An Enzyme-Linked Immunosorbent Assay for Erythropoietin Using Monoclonal Antibodies, Tetrameric Immune Complexes, and Substrate Amplification

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We recently reported the development of several monoclonal antibodies (MoAbs) to native human erythropoietin (Ep). In the present study we have used the two antibodies with highest affinity to develop a two-sided or sandwich enzyme-linked immunosorbent assay (ELISA) to measure Ep in human serum. In this assay Ep is incubated in microtiter wells precoated with the first (IgE) anti-Ep antibody. Assay wells are then incubated with the second (IgG) anti-Ep antibody, which is labeled noncovalently with the enzyme alkaline phosphatase (AP) by means of bispecific tetrameric antibody complexes consisting of IgG, anti-Ep cross-linked to IgG, anti-AP using rat MoAbs specific for mouse IgG. Application of this noncovalent labeling procedure, in combination with substrate amplification, results in a detection sensitivity of 0.5 to 1.0 mU/sample (5 to 10 mU/mL), which makes this assay suitable for measuring normal serum Ep levels. The validity of this ELISA for quantitating Ep in biological fluids was demonstrated by the parallelism obtained between pure recombinant Ep dose-response curves and those obtained with plasma and serum from healthy donors and patients with various hematologic disorders. Normal plasma Ep levels detected with this ELISA ranged from 9 to 101 mU/mL with a mean of 32 ± 23 (SD) mU/mL. Ep levels in sera from patients with polycythemia were in the low to normal range, whereas Ep levels in sera from patients with secondary polycythemia and patients with aplastic anemia were moderately to strongly elevated. These results demonstrate that the Ep-ELISA is a sensitive, reliable, and nonradioactive immunologic method for quantitating Ep levels and should prove useful in a variety of clinical and laboratory settings.

Erythropoietin (Ep) is the major in vivo regulator of RBC production and is produced, primarily in the kidney, in response to hypoxia in arterial blood. Serum Ep levels are normally increased by hypoxia and decreased by hyperoxia, and an inverse relation has been observed between hemoglobin concentration and Ep plasma levels. In some disorders, such as the anemia of renal disease, Ep levels are abnormally low with respect to the degree of anemia, whereas in other diseases Ep is overproduced. To diagnose and to investigate the role of Ep in hematologic disorders, it is important to obtain accurate measurements of Ep serum levels. Although in vivo and in vitro bioassays have been used to measure Ep levels in serum samples, these assays are not suitable for measuring normal to low levels of the hormone because of the presence of nonspecific stimulators and inhibitors in many sera. The validity of radioimmunoassays (RIAs) to measure elevated, normal, and even subnormal settings. should prove useful in a variety of clinical and laboratory settings.

MATERIALS AND METHODS

Purification of Ep. Urinary Ep (uEp) was purified from the urine of aplastic anemia patients as described. Recombinant human Ep (rEp) was purified by a similar, though simpler procedure, involving CM Affi Gel Blue chromatography and phenyl-HPLC of the culture supernatant from Baby Hamster Kidney cells stably transfected with the human Ep gene. The Ep preparations were calibrated against the Second International Reference Preparation using an in vitro bioassay measuring Ep-dependent H-thymidine incorporation into spleen cells from phenylhydrazinetreated mice, as described previously.

MoAbs. The production and characterization of the mouse MoAbs anti-Ep-16 and anti-Ep-26 have been described. The preparation of mouse MoAb to the enzyme AP (CLB-AP 2.84) and the rat MoAb specific for mouse IgG (CLB-mIgG) have also been reported. Mouse and rat IgG antibodies were purified to homogeneity by affinity chromatography over protein-A-Sepharose according to procedures described by Eym et al. Mouse anti-Ep 26 was affinity purified using a column of rat MoAbs specific for mouse Ig-kappa chains coupled to Sepharose 4B (Pharmacia, Dorval, PQ).
ELISA FOR ERYTHROPOIETIN

For some experiments crude preparations of rat antimouse IgG and anti-AP MoAbs were used instead of purified preparations. Assay results for pure and crude antibody preparations were essentially identical. Prior to use all antibody preparations were centrifuged at 14,000 g for five minutes to remove aggregates.

