A Specific In Vitro Bioassay for Measuring Erythropoietin Levels in Human Serum and Plasma

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The accurate measurement of biologically active erythropoietin (Ep) in human serum and plasma using present in vivo and in vitro bioassays is difficult because of the presence of both inhibitors and non-Ep stimulators of erythropoiesis. We have developed a simple procedure to quantitatively purify Ep from serum and plasma for subsequent testing in the phenylhydrazine-treated mouse spleen cell assay. The method involves absorption of Ep to an immobilized high-affinity anti-Ep monoclonal antibody and acid elution of the antibody-bound material. After neutralization, the eluted Ep is then tested directly in the in vitro bioassay without interference by other serum proteins. By using magnetic beads as a solid support for the antibody, washing and elution steps can be performed rapidly and efficiently. Recoveries of Ep after this procedure show very little sample-to-sample variation and are consistently between 45% and 55%, which is close to the maximum binding expected for the anti-Ep antibody. Coupled with the 7.4-fold concentration that this procedure affords, there is an overall increase in sensitivity of three- to fourfold, which makes this assay suitable for accurately measuring Ep levels in patients with below-average titers. Results with this magnetic bead assay indicate that accurate and reproducible estimates for Ep levels in the serum and plasma from healthy donors as well as from patients with hematologic disorders can be obtained. Titers of biologically active Ep in the sera from a group of patients with either leukemia or lymphoma were found to be elevated, and the values correlated well with titers of immunoreactive Ep measured in the Ep radioimmunoassay. Because of its specificity and high sensitivity, the magnetic bead assay is a valuable alternative to immunoassays for the measurement of elevated, normal, and even subnormal Ep levels in human serum and plasma.

THE GLYCOPROTEIN HORMONE erythropoietin (Ep) is a major physiologic regulator of red blood cell formation. Its level in serum varies inversely with oxygen tension in normal individuals and an accurate assessment of its concentration in serum is helpful in the diagnosis of various hematologic disorders, such as polycythemia vera and secondary polycythemia. Moreover, as more patients with chronic renal failure are treated with exogenous Ep to alleviate their anemia, there will be an increased demand for a simple accurate assay to monitor serum levels of biologically active Ep. Immunologic assays, such as the competitive radioimmunoassay (RIA)[1-3] and the sandwich enzyme-linked immunosorbent assay (ELISA),[4,5] while relatively easy to perform and capable of discriminating between elevated and normal levels of Ep, detect not only biologically active Ep molecules but also biologically inactive fragments and aggregates of Ep.[6] In addition, the lower limits of detection of present immunoassays are in the same range as normal Ep levels, making these assays less than ideal for discriminating between normal and subnormal levels.

In vitro bioassays, such as the fetal mouse liver cell (FMLC) assay[7] and the mouse spleen cell (MSC) assay,[8] which measure the incorporation of either ^{59}Fe or ^{3}H-thymidine into erythroid cells, have the advantage of detecting biologically active Ep. In addition, their lower limits of detection, especially those using ^{3}H-thymidine, ie, 1 to 3 mU/mL, make the accurate measurement of subnormal Ep levels possible. However, the usefulness of these in vitro bioassays to measure Ep levels in biologic fluids has been impeded by non-Ep components of serum and plasma that either stimulate or inhibit erythroid cell growth. Attempts to improve the specificity of these bioassays by, for example, the addition of saturating amounts of iron-transferrin,[9,10] correcting for the effects of serum iron on ^{59}Fe-incorporation,[11,12] or heat treating test sera to abolish complement-related toxic effects on murine cells by human immunoglobulin M (IgM) antibodies,[13-15] have only been partially successful. In addition, attempts to improve specificity by prior fractionation of sera using, for example, gel filtration[16] or wheat-germ agglutinin affinity chromatography,[16-18] have been disappointing due to variable recoveries of Ep and the copurification of interfering substances.

Previously, we reported the development of several high-affinity monoclonal antibodies (MoAbs) to human Ep and the application of two of these antibodies to a sensitive ELISA for immunoreactive Ep in human serum, plasma, and urine.[4,19] In this study we investigate the possibility of using our highest affinity MoAb, anti–Ep-26, in combination with the MSC assay to develop a new assay for Ep that can detect biologically active Ep in human serum and plasma with high specificity and sensitivity. The method involves specific and quantitative extraction of Ep from test samples by absorption to magnetic bead-immobilized anti–Ep-26. The Ep is eluted from the beads using a low pH buffer and, after pH neutralization, tested directly in the MSC bioassay without prior dialysis or concentration of the samples. Using this specific bioassay, it has been possible to measure Ep levels in serum and plasma without interference by non-Ep compo-

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nents and with a sensitivity high enough to allow measurement of subnormal Ep levels.

