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Development of radioimmunoassays for human erythropoietin using recombinant erythropoietin as tracer and immunogen

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Since the estimation of erythropoietin (EPO) by radioimmunoassay (RIA) has been limited by the availability of highly purified urinary (U) human (Hu) EPO, we investigated the use of recombinant (R)-HuEPO as a replacement for U-HuEPO in the preparation of ^{125}I-tracer and high affinity antisera. In each of two validated RIAs developed using U-HuEPO-derived reagents, dose-response lines and potency estimates for samples were compared when ^{125}I-U-HuEPO and anti-U-HuEPO antisera were sequentially replaced to give assay variants using R-HuEPO-derived reagents. Two U-HuEPO preparations, the 2nd International Reference Preparation and CAT-1, and R-HuEPO were variously used as standards. The samples tested included clinically relevant human sera and partially purified preparations of U-HuEPO and R-HuEPO. In each RIA and for each assay variant tested, samples gave dose-response lines whose slopes did not differ significantly from that of the standard. For each of the two variant RIAs, potency estimates for any sample were consistent and, where examined, RIA potency estimates agreed with in vivo bioassay determinations. These results, obtained independently in two laboratories, indicate that RIAs having appropriate specificity and sensitivity for the estimation of EPO in clinical samples can be developed using reagents derived from R-HuEPO.

Key words: Radioimmunoassay; Recombinant DNA; Erythropoietin; Erythropoiesis

Introduction

Erythropoietin (EPO), a sialylglycoprotein hormone with an apparent molecular weight of 36000, is produced mainly, although not exclusively, in the kidney and is responsible for regulating the production of red blood cells (Graber and Krantz, 1978; Spivak and Graber, 1980). Although serum EPO levels are normally increased by hypoxia or anemia and decreased by hyperoxia (Adamson and Finch, 1968; Abbrecht and Littell, 1972; Milledge and Cotes, 1985; Erslev and Caro, 1986), several abnormal production patterns have been identified. In renal disease, the increased EPO secretion in response to anemia is usually impaired and, in these patients, serum EPO levels are inappropriately low for the severity of anemia (Caro et al., 1979; McGonigle et al., 1984). Indeed, the anemia may be corrected by treatment with exogenous EPO (Adamson et al., 1986; Winearls et al., 1986; Zins et al., 1986; Eschbach et al., 1987). Inappropriate over-production of EPO, sometimes but not necessarily ectopic (Desablens et al., 1980) or familial (Adamson, 1975)

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Abbreviations: RIA, radioimmunoassay; EPO, erythropoietin; U, urinary; Hu, human; R, recombinant; IRP, International Reference Preparation.

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may occur and induce polycythemia. Recognition of these abnormalities may be important for their clinical management and requires the availability of assays for the estimation of EPO in clinical samples.

Radioimmunoassays (RIA) for EPO have already provided a useful tool in the investigation and diagnosis of hematological disorders (Sherwood and Goldwasser, 1979; Koeffler and Goldwasser, 1981; Cotes, 1982; Garcia et al., 1982; Rege et al., 1982; Wedzicha et al., 1985; Cotes et al., 1986). The widespread use of EPO RIAs, however, has been limited by the availability of both highly purified urinary (U) human (Hu) EPO for the preparation of ^{125}I-tracer and high affinity anti-U-HuEPO antisera. The recent cloning and expression of the human EPO gene (Jacobs et al., 1985; Lin et al., 1985) have provided large amounts of highly purified recombinant (R)-HuEPO, which is biologically active and indistinguishable from U-HuEPO in a variety of different assays (Egrie et al., 1985, 1986). The present study was carried out to determine whether RIAs based on reagents derived from R-HuEPO have the appropriate specificity for estimation of the natural hormone.

To validate the RIAs using the R-HuEPO-derived reagents, investigations were carried out independently in two laboratories. Test materials selected to be representative of a wide range of samples likely to be encountered in clinical practice were examined in either one or other of two already validated RIAs each based on reagents prepared from U-HuEPO. These two assays used different protocols and different anti-U-HuEPO antisera. In each RIA method, the slopes of RIA log dose-response curves and potency estimates for test samples were determined when either or both U-HuEPO tracer and antiserum against U-HuEPO were replaced by reagents prepared from R-HuEPO. In addition, to verify that the RIA measures biologically active EPO, selected samples were assayed in the polycythemic mouse in vivo bioassay.

Materials and methods

Purification of EPO

Urinary derived EPO was purified to apparent homogeneity from the urine of a patient with aplastic anemia using the procedure of Miyake et al. (1977). R-HuEPO was purified to apparent homogeneity from the cell-conditioned media of Chinese hamster ovary cells stably transformed with the human EPO gene using sequential column chromatography (Lin et al., 1985).