Preparation of anti-Ep anti-AP/AP tetrameric complexes. The tetrameric antibody complex technology developed by Lansdorp et al23 was used to enzyme label anti-Ep-16 while retaining its antigen-binding properties. IgG anti-Ep, at a concentration of 1 µg/mL, was mixed with IgG anti-AP antibodies at a concentration of 4 µg/mL, and immune complexes were then generated by the addition of rat antibodies specific for mouse IgG, at a concentration equimolar to the total concentration of mouse antibodies. The antibody ratio of 1:4:5 was used to ensure that more bispecific anti-Ep/anti-AP complexes were formed than monospecific anti-Ep/anti-Ep complexes and was found to give optimal results. The complexes were enzyme labeled by incubation with pure AP (Type XXX-T, Sigma, St Louis) at a concentration of 6 µg/mL. Similar results were obtained when crude AP (type 1, Sigma) at a concentration of 10 mg/mL, was instead used of the pure enzyme.

ELISA for Ep. Anti-Ep-26 was immobilized covalently in 96-well microtiter plates (Cobind, Micromembranes, Newark, NJ) by incubating each well with 100 µL anti-Ep-26 at 5 µg/mL in 10 mmol/L sodium phosphate, pH 7.4, 0.14 mol/L NaCl (PBS). After 18-hour incubation at room temperature, the wells were rinsed twice with PBS and incubated for one to two hours with a 1% (wt/vol) solution of bovine serum albumin (BSA; Sigma) in PBS to block unreacted sites. Wells were then rinsed twice with 10 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl (TBS), and used immediately. Pure AP or test samples were diluted in TBS containing 1% (wt/vol) BSA, 0.02% (wt/vol) sodium azide, and 0.1% (vol/vol) Tween 20. One hundred-microliter aliquots were added to triplicate wells of anti-Ep-26–coated assay plates and incubated at room temperature for a period of six hours. Plates were then washed four times with TBS and 100 µL of preformed anti-Ep-16 anti-AP/AP tetrameric immune complexes (see above) added to the wells. Plates were incubated overnight, washed four times with TBS, and bound AP activity measured. In some experiments a one-step procedure was used: 50 µL aliquots of Ep were added to anti-Ep-26–coated wells together with 50 µL of anti-Ep-16 anti-AP/AP tetrameric complexes containing anti-Ep-16 at a concentration of 2 µg/mL. Plates were then incubated for a minimum period of five hours, washed four times with TBS, and enzyme activity measured.

Measurement of AP activity. AP activity in the assay plates was measured using the substrate amplification system described by Selig12 and Stanley et al,29 with some modifications. Briefly, 100 µL of 0.1 mmol/L nicotinamide adenine dinucleotide phosphate (NADP, Boehringer, Mannheim, FRG) in 50 mmol/L diethanolamine buffer, pH 9.5, containing 1.0 mmol/L MgCl₂, was added to each well and incubated at room temperature for ten minutes. One hundred microliters of amplifier consisting of 0.165 mg/mL alcohol dehydrogenase (ADH, Sigma, No. A3263), 0.165 mg/mL diaphorase (Sigma No. D2381), and 1 mL L-p-iodonitro-tetrazolium-violet (INT-violet, Sigma, No. 18377) dissolved in 25 mmol/L Na phosphate buffer, pH 7.0, containing 3% ethanol, was then added, and color development was allowed to proceed for two to three minutes. The reaction was stopped by the addition of 50 µL of 0.2 mol/L sulphuric acid, and the absorption at 490 nm (A₄₉₀) was measured in an ELISA plate reader (Biotek Instruments, Winooski, VT). A₄₉₀ measurements from wells incubated with substrate and amplifier alone ranged from 0.09 to 0.12 and were subtracted from the A₄₉₀ measured in the assay wells.