MATERIALS AND METHODS

Purification of Ep. Urinary Ep (uEp) was purified from the urine of aplastic anemia patients as described.[20] Recombinant Ep (rEp) was purified by CM Affi Gel Blue chromatography (Bio-Rad, Richmond, CA) and phenyl-high performance liquid chromatography of the culture supernatant from baby hamster kidney cells expressing the human Ep gene.[21]

MoAbs to Ep. The production and purification of MoAb anti–Ep-26 has been described.[4,19] Anti–Ep-26 was coupled to tosyl-activated magnetic polystyrene beads (Dynabeads M450, Dynal, Oslo, Norway) according to the manufacturer's instructions, with modifications. Specifically, 0.3 mg of anti–Ep-26 was incubated with 30 mg of beads in 2 mL of 0.1 mol/L sodium borate buffer, pH 9.5. After incubation for 16 hours at 23°C by end-over-end rotation, the beads were washed for 3 × 30 minutes with 4 mL of 10 mmol/L sodium phosphate, 0.14 mol/L NaCl, pH 7.4 (phosphate-buffered saline [PBS]), containing 1% (wt/vol) bovine serum albumin (BSA) and using a magnetic separator (Dynal MPC-1). The beads were then incubated at 15 mg beads/mL for 16 hours at 4°C in PBS, 1% BSA, 0.1% (vol/vol) Tween-20, and 0.01% (wt/vol) sodium azide, washed, and resuspended in the same buffer at a concentration of 10 mg/mL and stored at 4°C. Before use, beads were washed twice with RPMI containing 0.1% BSA and resuspended in RPMI, 0.04% Tween-20, 0.1% (wt/vol) polyethyleneglycol (PEG; molecular weight: 8,000), pH 3.5, and incubated for 5 minutes at 23°C to elute noncovalently bound antibodies. The beads were then washed twice with RPMI containing 0.1% BSA and resuspended at 1 mg/mL. The capacity of the beads, as determined in competition experiments with ^{125}I-Ep and unlabeled Ep, was ~50 U of Ep per milligram of beads.

Magnetic bead bioassay for Ep. Five-milliliter Falcon tubes (Becton-Dickinson, Lincoln Park, NJ), each containing 1 mL of human serum or plasma and 0.1 mg of anti–Ep-26 beads in 0.1 mL of RPMI containing 0.1% BSA, were rotated end-over-end for 40 to 48 hours at 23°C. The beads were then washed once at 4°C with 1 mL of 0.15 mol/L NaCl in ddH_2O using a 60-tube magnetic separating unit (Cat No. 4103S; Advanced Magnetics Inc, Cambridge, MA) and bound Ep eluted with 100 μL of RPMI, 0.04% (vol/vol) Tween-20, and 0.1% PEG-8,000 made to a pH of 2.0 with concentrated HCl. After 15 minutes of vigorous shaking at 23°C, each eluate was removed and its pH brought back to neutrality by rinsing the beads with 35 μL of 150 mmol/L HEPES, pH 9.0, and adding the rinse to the eluate. Various amounts of each eluate (5 to 30 μL) were then added, in duplicate, to individual wells of 96-well microtiter plates containing 400,000 spleen cells from phenylhydrazine-treated mice, in a total volume of 100 μL, and ^{3}H-thymidine incorporation was measured after 24 hours of culture at 37°C as described.[8] In initial experiments, 1 mL samples of twofold dilutions of pure human Ep (specific activity: 100,000 U/mg), diluted in RPMI containing 0.1% BSA at concentrations ranging from 1.56 to 400 mU/mL, were also subjected to the same procedure. Dose-response curves obtained with magnetic bead eluates of the pure Ep samples were then compared with dose-response curves of untreated pure Ep samples to measure the recovery of Ep. In later experiments, only a single control sample, ie, pure Ep at a concentration of 50 mU/mL, was put through magnetic bead absorption together with the test samples, because the recovery of Ep was found to be constant for Ep concentrations ranging from 3 to 200 mU/mL (see Results). Ep concentrations in test samples were calculated using the following equation: $[Ep] = E \times D/R \times C$, in which E is the concentration of Ep measured in the ^{3}H-thymidine incorporation assay; D is the dilution of each eluate in the assay wells; R is the fraction of Ep recovered after magnetic bead treatment; and C is the concentration factor for each eluate (ie, 1 mL/0.135 mL = 7.4). ^{125}I-Ep used in experiments to optimize assay conditions was obtained from Amersham (Arlington Heights, IL) and had a specific activity of 700 Ci/mmol.