Anti-R-HuEPO antiserum production

Antibodies to R-HuEPO were raised in New Zealand White rabbits by multiple intradermal injections of a total of between ~ 40–1000 μg of highly purified R-HuEPO (emulsified with Freund's complete adjuvant) over a 3–4-month period. All animals raised antibodies to R-HuEPO. Anti-R-HuEPO antiserum 8C 295 no. 3, a neutralizing antiserum, was chosen for use in the present study. It was used in both RIA methods *a* and *b* detailed below at a final dilution of $1:1 \times 10^6$.

Radioimmunoassays

RIAs were performed in two laboratories, *A* and *B*, using already validated methods *a* and *b*, respectively. In each RIA method, originally developed using U-HuEPO-derived reagents, tracer and/or antiserum were replaced by R-HuEPO-derived reagents to generate the following four assay variants:

^{125}I-U-HuEPO and anti-U-HuEPO antiserum (U/anti-U)
^{125}I-U-HuEPO and anti-R-HuEPO antiserum (U/anti-R)
^{125}I-R-HuEPO and anti-U-HuEPO antiserum (R/anti-U)
^{125}I-R-HuEPO and anti-R-HuEPO antiserum (R/anti-R)

RIA method a: EPO sample or standard (CAT-1, a U-HuEPO preparation at 1140 U/mg, provided by Dr. Eugene Goldwasser, or highly purified R-HuEPO calibrated by RIA against CAT-1) were incubated at 4°C for 20–24 h with rabbit anti-U-HuEPO antiserum (8C 204 no. 3089) in a final volume of 1.0 ml. All assay tubes contained 100 U of Kallikrein inactivator (Calbiochem, La Jolla, CA). The assay diluent was 10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl, 0.1% bovine serum albumin and 0.025% sodium azide. ^{125}I-U-HuEPO prepared using either iodogen (Fraker and Speck, 1978) or chloramine-T

(Greenwood et al., 1963) was added and the incubation continued for an additional 18–20 h at 4°C. Antibody-bound ^{125}I-EPO was separated from free ^{125}I-EPO by the addition of 0.3 ml of Tachisorb (goat anti-rabbit IgG conjugated to *Staphylococcus aureus* cells, Calbiochem, La Jolla, CA), followed by a 45 min room temperature incubation. The Tachisorb pellets were collected by centrifugation at room temperature for 3 min at $12000 \times g$, washed twice and counted in a gamma counter. Responses were expressed as percent inhibition of tracer binding. The anti-U-HuEPO antiserum was generated by immunizing rabbits with a 1% pure U-HuEPO preparation. The antibody was used at a final concentration of 1:100000 at which 10–20% of the tracer ^{125}I-U-HuEPO was bound.

RIA method b: Method *b* was modification I (Cotes et al., 1983) of the assay described by Cotes (1982) using rabbit antiserum R78/15231179 at a final dilution of 1:100000. Responses were expressed as percent inhibition of tracer binding for comparability with results from method *a*. R-HuEPO was iodinated by the chloramine-T method of Greenwood et al. (1963). The 2nd International Reference Preparation (IRP) was used as standard.

In vivo EPO bioassay

In vivo biological activity was determined using five mice per dose in the exhypoxic polycythemic mouse bioassay as described by Cotes and Bangham (1961). Normal human serum (25 ml) was lyophilized and resuspended in deionized water to one-third the original volume before assay.

Samples tested

The preparations tested were representative of materials likely to be relevant to clinical and physiological studies in man. They included partially purified preparations of U-HuEPO and R-HuEPO and sera from normal subjects, patients with polycythemias of varied etiology, a patient with aplastic anemia, and extra-renal EPO in serum from an anephric subject.

Results

Slopes of RIA dose-response lines

Method a: In each of the four variants of method *a*, there were no significant differences between the slopes of log dose-response curves generated by the standard and all test preparations examined. These comparisons were made between partially purified preparations of R-HuEPO and U-HuEPO and serum from a patient with polycythemia as illustrated in Fig. 1*A*. In addition, in variant R/anti-R of assay *a*, the slopes of dose-response lines for normal human sera were examined in more detail because of their physiological relevance and to exclude serum matrix effects in the assay. As shown in Fig. 2, the slopes ($\pm$SE) of log dose-response lines produced by two individual serum samples and one serum pool (Irvine Scientific, Irvine, CA), each tested at five or six levels between 75 and 500 μl per assay tube were 60 ± 6, 68 ± 5 and 67 ± 7, respectively. In the same assay, the slope for the standard, a calibrated preparation of R-HuEPO, was 65 ± 3.