The conventional substrate, p-nitrophenylphosphate (PNPP, Sigma No. 104-0), was used at a concentration of 5.0 mmol/L in 1 mol/L diethanolamine buffer, pH 9.8, containing 1.0 mmol/L MgCl₂. Each well received 100 µL of PNPP solution and was incubated for 20 minutes at room temperature after which 100 µL of 2 mol/L sodium hydroxide was added to stop the reaction. Color development was measured at 405 nm.

RIA for Ep. The competitive RIA for Ep was performed according to published procedures29,30 with some modifications. Briefly, pure Ep or test samples (300 µL/tube) diluted in 50 mmol/L sodium phosphate, pH 7.5, containing 5% BSA and 0.02% sodium azide were mixed with 100 µL of a 1:4,000 dilution of rabbit anti-Ep antisum. The mixture was incubated for three days at 4°C before the addition of 100 µL 125I-labeled Ep (15,000 cpm) in 50 mmol/L sodium phosphate, pH 7.5, containing 1% BSA, and incubated for three days. One hundred twenty-five microliters goat antirabbit IgG (Calbiochem, LaJolla, CA) and 100 µL of a 1:30 dilution of normal rabbit serum were added to each tube to precipitate immune complexes, and after 20-hour incubation at 4°C, precipitates were collected by centrifugation and counted in a Beckman 5500 γ-counter. Anti-Ep antisum was obtained by immunizing rabbits with pure human uEp (specific activity ~80,000 U/mg).24 Pure rEp (specific activity: ~100,000 U/mg) was labeled with 125I to a specific activity of 900 to 2,700 Ci/mmol using the chloramine T method.21

Determination of epitope specificity for anti-Ep-16 and -26. The capacity of the two anti-Ep MoAbs to inhibit each other's binding to Ep was tested in 96-well assay plates coated with anti-Ep-16 or -26 (1 µg/mL in PBS, overnight incubation, 23°C). Non-specific binding was blocked by incubation with 1% BSA in PBS (two hours, 23°C). The wells were then incubated overnight with 125I-labeled Ep (40,000 cpm) in buffer alone (PBS+1% BSA) or in the presence of increasing concentrations of anti-Ep-16 or -26 diluted in the same buffer. After washing with PBS (four times), bound radioactivity was eluted from the wells with 100 µL of 2 mol/L NaOH, transferred to polystyrene tubes, and counted in a γ-counter.

Serum and plasma samples. Plasma samples were obtained from 18 normal subjects. Serum samples were obtained from six aplastic anemia patients with hemoglobin levels ranging from 85 to 110 g/L, seven secondary polycythemia patients with hemoglobin levels ranging from 162 to 208 g/L, and two polycythemia vera patients with hemoglobin levels of 159 and 200 g/L. Urine samples were obtained from 11 aplastic anemia patients screened previously for the presence of elevated Ep levels by in vitro bioassay.4

RESULTS

Epitope specificity of anti-Ep MoAbs. To develop a sandwich immunoassay for Ep in which Ep is first bound to an immobilized anti-Ep antibody and then detected by binding of a second enzyme-labeled anti-Ep, it was important to establish that the two anti-Ep MoAbs chosen for this assay were specific for different epitopes on the Ep-molecule and did not inhibit each other's binding to Ep. As shown in Fig 1, soluble anti-Ep-26 at concentrations as high as 50 µg/mL was not capable of inhibiting the binding of 125I-Ep to immobilized anti-Ep-16, nor was soluble anti-Ep-16 capable of inhibiting 125I-Ep binding to immobilized anti-Ep-26. As expected, binding of 125I-Ep to the immobilized antibodies was inhibited when the homologous antibodies were added, even at concentrations as low as 0.5 µg/mL (Fig 1). These results demonstrated that anti-Ep-16 and -26 were capable of binding to the same Ep-molecule and could thus be applied to a sandwich-immunoassay for human Ep.