RIA for Ep. The competitive RIA for Ep was performed according to published procedures,[1-3] with some modifications. For this procedure, anti-Ep antiserum was prepared by immunizing rabbits with pure human urinary Ep (specific activity = 80,000 U/mg). Pure recombinant Ep (specific activity = 100,000 U/mg) was labeled with ^{125}I to a specific activity of 900 to 2,700 Ci/mmol using the chloramine T method.[22] Test samples or pure Ep samples (200 μL/tube) diluted in PBS, 1% BSA, 2.5 mmol/L EDTA, and 0.01% NaN_3 were mixed with 100 μL of a 1:4,000 dilution of rabbit anti-Ep antiserum and 400 μL of the above buffer such that the total reaction volume was 700 μL. The mixtures were incubated for 3 days at 23°C, and then 100 μL of ^{125}I-labeled Ep (8,500 cpm) was added and the samples incubated for a further 24 hours. Goat anti-rabbit IgG (100 μL) (Calbiochem, La Jolla, CA) and 100 μL of a 1:30 dilution of normal rabbit serum were then added to each tube to precipitate immune complexes. After 4 hours of incubation at 23°C, 500 μL of a 10% (wt/vol) solution of PEG-4,000 in 10 mmol/L Na-phosphate, pH 7.6, was added and precipitates were collected by centrifugation and counted in a Beckman 5500 γ-counter (Beckman, Fullerton, CA).

ELISA for Ep. The sandwich ELISA for Ep was performed as described previously.[4]

Serum and plasma samples. Plasma samples were obtained from 32 healthy blood donors. In addition, serum samples were obtained from 4 patients with non-Hodgkin's lymphoma, 2 patients with acute myeloid leukemia, and 1 patient with acute lymphocytic leukemia during a 37-day period before and during treatment with cytostatic drugs.

RESULTS

Development and optimization of the magnetic bead assay procedure. In preliminary experiments, the optimal time and temperature for binding of Ep to anti-Ep MoAbs covalently linked to magnetic beads were investigated. For this purpose, ^{125}I-labeled Ep was diluted in either RPMI supplemented with 0.1% BSA or in normal human plasma. One-milliliter aliquots were then incubated with 0.1 mg of beads for different times and at different temperatures, and the amount of radioactivity bound to these anti-Ep magnetic beads was measured. At 23°C, maximal binding of ^{125}I-Ep in both RPMI/BSA and in plasma occurred between 24 and 48 hours (Fig 1). Longer incubations did not lead to increased binding (data not shown). At 4°C, as expected, binding was slower, and by 48 hours still substantially lower than at 23°C. At 37°C, binding was much faster with maximal binding being achieved after only 6 hours of incubation. However, at this temperature the maximal amount of ^{125}I-Ep bound was less than that achieved at 23°C, and ^{125}I-Ep binding actually decreased with time after 6 hours (data not shown). For this reason, a binding period of 40 to 48 hours at 23°C was used in all subsequent experiments.

As is also shown in Fig 1, similar binding kinetics were observed when Ep was diluted in RPMI/BSA or in plasma, but the maximal amount of ^{125}I-Ep bound was lower in plasma (36%) than in RPMI/BSA (52%). However, when elution conditions were investigated it was found that ^{125}I-Ep

