Rapid and Sensitive Method for Erythropoietin Determination in Serum

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An enzyme-linked immunosorbent assay (ELISA) method for erythropoietin determination has been established by using two kinds of monoclonal antibodies with specific affinities to erythropoietin. Besides being rapid (2.5 h) and sensitive (detection limit 0.3 int. unit/L), the present method gives accurate results and is easy to perform. The method may be clinically applicable for discriminative diagnosis of polycythemia and analyses of various anemic conditions.

Addtional Keyphrases: enzyme-linked immunosorbent assay · anemia · polycythemia

Measuring erythropoietin concentration in serum is useful for evaluating various anemic conditions, for making a discriminative diagnosis of polycythemia, and for monitoring erythropoietin administered as a drug (1–5). Various assay methods for this have been developed: in vivo methods such as the polycythemic (extrapoxic or blood-transfused) mouse assay (6–8), in vitro methods such as the mouse fetal-liver cell assay (9–11), radioimmunoassay (RIA) with natural erythropoietin (12–15), and RIA with recombinant erythropoietin (16–19). However, a well-equipped laboratory and relatively large amounts of sample (100–200 μL) are required for RIA, which also involves the use of a radioisotope. Furthermore, most RIAs take from several hours to a few days to quantify erythropoietin: 3 days (4); 5 days, with a sensitivity (detection limit) of 6.6 international units (IU)/L (14); 3 days, with a sensitivity of 3.0 IU/L (17); 3 days, with a sensitivity of 2.1 IU/L (18); 2 days, with a sensitivity of 3.0 IU/L (19); and 4 h, with a sensitivity of 1.0 IU/L (Epo-Coatria; BioMérieux, Lyon, France).

Here we present a very rapid and sensitive enzyme-linked immunosorbent assay (ELISA) involving two kinds of monoclonal antibodies, which have high and specific affinities to erythropoietin.

Materials and Methods

Materials

Bovine serum albumin, sodium citrate, potassium dihydrogen phosphate, disodium hydrogen phosphate, o-phenylenediamine dihydrochloride, Tween 20, hydrogen peroxide, and sulfuric acid were from Nakarai Chemical Co. (Kyoto, Japan). Horseradish peroxidase (grade I-C) was obtained from Toyobo Co., Ltd. (Osaka, Japan). Microplates were obtained from Costar Corp. (Cambridge, UK).

Purified erythropoietin from human urine was obtained from Toyobo Co. Beta-type erythropoietin and erythropoietin RIA Chugai were purchased from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Alpha-type erythropoietin was purchased from Sankyo Co., Ltd. (Tokyo, Japan). Erythropoietin WHO Second International Reference Preparation (no. 67/343) was purchased from the National Biological Standard Board (Herts., UK).

Antibodies. Spleen cells from a Balb/c mouse immunized with purified erythropoietin from human urine and osteoma cells (NS-1) were fused (20), and two hybridomas secreting anti-erythropoietin antibody were selected. Monoclonal antibodies MC-R-2 and MC-R-6 were purified from the culture supernate of individual hybridomas. The antibodies were obtained from Toyobo Co.

Preparation of solid phase. We added 1 μg of MC-R-6 monoclonal antibody in 100 μL of 0.3 mol/L carbonate buffer, pH 9.5, to each well and incubated the mixture for 2 h. After incubation, the plate was washed three times in phosphate-buffered saline (PBS) containing 0.5 mL of Tween 20 per liter (pH 7.2; Tween 20/PBS). We then added 200 μL of PBS containing 10 g of bovine serum albumin per liter (pH 7.2) to each well and incubated the plate for 2 h to block unbound sites. We dried the plate, packed it in an aluminum bag with desiccant, and stored it at <10 °C unfrozen. The microplate was stable under these conditions for one year.

Preparation of conjugate. The conjugate of MC-R-2 monoclonal antibody and horseradish peroxidase was prepared by the method described by Nakane and Kawaoi (21). The conjugate was diluted with PBS containing 50 mL of normal equine serum per liter (pH 7.2) and stored at <10 °C unfrozen. The conjugate was stable under these conditions for one year.