ELISA procedure and sensitivity. A recently described method for the noncovalent enzyme labeling of murine IgG,
shown in Fig 2B, application of the amplification system to the system developed by Self et al and Stanley et al (Fig 2A). As captured Ep then binds the preformed anti-Ep-16/anti-antibodies by means of tetrameric antibody complexes was washed. and the radioactivity bound to the wells was measured. Each point is the mean ± SE of triplicate samples. Bars are not shown where the SE was smaller than the symbol used to represent the mean.

antibodies by means of tetrameric antibody complexes was used to obtain an anti-Ep-enzyme conjugate that fully retained its antigen-binding properties. The principle of the Ep-ELISA is outlined schematically in Fig 2A. Ep is first captured by immobilized anti-Ep-26 in microtiter wells. The captured Ep then binds the preformed anti-Ep-16/anti-AP/AP tetrameric antibody complexes, resulting in the formation of a sandwich consisting of immobilized anti-Ep-26, Ep, and enzyme-labeled anti-Ep-16. Bound AP activity is then detected by applying the substrate amplification system developed by Self and Stanley et al (Fig 2A). As shown in Fig 2B, application of the amplification system to the Ep-ELISA resulted in a significant increase in signal intensity and detection sensitivity over that achievable with the conventional substrate PNPP. When PNPP was used as substrate, the detection limit, defined here as twice the SD above the mean of the background signal, was only 50 mU/mL. However, using the substrate amplification procedure, this limit dropped to 5 mU/mL (0.5 mU per sample), indicating a tenfold increase in sensitivity over that achievable with PNPP (Fig 2B).

Specificity of the Ep-ELISA. To demonstrate the specificity of the Ep-ELISA for Ep, assays were performed using bispecific tetrameric antibody complexes in which anti-Ep-16 was replaced with an irrelevant IgG, antibody and by using monospecific anti-AP/anti-AP/anti-AP tetrameric complexes. No signal significantly above background was obtained in either of these experiments (data not shown). In addition, a significant signal was also not obtained when the assay was performed in wells not coated with anti-Ep-26. These results indicated that color development in the Ep-ELISA was strictly dependent on the specific immunologic interaction of Ep with both anti-Ep MoAbs.

To further establish the specificity of the ELISA for Ep, serum and urine obtained from aplastic anemia patients and containing 3.3 U/mL and 7.6 U/mL, respectively, were tested in the ELISA after preincubation with saturating amounts of polyclonal rabbit anti-Ep antibodies. The rabbit antiserum, at a dilution of 1:10, completely blocked color development in the ELISA, indicating that the only ELISA-reactive molecule in the serum and urine was Ep itself. An additional proof of specificity was provided by experiments in which aplastic anemia serum was subjected to size-exclusion chromatography on Sephadex G-150 and ELISA-positive fractions were found to co-elute with a pure 125I-Ep (data not shown).

As shown in Fig 3, dose-response curves obtained with pure rEp (specific activity = 100,000 U/mg) and partially purified uEp (TFL Step I; specific activity = 1,000 U/mg) were virtually superimposable, demonstrating that native and recombinant Ep react identically in the Ep-ELISA.

Accuracy of the Ep-ELISA. The intra-assay coefficient of variation (CV) was calculated by carrying out dose-response curves using pure Ep. When the intra-assay CV of the dose-response curves from six experiments were averaged, a precision profile, as shown in Fig 4, was generated. The intra-assay CV ranged from 2.1% to 18.5%, with highest variance being, as expected, in the low Ep-concentration range.