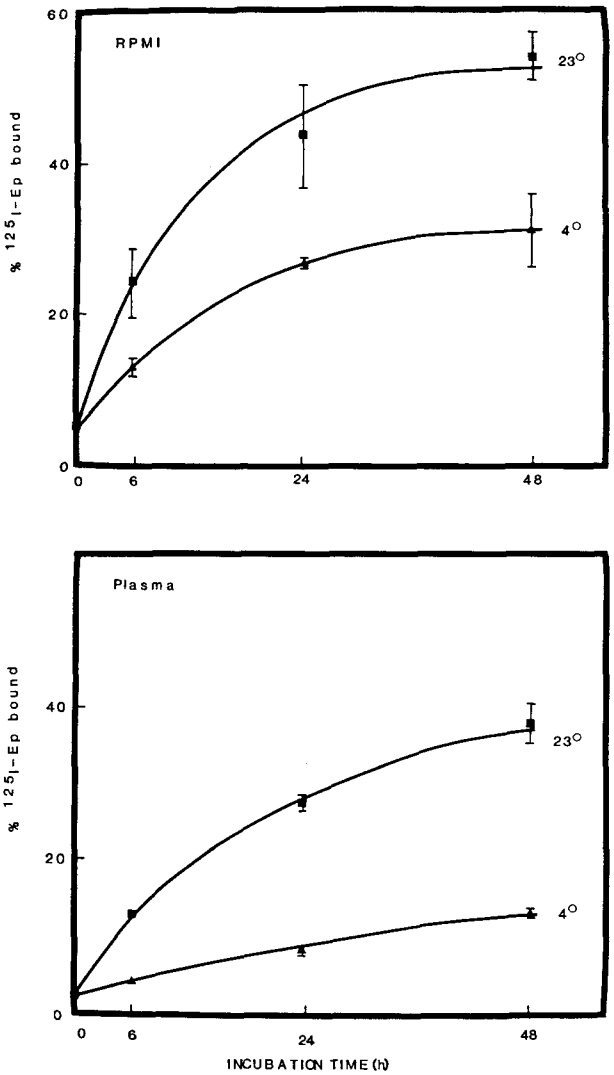

Fig 1. Binding of ^{125}I-Ep to anti-Ep magnetic beads. ^{125}I-Ep was diluted in RPMI/0.1% BSA (top) or in normal human plasma (bottom). One-milliliter aliquots containing 12,500 cpm of ^{125}I-Ep were then incubated at 4°C (▲) or 23°C (■) for the indicated time periods, after which the beads were washed and the amount of radioactivity bound to the beads measured. Results are the mean ± SE of duplicate samples.

was eluted more efficiently from beads incubated with plasma than from beads incubated with RPMI/BSA. As shown in Fig 2, incubation of the beads with pH 2 buffer resulted in a rapid elution of the ^{125}I-Ep from the beads. After 15 minutes of incubation with elution buffer, 90% of the ^{125}I-Ep bound in the presence of plasma, but only ~70% of the ^{125}I-Ep bound in the presence of RPMI/BSA was released by the beads. When higher pH elution buffers were tested, release of Ep was much lower, with only 30% released at pH 3 and 10% released at pH 4 (data not shown). Moreover, elution buffers with pH values below 2 did not release more ^{125}I-Ep than pH 2 buffers, and therefore were not used to keep pH-induced damage of Ep to a minimum. No change in Ep activity was observed, as assessed by the MSC assay, when control Ep preparations were exposed for 15 minutes to the pH 2 buffer used for elution.

As a result of the differences in binding and elution efficiencies between Ep in RPMI/BSA and in plasma, the overall recoveries of Ep after elution at pH 2 were very similar; ie, 36% and 32%, respectively, for the experiment shown in Figs 1 and 2. To determine whether Ep recovery (ie, binding and release) varied among individual plasma samples, ^{125}I-Ep was absorbed to anti-Ep magnetic beads in the presence of plasma samples obtained from five different donors. From these experiments it was found that the amount of ^{125}I-Ep that was bound to magnetic beads did not vary significantly from one plasma sample to another, with an average ± SEM of 40% ± 2%. (Slightly lower levels of ^{125}I-Ep were bound when sera were used instead of plasma, but the difference was not significant.) After pH 2 elution, 89% ± 3% of the bound ^{125}I-Ep was released, and again no difference was observed between serum and plasma. Therefore, these results indicate that the recovery of Ep after binding to and elution from anti-Ep magnetic beads is not significantly influenced by the composition of individual serum or plasma samples.

Because overall recoveries of radiolabeled Ep were not significantly affected by the composition of Ep samples, we thought it reasonable to use the recovery of standard Ep preparations in RPMI/BSA as an estimate for the recovery of Ep in serum and plasma samples tested in the same experiment. To measure the recovery of biologically active Ep after absorption to and elution from the anti-Ep magnetic beads, 1-mL samples of twofold dilutions of pure Ep in RPMI/BSA were incubated with the anti-Ep magnetic beads. The beads were then washed and bound Ep eluted with 100 μL of pH 2 adjusted RPMI culture medium. After neutralization of the pH by addition of 35 μL 150 mmol/L Hepes, pH 9.0, the eluates for each sample were measured for their biologic activity in the standard MSC assay. In Fig 3, the Ep concentrations in the original 1-mL samples of twofold diluted pure Ep are plotted against the ^{3}H-thymidine incorporation obtained when 20 μL of the eluates for each