Standard. The standard solution was prepared by diluting purified erythropoietin from human urine with dilution buffer. Expression of the units was corrected for the WHO Second International Preparation (no. 67/343). The standard solution was stored at <10 °C unfrozen; it was stable for one year.

Color reagent. Color reagent was prepared just before use by dissolving 3 g of o-phenylenediamine dihydrochloride in 1 L of 0.1 mol/L citrate buffer, pH 5.7, containing 0.2 mL of hydrogen peroxide per liter.

Erythropoietin Assay

We added 25 μL of sample and 75 μL of conjugate to each well and incubated the plate for 2 h at 10–30 °C. We washed the plate three times with 300 μL of Tween 20/PBS. We then added 100 μL of the color reagent and allowed the enzyme reaction to proceed in the dark for 30 min at room temperature. We added 100 μL of 4 mol/L sulfuric acid to each well to stop the enzyme reaction and then measured absorbance at 490 and 650
nm. The erythropoietin concentration was obtained by using the calibration curve for $A_{490} - A_{660}$ for the standard.

Assay Validation Studies

Within-run precision was evaluated by analyzing 12 serum replicates in one run. Between-run precision was calculated by analyzing sera in seven different runs. To measure analytical recovery, we added a known amount of purified erythropoietin from human urine to serum obtained from healthy subjects, measured the total amount of erythropoietin, and calculated the recovery. To assess the linearity of the assay, we measured erythropoietin concentrations in serially diluted serum. We measured erythropoietin concentrations in sera containing potentially interfering compounds to examine the effect of such compounds. Known concentrations of $\alpha$- and $\beta$-types of recombinant erythropoietins were measured to examine the cross-reactivity. Correlation of our ELISA method with the conventional RIA method was examined by using sera from healthy subjects and from patients with various diseases. Erythropoietin RIA Chugai was used as the comparison method, according to the manufacturer's instructions.

Clinical Studies

Sera used to determine the normal reference value were obtained from healthy subjects who had a normal hematocrit value or a normal value for specific gravity of blood. The patients' sera used in clinical studies were obtained from Kyoto University Hospital, Hyogo College of Medicine, Osaka Red Cross Hospital, National Osaka Hospital, Osaka City University Hospital, and Tenri Hospital.

Results

Assay validation studies. A linear calibration curve was obtained between 0 and 140 IU of erythropoietin per liter (Figure 1, left). The detection limit of the present ELISA, determined as the erythropoietin concentration corresponding to the mean + 2 SD of five blank determinations, was 0.3 IU/L (Figure 1, middle). No high-dose "hook" effect was observed at erythropoietin concentrations up to 21 000 IU/L (Figure 1, right).

Within-run and between-run precisions are shown in Table 1. Mean analytical recoveries from three different sera were 99.5%, 96.1%, and 98.3%. Erythropoietin concentrations in dilutions of three different sera were linear (Figure 2).

We observed no interference with our method when reducing compounds (ascorbic acid ≤0.5 g/L and glutathione ≤0.5 g/L), anticoagulant compounds (heparin sodium salt ≤0.5 g/L, EDTA disodium salt ≤5 g/L, NaF ≤30 g/L, and oxalic acid ≤10 g/L), internal hemoglobin (≤4 g/L), bilirubin (≤0.2 g/L), or rheumatoid factor (≤100 IU/L) were added. Cross-reactivities of recombi-

| Table 1. Precision of Erythropoietin Determination by the Present ELISA |
|-----------------------------|---------------------|---------------------|
|                            | Mean    | SD     | CV, %    |
| Within-run (n=12)          |         |        |          |
| Serum 1                    | 10.01   | 0.32   | 3.2      |
| Serum 2                    | 59.15   | 0.57   | 1.0      |
| Serum 3                    | 121.54  | 1.71   | 1.4      |
| Between-run (n=7)          |         |        |          |
| Serum A                    | 29.69   | 0.74   | 2.5      |
| Serum B                    | 72.30   | 2.06   | 2.8      |
| Serum C                    | 128.27  | 2.37   | 1.8      |

Fig. 2. Dilution linearity of our ELISA method for three serum samples.
nant α- and β-type erythropoietin were 106% and 112%, respectively (data not shown). Correlation of the present ELISA (γ) and the conventional RIA (x) was $y = 0.807x - 6.136$ ($n = 52; r = 0.988, S_{yx} = 4.81$).