The accuracy of the Ep-ELISA in the presence of human plasma was determined by serially diluting rEp in 50% human plasma (containing a known concentration of Ep, as determined by both RIA and ELISA). The measured Ep levels were then compared with the theoretic Ep levels contributed by both the added rEp and the Ep in the plasma. As shown in Table 1, measured Ep levels varied from 84% to 94% of expected values with a mean of 90% ± 4% (SD), similar to results reported for the Ep-RIA.1022 Ep measurements were also carried out using plasma concentrations of 25% and 10%. The mean percentage recovery in these experiments was 94 ± 8 and 104 ± 10, respectively. These results indicate that Ep can be measured with acceptable accuracy in the presence of high concentrations of human plasma.

Ep levels in urine, serum, and plasma samples. Sera from three patients with aplastic anemia, one patient with secondary polycythemia, and plasma from one healthy donor were titrated in the Ep-ELISA. As shown in Fig 5, Ep could be readily detected in these samples using the ELISA. The serum dose-response curves were parallel with the pure Ep dose-response curve, and Ep titers could be calculated from the displacement of the serum curves from the pure Ep curve.
Fig 2. (A) Schematic representation of the ELISA procedure. Anti-Ep-26, an IgE antibody immobilized in microtiter wells, is used to capture Ep. Preformed anti-Ep-16/anti-AP/AP bispecific tetrameric antibody complexes bind to the captured Ep after which the bound AP activity is detected enzymatically using the substrate amplification procedure described by Seft.19 (B) Dose-response curves for pure rEp titrated in the ELISA using two different substrates to measure bound AP activity. Enzyme activity assayed with the substrate amplification system was measured by the absorbance of the reaction product at 490 nm (●). The reaction product of the conventional substrate PNPP was measured by its absorption at 405 nm (○). Results are expressed as mean ± SE of triplicate wells.

The Ep titers presented in Table 2 are the mean from the Ep titers obtained from Fig 5 and from two to three additional assays for each sample, and the standard errors (SE) therefore indicate interassay variation. As shown in Table 2, the interassay CV varied from 8.4% to 34.8%. To compare the ELISA with the RIA, the same five samples were also quantitated in the RIA, using rabbit anti-Ep antiserum and 125I-Ep as tracer (Table 2). The RIA results corresponded well with those obtained with the ELISA.

Ep titers in the plasma of 18 normal donors, as measured in our ELISA, ranged from 9 to 101 mU/mL (Fig 6, column 4). The mean ± SD was 32 ± 23 mU/mL, comparable to, although somewhat higher than, the 15 to 25 mU/mL mean values for normal plasma or serum Ep levels reported with the RIA.21,22,23 To study the usefulness of the Ep-ELISA in the differential diagnosis of anemias and polycythemias, sera from two patients with polycythemia vera, seven patients with secondary polycythemia, and six patients with aplastic anemia were also tested in this assay. The Ep levels measured in the serum of patients with polycythemia vera were within the normal range (Fig 6, column 3, open symbols) but were lower than the mean for normal donors. Serum Ep titers in the patients with secondary polycythemia ranged from 30 to 1,300 mU/mL (lane 3; closed symbols) with a mean value (ie, 291 mU/mL) that was significantly above the normal plasma mean (P < .001, one-tailed t test). As expected, Ep titers for the aplastic anemic sera were also markedly elevated when compared with normal values (column 2). Ep levels in these sera ranged from 89 to 3,260 mU/mL, with a mean value of 1,122 mU/mL. The Ep-ELISA was also used to measure Ep levels in urine samples from 11 aplastic anemia patients. ELISA titers in these urines ranged from 0.4 to 17.4 U/mL (column 1), and a highly significant correlation (y = 0.94x + 0.19; r = .94) was observed when the ELISA-determined Ep titers were compared with Ep titers determined by the RIA. However, a lower correspon-
staining efficiencies at least as good as those achievable with covalently labeled antibodies,13,24 and in this article we show that this technique can also be used to enzyme label antibodies for use in immunoassays. Application of the tetrameric antibody technique yielded directly labeled anti-Ep antibodies, which, because of the noncovalent nature of the cross-linking, fully retained their antigen-binding capacity. Additional advantages of this noncovalent labeling procedure, as compared with covalent antibody-enzyme conjugates, are the lack of batch-to-batch variation, the simplicity of conjugate formation, and the possibility of using crude enzyme and antibody preparations.