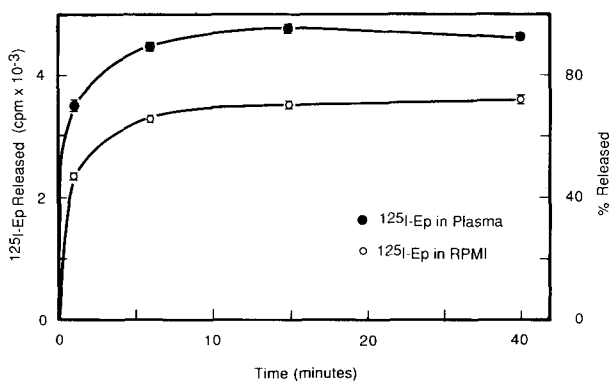

Fig 2. Release of ^{125}I-Ep by anti-Ep magnetic beads. ^{125}I-Ep, diluted up to 1 mL with either RPMI/0.1% BSA (○) or normal human plasma (●), was bound to 0.1 mg of anti-Ep magnetic beads. After washing, the beads were incubated with 100 μL RPMI, pH 2, for the indicated time periods, and the radioactivity in the eluates was measured. Results are the mean ± SE of duplicate samples.

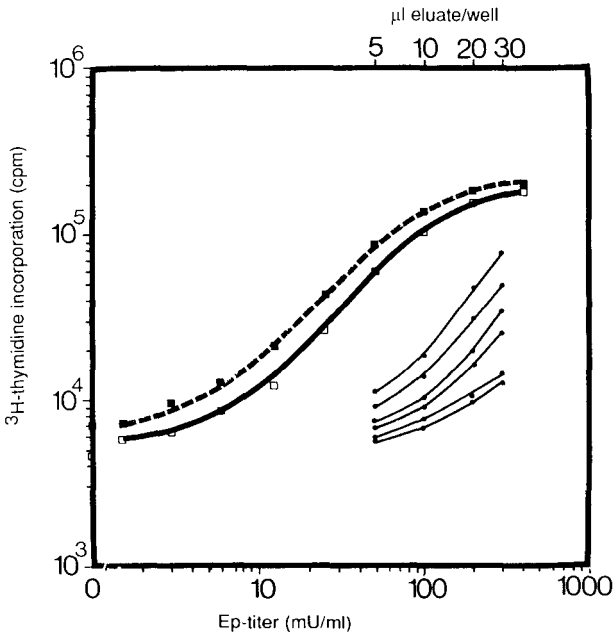

Fig 3. Dose-response curves of pure rEp and normal human plasma samples in the magnetic bead assay. One-milliliter samples of twofold dilutions of pure rEp (1.56 to 400 mU/mL) in RPMI/BSA were incubated with anti-Ep magnetic beads. The beads were washed and bound Ep was eluted with RPMI, pH 2. After pH neutralization, 20 µL of each eluate was tested in duplicate in the MSC assay. ^{3}H-thymidine incorporation of 20 µL of each eluate is plotted against the Ep concentration in the original sample (—). Dose-response curve of pure Ep tested directly in the MSC assay (---). Plasma samples from six healthy donors were also tested in the magnetic bead assay: ^{3}H-thymidine incorporation is plotted against the volume of eluate tested in the MSC assay (–●–). Results are the mean of duplicate determinations. SE was smaller than 10% for all points.

sample were tested in the MSC assay. The dose-response curve for the eluates was virtually parallel with the dose-response curve for untreated Ep between 3 and 200 mU/mL, and, from the shift of the curves, the recovery of Ep after magnetic bead absorption could be calculated as 52%. This was significantly higher than that obtained with ^{125}I-Ep (30% to 40%), perhaps because of damage to some Ep molecules as a result of iodination. The parallelism indicated that the efficiency of binding and elution of Ep did not vary with the amount of Ep added to the magnetic beads. This experiment was repeated four times; in all cases complete parallelism was obtained and Ep recoveries ranged from 45% to 55%. For this reason, only a single Ep standard was put through the magnetic bead procedure in all subsequent assays to determine Ep recovery. Because of the 7.4-fold concentration of Ep using the magnetic bead procedure (ie, from 1 mL to 0.135 mL), the sensitivity of the magnetic bead assay was approximately three to four times that of the standard MSC assay itself, ie, giving it a lower limit of detection in the original sample of approximately 0.25 mU/mL.