Clinical studies. We measured erythropoietin concentrations in 859 normal subjects and obtained a normal logarithmic distribution of erythropoietin concentrations: mean log value was 0.842 (SD 0.197); the 95% confidence interval was 2.8–17.2 IU/L (log values were 0.448–1.236) (Figure 3). In 155 patients with renal failure caused by chronic glomerulonephritis and diabetic nephrosis, 69 patients (44.5%) had serum erythropoietin concentrations lower than the lower normal reference value. Patients with aplastic anemia, iron-deficiency anemia, leukemia, myelodysplastic syndrome, malignant lymphoma, and myeloma had erythropoietin concentrations higher than the upper normal reference value (Figure 4).

In anemic patients without renal failure, we found an inverse logarithmic correlation between hematocrit (x) and erythropoietin concentrations (γ): in aplastic anemia, $\log y = -0.093x + 5.574$ ($n = 52, r = 0.887, P < 0.01$); in iron-deficiency anemia, $\log y = -0.092x + 4.381$ ($n = 39, r = 0.739, P < 0.01$); in patients with renal failure caused by chronic glomerulonephritis, $\log y = -0.015x + 0.896$ ($n = 129, r = 0.184, NS$) (Figure 5). In nine patients with polycythemia vera who were not receiving therapy, the mean erythropoietin concentration was 0.37 (SD 0.17) IU/L; in seven patients, the concentrations were less than the detection limit. Ten patients with secondary polycythemia who were not receiving therapy had erythropoietin concentrations of 10.07 (SD 14.10) IU/L (Figure 6).

Discussion

We established a very rapid (2.5 h) and sensitive (0.3 IU/L) ELISA for erythropoietin determination in serum with only 25 μL of sample. Precision, recovery, and linearity were excellent, and the assay was not disturbed by possible interfering compounds. The results also were highly accurate. The present ELISA correlated well with the conventional RIA ($r = 0.988$) but the ELISA-measured concentration was slightly lower than that obtained by the RIA (slope = 0.807).

The distribution of serum erythropoietin concentrations in 859 normal subjects could be regarded as a
normal logarithmic distribution rather than a normal distribution. Therefore, the calculated 95% confidence interval of erythropoietin concentration in serum was 2.8–17.2 IU/L. In the RIA, reported normal reference values include 15.2 (SD 5.5) (2), 21.9 (SD 12.0) (3), 14.9 (SD 3.3) (4), 14.9 (SD 4.7) (14), 13.3 (15), 19.6 (SD 15.6) (17), 22.5 (SD 4.4) (18), and 13.5 IU/L (19).

The reason why the slope of the equation for the correlation between our ELISA and the RIA was <1.0 and why the normal reference value by the ELISA tended to be lower than in the RIA may be the higher specificity of the two kinds of monoclonal antibodies used.

The serum erythropoietin concentration in patients with aplastic anemia, iron-deficiency anemia, hemolytic anemia, leukemia, myelodysplastic syndrome, malignant lymphoma, or myeloma was often above the upper normal reference value. For such cases, others have reported an inverse correlation between hematocrit and erythropoietin concentration (5, 6, 18, 19). We obtained a similar finding. Almost 50% of the patients with renal failure caused by chronic glomerulonephritis and diabetic nephrosis showed a concentration less than the lower normal reference value. Their erythropoietin concentrations were significantly lower than those observed in the above cases for a comparable hematocrit, and no significant correlation of erythropoietin with hematocrit was observed.

In patients with polycythemia vera, the erythropoietin concentration of most patients was below the detection limit. Patients with secondary polycythemia had a relatively higher concentration than did patients with polycythemia vera. These findings agree with those obtained by the RIA (19).

The present ELISA showed almost quantitative reactivity with the recombinant erythropoietins. Therefore, this ELISA may be used to monitor the concentrations of recombinant erythropoietins administered as a drug.

In conclusion, we consider the present ELISA useful for diagnosis of the anemic condition, for discriminative diagnosis of polycythemia, and for monitoring erythropoietin administered as a drug.

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