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Correlation between Bioassay and Radioimmunoassay for Erythropoietin in Human Serum and Urine Concentrates[1] (42099)

RUTH A. COHEN, GISELA CLEMONS, AND SHIRLEY EBBE[2]

Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720, and Department of Laboratory Medicine, University of California, San Francisco 94143

Abstract. Both immunoreactive erythropoietin (Ep) and biologically active Ep were measured in 23 samples of human serum and 21 concentrates of human urine. Immunoreactive Ep was measured by radioimmunoassay (RIA). Biological activity was determined in the plethoric mouse bioassay in which ^{59}Fe incorporation was converted to units of Ep from standard reference curves. Low values for Ep were determined from standard curves plotted as probits to improve sensitivity for levels of Ep as low as 30 mU/ml. Ep levels in 35 samples ranged between 30 and 1000 mU/ml by both assays; in 9 samples Ep was 15.2–37.5 mU/ml by RIA but was not detectable by bioassay. Analysis of the data for the 35 samples in which Ep could be measured by both assays showed a strong correlation between the values obtained by the two assays. These results indicate that the RIA used in these experiments detects biologically active Ep in human serum and urine when it is present in amounts only moderately higher than normal. The ultrafiltration method used for preparation of urine samples was effective in concentrating Ep in some urines, but the results were too erratic and nonquantitative to permit its use as a method for quantifying human urinary Ep excretion.

The development of radioimmunoassays (RIA) for erythropoietin (Ep) has expanded the potential clinical and research applications for measurement of Ep by introducing the possibility to accurately measure low, normal, and slightly elevated levels of Ep, in addition to the higher levels that could previously be detected by bioassay *in vivo*. However, when Ep is measured by RIA, it is possible that molecules not having the characteristic erythropoietic biological activity of Ep *in vivo* might be detected, depending on the specificity of the antibody used in the RIA. Therefore, it is important to compare the extent to which the two assays measure the same or different Ep molecules.

The RIA developed by Garcia (1, 2) is in use in this laboratory. It (2) and the RIA described by Rege *et al.* (3) have both been reported to give values that correlate with bioassay values for very high levels of Ep (1–20 U/ml) in plasma or serum of anemic patients. These levels are at least 50 times as high as those found in normal human serum (2), so it is of interest to know if the correlation is valid for Ep secreted under more nearly physiological conditions. Using Garcia's antibody, Birgegård *et al.* (4) showed good correlation between Ep values by RIA and *in vivo* bioassay in patient samples with lower levels of Ep (about 25–750 mU/ml). Sherwood and Goldwasser (5) used an independently generated antibody and, likewise, showed concordance between the two types of assays for Ep in human serum over a wide range of concentrations (10–2000 mU/ml), save for two patients with chronic renal disease whose sera contained considerably more immunoreactive material than was found with the bioassay. Their antibody, therefore, probably differs from that used by us, because a discrepancy in Ep values in serum from anephric patients disappeared when pure Ep was provided to Garcia for labeling and use in the RIA (1).

The present studies were done to further test the specificity of the RIA, done as described by Garcia, for biologically active Ep in human serum and urine for concentrations as low as could be detected by bioassay *in vivo*.

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[2] To whom reprint requests should be sent.

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Materials and Methods. *Human subjects.* There were five patients with hemochromatosis, one patient with mild secondary (to smoking) erythrocytosis, one patient with Fanconi's aplastic anemia, and five normal adults. Serum and 24-hr urines were collected before and after phlebotomy of 500 ml from all except the anemic patient. Human participation in these studies was approved by the Human Use Committee of the Lawrence Berkeley Laboratory and the Committee for the Protection of Human Subjects of the University of California, Berkeley.

Urine concentrates. Urine was collected in 1 liter sterile bottles which contained 50,000 U penicillin and 50 mg streptomycin, and then stored frozen. It was thawed, clarified by filtration through double 588 S&S filter paper, and 24-hr aliquots were pooled. The urine was processed in an Amicon DC-2 system with hollow fiber cartridges with a nominal 10,000 mol wt cut-off (6). Ultrafiltration was done in the cold; the flow rate of effluent was set at 30 ml/min; the 24-hr volume was concentrated to 600 ml, dialyzed against water (2 hr/liter of original volume), and concentrated to 100 ml. It was then lyophilized and frozen. Each lyophilized sample was resuspended in 10 ml saline or 5% phosphate buffered bovine serum album (BSA) for assay. On samples from the patient with Fanconi's anemia, protease activity was estimated in the reconstituted samples with agar diffusion plates (Bio-Rad Protease Substrate Gel Tablets). Protein was measured in her raw urine and final concentrates (Pierce Microprotein Rapid Stat Kit; Chemetrics Analyzer II).