Measurement of Ep in plasma and serum. To examine the usefulness of this assay procedure to measure Ep in biologic fluids, plasma samples obtained from six healthy donors were absorbed to anti-Ep magnetic beads, and the pH 2 eluates titrated in the bioassay. As shown in Fig 3, dose-response curves obtained for 5 to 30 µL of the eluates were virtually parallel with each other and with the pure Ep dose-response curve, suggesting that the assay was specific for Ep. To examine if non-Ep stimulators were present in the test samples, the eluates of these six plasma samples were also tested in the presence of a saturating amount of a rabbit antiserum against Ep. In the presence of the polyclonal antibody preparation, ^{3}H-thymidine incorporation did not exceed background levels, indicating that Ep was the only stimulator present in the eluates (data not shown). To examine the possibility that the eluates contained inhibitors of cell growth, additivity experiments were performed. For this purpose, 20-µL samples of eluates from 10 normal donors were tested in the MSC assay in the presence of pure Ep at a concentration of 20 mU/mL. The amount of Ep measured in the assay wells was then compared with the predicted amount of endogenous (previously determined) plus exogenous Ep (25 mU/mL) present. As shown in Table 1, values ranged from 99% to 114%, with an average value (±SD) of 106% ± 4%, demonstrating no significant difference between expected (calculated) and measured Ep levels. This, in turn, suggested that inhibitors were not present in the magnetic bead eluates. This was substantially different

Table 1. Additivity of Endogenous Plus Exogenous Ep in the Magnetic Bead Assay

Plasma	Endogenous Ep* (mU/mL)	Exogenous Ep (mU/mL)	Total Ep Calculated (mU/mL)	Total Ep Measured (mU/mL)	Recovery (%)
1	10.0	25	35.0	34.5	99
2	7.4	25	32.4	35.8	110
3	8.9	25	33.9	37.2	110
4	16.4	25	41.4	43.2	104
5	20.0	25	45.0	45.9	102
6	15.5	25	40.5	43.2	107
7	18.9	25	43.9	46.6	106
8	18.5	25	43.5	45.9	106
9	13.5	25	38.5	39.9	104
10	28.4	25	53.4	60.8	114

*Endogenous Ep values were calculated from results similar to those shown in Fig 3, assuming a recovery of 50% through the magnetic bead procedure.

from results obtained with these plasma samples before magnetic bead treatment. The untreated plasmas showed varying degrees of nonparallelism and no additivity (data not shown). These results indicate that the magnetic bead bioassay procedure is specific for Ep.

To determine the accuracy of the magnetic bead assay, a serum sample obtained from an aplastic anemia patient was assayed in quintuplicate in the same assay. The mean Ep titer ± SEM was 489 ± 9 mU/mL, and from these data a value of 4% was obtained for the intra-assay coefficient of variation (CV). A different serum sample was also tested in five different assays to study inter-assay variation. The mean Ep titer ± SEM was 822 ± 37 mU/mL and the inter-assay CV was 10%.

To substantiate the validity of this magnetic bead assay, the relationship between magnetic bead assay-determined Ep levels and hemoglobin concentrations was determined for 33 blood samples from normal donors and from anemic, nonuremic patients. As can be seen from Fig 4, there was a significant negative correlation with a correlation coefficient of 0.79 ($P < .001$).

Ep levels were then measured in plasma samples from 32 healthy donors. As shown in Table 2, Ep levels ranged from 5.9 to 39.2 mU/mL with a mean value (±SD) of 16.6 ± 7.9 mU/mL. Ep levels in female donors were not significantly different from those in male donors (Table 2).

Comparison with RIA and ELISA. To further substantiate the validity of this new bioassay, titers of biologically active Ep were determined for 22 serum samples from patients with either leukemia or lymphoma at various stages of treatment with chemotherapy, and the results were compared with Ep titers measured using the competitive RIA and the recently developed Ep ELISA.[4] As shown in Fig 5 (top), a significant correlation was observed when the Ep titers measured in the magnetic bead assay were compared with those measured using the Ep RIA, although titers determined with the RIA were consistently lower. The equation for the logarithmic transformation of the data was $y = 0.85x + 0.19$, and the correlation coefficient was 0.98. Correlation was also determined between the magnetic bead assay and the Ep ELISA. As shown in Fig 5 (bottom), the correlation was not as good, with an equation for the log-transformed data of $y = 1.04x - 0.32$ and a correlation coefficient of 0.91.

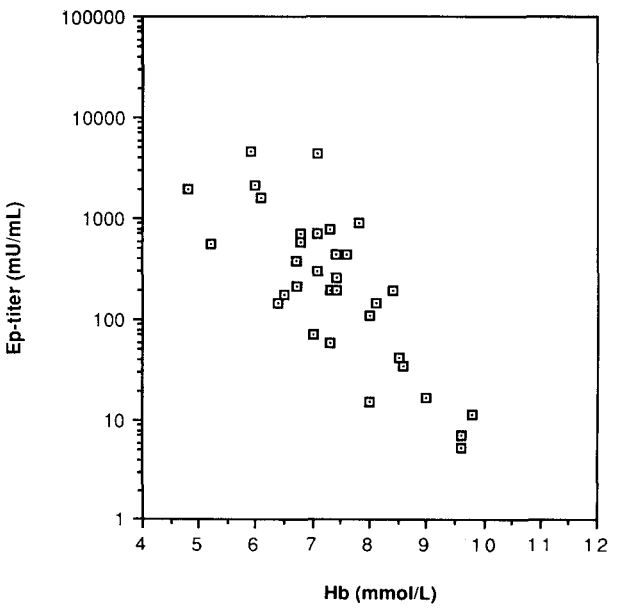

Fig 4. Relationship between magnetic bead assay determined Ep levels and hemoglobin levels in blood samples obtained from 33 patients with hemoglobin levels between 4.8 and 9.6 mmol/L.