The second important feature of this Ep-ELISA is the use of a substrate amplification system for the enzyme AP.19,20 This system amplifies the amount of reaction product generated per molecule of AP, resulting in increased detection sensitivities over those achievable with conventional substrates, as has previously been demonstrated in assays for thyroid stimulating hormone (TSH) and progesterone.20 The combination of these two important features with two of the anti-Ep MoAbs that we have isolated12 allowed us to develop a sensitive and nonradioactive procedure that could be used to quantitate Ep levels in human serum, plasma, and urine.

The validity of the Ep-ELISA to measure Ep levels in biological fluids was demonstrated in several ways. First, the

DISCUSSION

In this report we describe the development of a sensitive ELISA procedure for human Ep. Apart from the application of MoAbs, the assay has two important features. First, enzyme labeling of the detecting antibody was not achieved by covalent conjugation techniques but by the formation of noncovalent tetrameric antibody complexes. We have previously demonstrated that this tetrameric antibody technique can be used for the enzyme and fluorochrome labeling of antibodies specific for cell-surface antigens, resulting in staining efficiencies at least as good as those achievable with covalently labeled antibodies,13,24 and in this article we show that this technique can also be used to enzyme label antibodies for use in immunoassays. Application of the tetrameric antibody technique yielded directly labeled anti-Ep antibodies, which, because of the noncovalent nature of the cross-linking, fully retained their antigen-binding capacity. Additional advantages of this noncovalent labeling procedure, as compared with covalent antibody-enzyme conjugates, are the lack of batch-to-batch variation, the simplicity of conjugate formation, and the possibility of using crude enzyme and antibody preparations.

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Table 1. Measurement of Ep in 50% Human Plasma

<table>
<thead>
<tr>
<th>50% Human Plasma</th>
<th>Exogenous Ep</th>
<th>Total Ep</th>
<th>Total Ep</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mU/mL)</td>
<td>Present/Sample (mU/mL)</td>
<td>Measured/Sample (mU/mL) %</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Exogenous Ep/Sample (mU/mL)</td>
<td>500</td>
<td>517</td>
<td>460</td>
<td>89</td>
</tr>
<tr>
<td>17</td>
<td>250</td>
<td>267</td>
<td>250</td>
<td>94</td>
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<td>91</td>
</tr>
<tr>
<td>17</td>
<td>8</td>
<td>25</td>
<td>21</td>
<td>84</td>
</tr>
</tbody>
</table>

rEp was serially diluted in 50% human plasma (assayed previously both by RIA and ELISA; levels were 39 and 35 mU/mL in these assays, respectively) and tested using rEp diluted in buffer as standard. The recovery was calculated as the percent of the total Ep present in each sample measured by ELISA.
Serum Dilution

Table 2. Comparison of the ELISA With the RIA for Measuring Ep Titers in Biologic Fluids

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of Assays (ELISA)</th>
<th>Mean ± SE (mU/mL)</th>
<th>CV (%)</th>
<th>No. of Assays (RIA)</th>
<th>Mean ± SE (mU/mL)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>3260 ± 140</td>
<td>8.4</td>
<td>6</td>
<td>2950 ± 238</td>
<td>19.8</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1750 ± 200</td>
<td>22.7</td>
<td>6</td>
<td>1161 ± 146</td>
<td>30.8</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>360 ± 30</td>
<td>15.0</td>
<td>4</td>
<td>458 ± 24</td>
<td>10.5</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>42 ± 8</td>
<td>34.8</td>
<td>2</td>
<td>49 ± 3</td>
<td>8.7</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>34 ± 2</td>
<td>13.1</td>
<td>6</td>
<td>35 ± 4</td>
<td>28.0</td>
</tr>
</tbody>
</table>

ELISA titers shown are the mean ± SE of three to four separate assays, one of which is presented in Fig 5.