Method b: For each of the four variants of method *b*, analysis of variance showed no significant difference between the slopes of log dose-response lines generated by the IRP (an impure preparation of U-HuEPO) and representative sera from a normal subject, an anephric patient (extrarenal EPO) and from three patients with polycythemia variously rubra vera, unknown etiology and inappropriate overproduction of EPO (shown in Fig. 1*B*). The number of dose levels tested for the serum samples (three or, from serum 13, four), was more than might be used in routine clinical analyses, but too few for precise estimation of slopes.

Potency estimates by RIA using different assay variants

Another requirement for the validation of assay methods based on R-HuEPO-derived reagents is that potency estimates be equivalent to those obtained using U-HuEPO-derived reagents. As illustrated in Table I, for RIA methods *a* and *b*, potency estimates were consistent when any one of a wide variety of different samples was assayed in each of the four assay variants. For method *a*, the listed samples were assayed in between 1–15

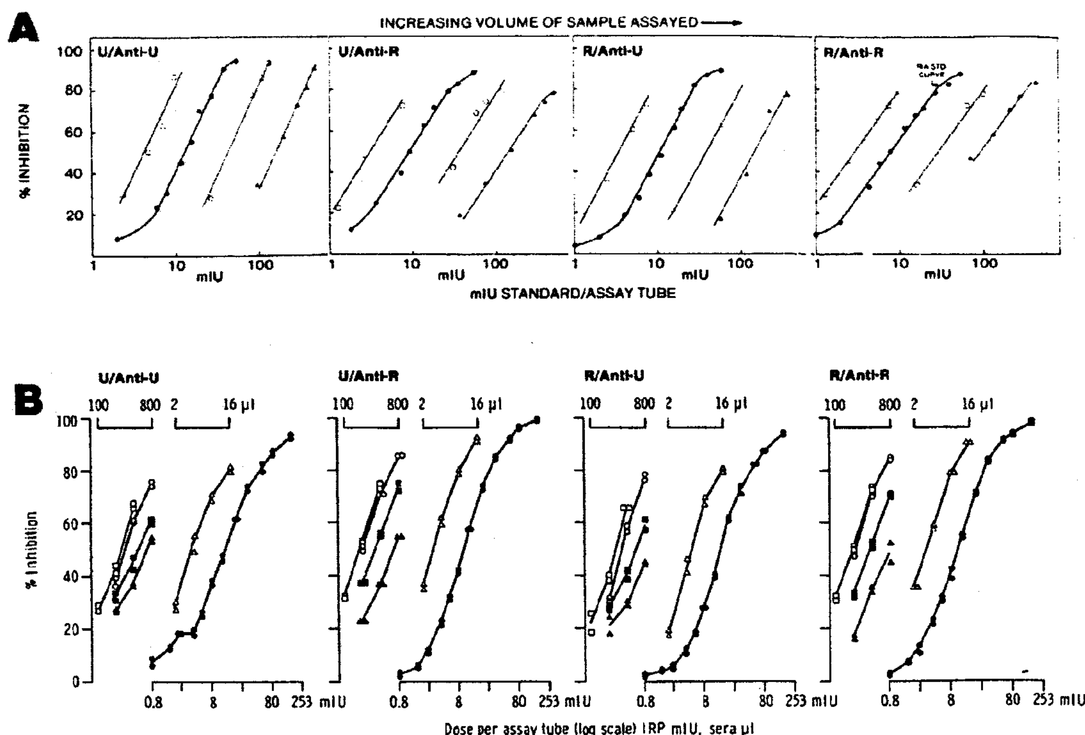

Fig. 1. Log dose-response lines for EPO standard and samples assayed by each of the four variants of RIA methods *a* (*A*. smoothed lines) and *b*(*B*, mean responses connected by straight lines). *A*: The standard (●) for the U/anti-U assays was CAT-1 and for the other three assay variants was a highly purified preparation of R-HuEPO calibrated against CAT-1. Samples were a subset of those listed in Table I: partially purified R-HuEPO (○), and U-HuEPO (▲) and polycythemic patient serum (□). *B*: The standard was the IRP (●) and samples were human sera listed in Table I: 9 (○), 10 (■), 11 (□), 12 (▲), 13 (△).