Table 2. Plasma Ep Titers in Normal Human Subjects

Sex	N	Range (mU/mL)	Mean ± SD (mU/mL)
M + F	32	5.9-39.2	16.6 ± 7.9
M	15	6.3-28.4	15.5 ± 6.2
F	17	5.9-39.2	17.6 ± 9.5

DISCUSSION

In this report we present a method for pretreating human serum or plasma with an immobilized high affinity MoAb to Ep to permit the accurate assessment of biologically active Ep. Several technical features contribute to the specificity, reproducibility, and sensitivity of this assay. The specificity is, of course, contributed by the high-affinity MoAb to Ep that we have produced and characterized in detail previously.[19,23] The use of magnetic beads allows for a more reproducible and efficient elution of Ep in smaller and better-controlled volumes than would be possible using nonmagnetic supports (eg, Sepharose beads; Pharmacia, Uppsala, Sweden), which require centrifugation. In addition, the use of magnetic polystyrene beads as the solid support for anti–Ep-26 and the use of a magnetic separator capable of holding up to 60 tubes simplifies the assay procedure by allowing the testing of large numbers of samples in a single assay with relative ease and speed. Also, the use of RPMI culture medium, adjusted to pH 2, to elute antibody-bound Ep and neutralization of the pH of the eluate with 150 mmol/L HEPES, pH 9, allows direct testing of the eluates in the MSC assay in volumes up to 30 μL without prior dialysis or ultrafiltration. In preliminary experiments it was found that other buffers, such as pH 2 adjusted PBS and pH 2 glycine buffers, inhibited (after neutralization) ^{3}H-thymidine incorporation in the MSC assay, probably by influencing the osmolality of the assay medium. Finally, we found that the reproducibility of the assay was greatly improved by pretreating the magnetic beads before the addition of test sera with a pH 3.5 buffer to remove noncovalently bound antibody (see Materials and Methods). In this regard we discovered that while 0.2 mg of beads bound slightly more ^{125}I-Ep than 0.1 mg per sample, more noncovalently coupled antibody was released, and this had a detectable inhibitory effect when more than 20 μL of sample was tested in the MSC assay. Based on our finding that 0.1 mg of magnetic beads were capable of binding approximately 5 U (1.4 pmol) of Ep and that the kd for anti–Ep-26 is ≤0.7 nmol/L,[19] the theoretical binding maximum for the levels of Ep present in

