified Ep preparation as the tracer. Also, it now appears that when a large group of values are analyzed, the female mean is significantly increased over that seen for the male, which is the reverse of what was found with the previous RIA. Although EDTA plasma values were significantly decreased from their respective serum values, no significant sex difference was seen. Preliminary results on plasma collected with heparin as an anticoagulant do not differ from Ep RIA results obtained on serum collected at the same time. Using a similar Ep RIA, Koeffler and Goldwasser[28] have recently obtained normal human serum values similar to those presented here, although the data are not separated into male and female groups. Extracts of normal plasma assayed in the polycythemic mouse bioassay show a mean of 7.8 mU/ml.[3] Using an in vitro bone marrow bioassay, Krystal et al.[29] recently obtained the very high average human serum Ep concentration of 800 mU/ml. In a review of normal human serum Ep concentration, a value of approximately 40 mU/ml was considered normal.[30] Perhaps the absolute value obtained with a particular assay system is not as significant as the ability to demonstrate appropriate relative changes in Ep concentration correlated with physiological and clinical conditions known to be associated with stimulation or depression of Ep secretion. More extensive use of a variety of Ep assay systems will ultimately determine the most appropriate absolute value for normal individuals.

The Ep concentrations seen in the plasmas from secondary polycythemia patients were considerably increased over the levels seen in the plasmas of normal subjects and polycythemia vera patients as measured by RIA. This confirms the recent observations of Koeffler and Goldwasser[28] on a large series of such patients, using a similar Ep RIA. Such relationships have also been observed from a plasma Ep extraction technique[3] and a urinary Ep extraction technique,[31] with Ep measured by the polycythemic mouse bioassay. The mean Ep concentrations from both male and female polycythemia vera patients were significantly decreased as compared to the plasma levels in the normal subjects. In the present study, male patients with treated polycythemia vera had hemoglobin concentrations comparable to those of normal males; however, the treated female polycythemia vera patients had a mean hemoglobin concentration of 15.6 gm/dl as compared to a normal female hemoglobin concentration of 13.3. Lower concentrations of plasma Ep in female patients with higher hemoglobins suggest that the inverse hemoglobin–Ep concentration relationship also exists for these patients. Unfortunately, plasma samples from untreated polycythemia vera patients were not available in this study. Such samples, with higher

hemoglobin concentrations, could be expected to have even lower Ep concentrations. Ep excretion studies in patients with polycythemia vera indicate that although Ep output is absent or markedly decreased at high hemoglobin concentrations, the Ep excretion increased when the hemoglobin concentration was reduced to normal levels.[31] The high Ep concentrations in the polycythemia vera patient with myelofibrosis shown in Fig. 4 are also supportive of an inverse hemoglobin-Ep relationship in such patients.

Use of pure Ep labeled with radioiodine has removed all discrepancies seen in a previous Ep RIA attempt.[27] The problem was not with the anti-Ep antiserum, as has been suggested,[32] since the antiserum used here is the same antiserum as that used in the previous RIA. The fact that this antiserum is a crude mixture of antibodies against other proteins as well as antibody specific for Ep[33] does not invalidate the RIA. There are several publications in which antibodies to two different hormones have been purposefully mixed in RIAs simultaneously measuring both hormones in the same sample of serum.[34, 35] One hormone was labeled with ^{131}I and one with ^{125}I, and care was taken in counting each isotope separately. Results obtained by the simultaneous RIA were equivalent to those obtained by RIA of each hormone separately.

As suggested by Erslev,[36] a fast, accurate Ep assay should be of considerable practical importance in the hematology clinic. It is believed that the Ep RIA in its present form fits this need. Large numbers of samples can be handled rapidly. Only a small volume of plasma or serum, with no extraction, is required and the assay is extremely sensitive and reproducible.

We thank Dr. John H. Lawrence for his long-standing encouragement throughout this work, Dr. Gisela K. Clemons for her help in the computerization of the erythropoietin radioimmunoassay, and Dr. Giuseppe Moccia for help in collecting some of the normal serum samples. Thanks are also due to Mr. David Wei, Ms. Greta Duke, Ms. Patricia Garbutt, Ms. Cathryne Allan, and Ms. Carol Bohlen for valuable technical assistance and to Ms. Karen Kalman for help in preparation of the manuscript.

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