RIA titers shown are the mean ± SE from two to six experiments for each sample. All samples were tested at two to four doses.

Fig 5. Comparison of dose-response curves for pure Ep (O) with that for sera from three aplastic anemia patients (samples 1 to 3), one secondary polycythemia patient (sample 4), and plasma from a normal donor (sample 5). Results are the mean ± SE of triplicate wells.

Ep-ELISA was found to have a lower limit of detection of 0.5 to 1.0 mU of Ep per sample, or 5 to 10 mU/mL, which means that it could be used to measure Ep levels in most serum samples without the need for concentrating the serum first. Second, dilution curves of urine and serum samples from patients with aplastic anemia and other hematologic disorders were parallel with dose-response curves generated with pure Ep. Third, the Ep-ELISA could be used to discriminate between the low Ep levels in sera from polycythemia vera patients and the elevated levels in sera from secondary polycythemia and aplastic anemia patients, thereby demonstrating the clinical usefulness of the assay. Finally, Ep titers measured with the Ep-ELISA corresponded well with those measured with the Ep-RIA, in which polyclonal antibodies against Ep are used.

The Ep-ELISA provides some clear advantages over the Ep-RIA. The titer and affinity of the polyclonal antibodies to Ep in rabbit antisera may vary from batch to batch. In addition, the specificity of the Ep-RIA can be affected by the presence of anti-Ep antibodies that are cross-reactive with plasma proteins other than Ep, and this can result in unreliable measurements, unless these antibodies are removed by adsorption to normal human serum proteins.0 In contrast, the MoAbs used in the ELISA are of constant quality and affinity and are monospecific for Ep,12 properties that make this new assay very suitable for standardizing Ep measurements between laboratories. An additional advantage of the Ep-ELISA as compared with the Ep-RIA is its relative speed. Whereas RIA procedures usually require incubation periods of at least one day25 and often 4 to 10 days,26,27 the Ep-ELISA can be completed within 24 hours or, when maximum sensitivity and accuracy are not required, even within a few hours.

Despite the monospecificity of the MoAbs used in the
Ep-ELISA, a small number of serum and plasma samples displayed a lack of parallelism between their dose-response curves and that of pure Ep when serum or plasma samples were tested at high doses in the one-step procedure, in which the Ep sample and the tetrameric complexes are added at the same time (data not shown). However, parallel curves were obtained with most of these samples when the ELISA was performed as a two-step procedure in which the incubation steps with the sample and anti-Ep/anti-AP/AP complexes were separated by a washing step or when the samples were adsorbed on murine IgG,-Sepharose beads, suggesting that the interference was caused by human antibodies capable of binding to mouse immunoglobulins.

As with the RIA,10,22 the accuracy of the ELISA to measure subnormal Ep levels is less than ideal (Fig 4). Application of an IgG, MoAb with a higher affinity for Ep than the anti-Ep-16 used in the present procedure could, once available, improve the accuracy of the ELISA for measuring low Ep concentrations by further increasing the detection sensitivity of the assay. Nevertheless, our results clearly demonstrate that the Ep-ELISA in its present form has a sufficiently high sensitivity and accuracy to allow measurement of Ep levels that are within the normal range. For this reason the Ep-ELISA should prove useful as a simple, rapid, and nonradioactive alternative to bioassays and RIA procedures for quantitating human Ep. The further development and application of this ELISA and additional nonradioactive Ep assays based on MoAb technology is especially important, since therapeutic application of Ep for the treatment of patients with a variety of hematologic disorders and the accompanying assessment of Ep levels in these patients is likely to become more widespread in the near future.

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An enzyme-linked immunosorbent assay for erythropoietin using monoclonal antibodies, tetrameric immune complexes, and substrate amplification

AW Wognum, PM Lansdorp, AC Eaves and G Krystal