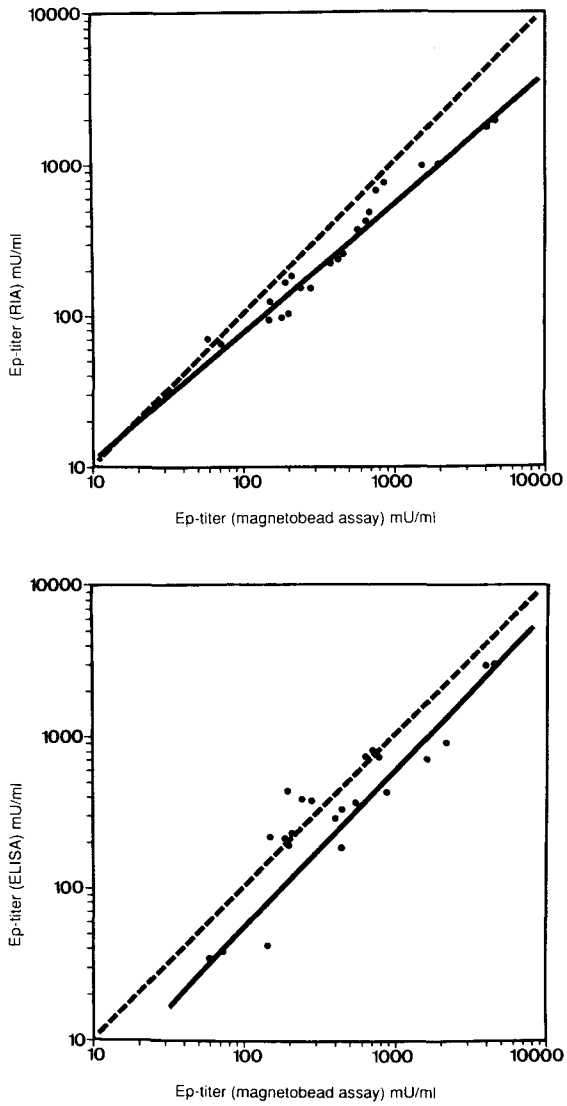

Fig 5. Correlation of magnetic bead assay results with results obtained from the RIA (top) and the ELISA (bottom) using 22 serum samples from patients with either acute myeloid leukemia or lymphoma.

normal serum is approximately 50%. This correlates very well with the actual recoveries of Ep through the magnetic bead procedure (ie, 45% to 55%). Moreover, the actual recovery is very consistent within each experiment and shows no significant sample-to-sample variation. Coupled with the 7.4-fold concentration of Ep as a result of the magnetic bead procedure, this concentrates the Ep in the original test sample three- to fourfold, resulting in a detection sensitivity (~0.25 mU/mL) high enough to measure subnormal Ep levels more accurately than with current immunoassays. Not unexpectedly, recoveries were substantially lower (20% to 30%) and somewhat more variable when the beads were washed more than once, after incubation with serum or plasma and before elution, and when the beads were allowed to remain in the 0.15 mol/L NaCl wash solution for more than 1 minute, since this resulted in some release of Ep into the wash solution due to re-equilibration.

The specificity of the magnetic bead bioassay procedure for Ep in serum and plasma was demonstrated by the parallelism of the dose-response curves of eluates derived from normal human plasma samples with the dose-response curve for pure recombinant Ep (Fig 3). Additional evidence for the specificity of this method for Ep was obtained from two other experiments. First, it was demonstrated that nonspecific stimulators of erythropoiesis were not present in the plasma eluates, as indicated by the ability of polyclonal anti-Ep antibodies to neutralize all stimulatory activity in the plasma eluates. Second, it was concluded from additivity experiments that inhibitors of cell growth also were not present in these eluates (Table 2).

The validity of the magnetic bead assay for measuring Ep levels in serum and plasma was also suggested by the negative correlation obtained between Ep titers determined using this assay and hemoglobin concentrations. Moreover, the high correlation between magnetic bead assay results and those obtained with the competitive RIA for Ep, using sera from a group of patients with either leukemia or lymphoma, was an additional indication of the validity of the magnetic bead assay for measuring biologically active Ep in serum. Surprisingly, however, RIA levels were consistently lower than those measured with the magnetic bead assay in the same serum samples, as evidenced by the regression line with a slope unequal to one for the relation between the log-transformed Ep titers measured with these assays (Fig 5). If these sera contained biologically inactive, but immunoreactive aggregates or fragments of Ep, RIA titers would be expected to be higher instead of lower than the Ep titers measured with the magnetic bead assay. Further investigation will be required to determine whether the observed differences are caused by an overestimation of Ep levels measured with the magnetic bead assay or by an underestimation of Ep titers measured with the RIA. A comparison was also made between the magnetic bead assay and the Ep ELISA. Correspondence between these two assays was lower than between the magnetic bead assay and the RIA, but the slope of the regression line for the log-transformed data was virtually equal to 1 (Fig 5), suggesting that, in general, very similar results are obtained with the magnetic bead assay and the ELISA.

Our finding that Ep levels are not significantly different in males and females is in agreement with several other studies using the RIA.[2,3,24] However, Garcia et al,[1] also using an RIA, found higher levels in female donors while de Klerk et al[12] measured significantly higher levels in males using a modified FMLC bioassay. Because of the small sample size used in our study (15 males and 17 females), further studies will be required to resolve this issue.

In conclusion, we have modified the standard in vitro bioassay measuring ^{3}H-thymidine incorporation into spleen cells from phenylhydrazine-treated mice to more specifically measure biologically active Ep in human serum and plasma. Anti–Ep-26, the MoAb used in this assay to extract Ep from the test samples, is highly specific for human Ep and does not

cross-react with murine and ovine Ep.[23] For this reason, the magnetic bead assay can be particularly useful in studying the metabolism and clearance of human rEp after injection into laboratory animals without interference by endogenous Ep. In the clinic, this novel assay may be a valuable alternative to immunoassays for measuring levels of biologically active Ep in patients with hematologic disorders, and to monitor these levels before, during, and after treatment of patients with rEp.

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