Radioimmunoassay for Ep was done as previously described in detail. Normal values in human serum by this method were previously reported by Garcia *et al.* at 18.8 ± 6.2 mU/ml for women and 17.2 ± 5.5 for men (2).

Bioassay for Ep was done in hypertransfused, plethoric CF_1 female mice, measuring 24-hr ^{59}Fe incorporation after injection of 1.0 ml serum or 0.5 ml urine concentrate on 2 consecutive days (7). A reference curve, calibrated with the second IRP Ep, of human urinary origin (obtained from the National Institute of Biological Standards, Mills Hill, England), was run with each of the six bioassays using the house standard (4 U/mg, human urinary) in amounts from 10 to 1000 mU/ml. The percentage ^{59}Fe uptake in the red cells of assay mice was converted to units of Ep/ml from the reference curves.

Results. The dose–response relationship in the bioassay, percentage ^{59}Fe uptake versus the Ep concentration, was approximately linear when fitted on semilogarithmic coordinates between 100 and 1000 mU/ml, but nonlinearity occurred at less than 100 mU/ml (Fig. 1). When the concentration of Ep versus the percentage ^{59}Fe uptake was plotted on logarithmic probit paper, a linear relationship for Ep values greater than 30 mU/ml resulted; a standard curve which utilized the IRP, Ep, human urinary, is shown in Fig. 2. In addition, three points from a different assay which utilized the house standard (30, 50, and 70 mU/ml) as the Ep source are shown to illustrate the reproducibility of the linearity of the dose–response relationship at these relatively low levels. All Ep values less than 100 mU/ml were determined from a log-probit standard curve and higher values from either type of curve. No difference was detected in the percentage ^{59}Fe uptake in hypertransfused mice injected with either saline alone, phosphate buffered BSA alone, or 10 or 25 mU/ml Ep in saline or phosphate buffered BSA. Ep values less than 30 mU/ml by bioassay were considered to be too low to measure by this method.

Figure 3 presents the results for 23 sera and 21 urine concentrates for which Ep was measured by both RIA and bioassay. Correlation of the data for both assays was analyzed

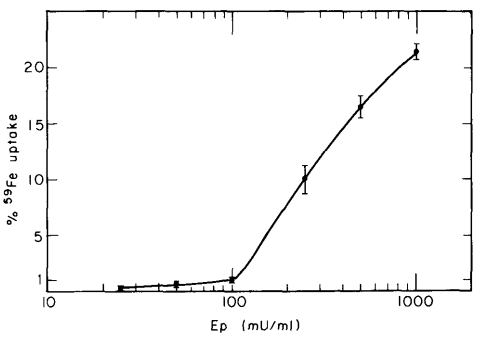

FIG. 1. Dose–response curve showing red cell ^{59}Fe incorporation in hypertransfused mice as a function of the dose of erythropoietin injected.

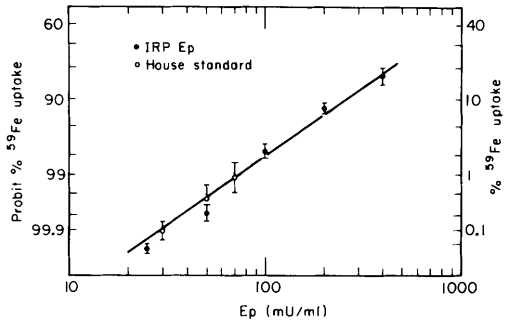

FIG. 2. Probit plot of red cell ^{59}Fe incorporation in hypertransfused mice as a function of the dose of erythropoietin injected.

separately for sera and urine with the formula $y = ax^b$. For sera and urine, respectively, $r^2 = 0.64$ and 0.64, $a = 0.88$ and 0.93, $b = 1.03$ and 1.00. The similarities suggested that both kinds of samples belonged to the same population, so the data were pooled and the following results obtained: $r^2 = 0.81$, $a = 1.09$, $b = 0.97$. The closeness of a and b to 1 suggests that, in fact, $x = y$ (i.e., bioassay = RIA). There were 9 additional serum samples which had Ep levels that were undetectable by bioassay; Ep levels determined by RIA on these samples were 15.2, 16.1, 17.9, 22.2, 22.5, 28.5, 30.0, 33.5, and 37.5 mU/ml, respectively.

The urine concentrates were prepared as a part of a study to try and quantify the 24-hr excretion of Ep in human subjects, since it was not possible to measure Ep in unconcentrated urine from normals or nonanemic patients by RIA. To monitor the efficiency of the process, aliquots of urine from a patient with Fanconi's anemia were processed frequently over a period of time. Ep levels in her urine were high enough (700–2000 mU/ml), so they could be measured in both raw urine and concentrates; from these values, recovery of Ep was calculated. In 63 samples, Ep recovery from the concentrating procedure was 0–131% with differences occurring unpredictably. This patient had proteinuria and protease activity in many of her urine concentrates. In 23 samples in which Ep recovery was 0.2–104%, recovery of protein was 66–99% and protease activity varied from absent from one sample to strongly positive in most of the other samples. There was no correlation between protein recovery or amount of protease and Ep recovery.

Discussion. In the present study, concentrations of Ep were measured by two different methods in serum and in urine concentrates from human subjects who did not have hemopoietic or renal disease. They were either in a steady state (except for the effects of cigarette smoking in three), or their erythropoietic system was moderately stressed by removal of about 7–14% of their estimated blood volume (7% of body wt). There was a strong correlation between the levels of immunoreactive Ep and biologically active Ep. This RIA has been reported to detect desialated Ep (1), but the present data support the conclusion that the immunoreactive Ep that is present in human serum and urine under nearly normal conditions is intact, as shown by its ability to recirculate and stimulate erythropoiesis upon transfer into plethoric mice. Had it been desialated, it would have lacked biological activity *in vivo* (8).

The correlation coefficient of 0.9 for the two assays was identical to that reported by

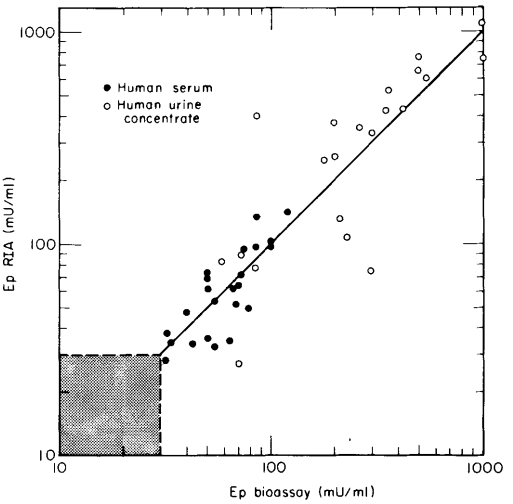

FIG. 3. Correlation between radioimmunoassay and bioassay for erythropoietin in human serum and urine concentrates. Sera were from six patients with hemochromatosis or secondary erythrocytosis; each patient contributed two to seven sera. Urines were collected from four of these patients and from five normals; there were one to six samples/person. Sera and urines were collected before and/or after phlebotomy. Shaded area indicates values too low to measure by bioassay.

Garcia *et al.* (2) for human plasma with very high levels of Ep due to severe anemia, and their data line up almost perfectly with the data in Fig. 3. Considered as a group, the present data and those of Garcia *et al.* (2) indicate that this RIA and *in vivo* bioassays probably detect the same substance in human serum, plasma, and urine over a wide range of concentrations (30–20,000 mU/ml). It seems probable that lower levels which could be measured by RIA would also be indicative of biological activity. It should be noted that, in spite of concordance of the measurements on the population of samples, there may occasionally be marked variation between the two assays in individual samples.

The technique we used to concentrate urine samples proved to be too erratic to be useful for quantifying excretion of Ep (9). It did, however, provide some samples with Ep levels that were high enough to measure by both assays, but the results did not suggest that the crude urine concentrates contained more than one species of Ep. In contrast, other investigators have found that highly purified preparations from human urine may contain two species of Ep which have different physical characteristics (10, 11, 12) and may also show different reactivities in RIA and bioassay (12). It cannot be determined at this time if these apparently discrepant results are due to our inability to detect a minor subspecies of Ep which might be concentrated sufficiently for detection by further processing, if the more extensive concentration techniques themselves may induce changes in some Ep molecules, or if a subspecies might have been lost by our procedure.

The plethoric mouse bioassay has been found by others to be sensitive to 50 mU of Ep or to show linearity of the dose–response curve to amounts of Ep greater than 50 mU (2–4, 7). By plotting ^{59}Fe incorporation values as probits, linearity of the dose–response curve with our bioassay could be extended from 100 mU down to about 30 mU/ml. This treatment of the data appears to improve the sensitivity and accuracy of the *in vivo* bioassay for low levels of Ep.

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