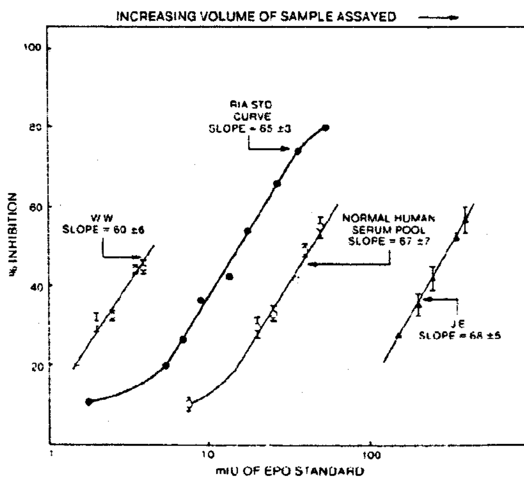

Fig. 2. Log dose-response lines for R-HuEPO standard (●——●), two individual normal human serum samples (□——□, ▲——▲), and one normal serum pool (○——○) assayed by variant R/anti-R of assay method *a*. Slopes are detailed ± SE.

different assays. Within each assay, samples were tested at between three and seven doses. The variability of estimates (shown by the SEM in the upper portion of Table I) therefore reflects inter-assay variability. In contrast, the comparisons presented for the four variants of method *b* were obtained in a single assay in which all samples were tested simultaneously in each variant. The variability of estimates is, in this case, shown by the within assay 95% fiducial interval (lower part of Table I).

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TABLE I

ESTIMATES OF ERYTHROPOIETIN BY VARIANTS OF TWO RIAs AND BY BIOASSAY IN VIVO

	Radioimmunoassay variant				Bioassay in vivo
	U/Anti-U	U/Anti-R	R/Anti-U	R/Anti-R	
Partially purified EPO	*Estimates from laboratory A as mean ± SEM (n = number of independent assay determinations)* [a]				
Partially purified R-HuEPO (IU/ml)	42815 ± 2302 (n = 5)	39896 ± 3979 (n = 2)	46248 ± 4207 (n = 2)	44323 ± 1230 (n = 15)	45018 ± 8164 (n = 4)
Partially purified U-HuEPO (IU/ml)	19.0 ± 1.0 (n = 5)	15.3 ± 2.6 (n = 2)	14.7 (n = 1)	16.1 ± 1.1 (n = 3)	19.9 ± 2.7 (n = 2)
Human serum samples					
(1) Polycythemic patient (mIU/ml)	269 ± 17 (n = 3)	262 (n = 1)	270 ± 24 (n = 2)	255 ± 12 (n = 7)	290 ± 14 (n = 2)
(2) Aplastic anemia (IU/ml)	17.3 ± 1.5 (n = 2)	17.0 ± 1.7 (n = 2)	13.8 (n = 1)	14.9 (n = 1)	18.6 (n = 1)
(3) Normal pool I (mIU/ml)	27.1 ± 1.1 (n = 4)	26.6 ± 3.6 (n = 3)	29.6 ± 2.4 (n = 2)	30.3 ± 1.1 (n = 12)	
(4) Normal, male I (mIU/ml)	17.1 (n = 1)	15.8 (n = 1)	16.0 (n = 1)	17.7 (n = 1)	
(5) Normal, male III (mIU/ml)	20.4 (n = 1)	24.1 (n = 1)	25.0 (n = 1)	27.0 (n = 1)	
(6) Normal, female I (mIU/ml)	20.4 (n = 1)	14.9 (n = 1)	18.0 (n = 1)	20.9 (n = 1)	
(7) Normal, male IV (mIU/ml)	26.5 ± 2.8 (n = 2)	24.8 ± 4.1 (n = 2)	21.6 ± 4.4 (n = 2)	26.9 ± 1.3 (n = 2)	
(8) Normal pool II (mIU/ml)				24.0 ± 1.9 (n = 2)	24.5 ± 2.0 (n = 2)
	Estimates from a single assay in laboratory B, mIU/ml (within assay 95% fiducial interval) [b]				
(9) Polycythemia (etiology unknown)	45 (39–50)	50 (44–56)	46 (39–54)	51 (44–58)	
(10) Normal, female	27 (23–30)	30 (27–33)	27 (23–32)	28 (24–31)	
(11) Anephric	56 (49–64)	55 (50–60)	59 (50–69)	55 (48–62)	
(12) Polycythemia rubra vera	20 (17–23)	15 (13–17)	18 (15–22)	15 (13–17)	
(13) Polycythemia associated with inappropriate EPO production	3322 (2923–3754)	3323 (3024–3655)	3150 (2741–3623)	3338 (2937–3772)	

[a] Samples were assayed at between three and seven doses.

[b] Obtained from the fiducial intervals for the log potency estimates which were determined as the displacement of the linear part of the log dose-response lines with a common slope fitted for standard and samples in each variant of the assay.

Comparison of EPO estimates by RIA and in vivo bioassay

Selected human serum samples and partially purified U-HuEPO and R-HuEPO preparations were assayed in the polycythemic mouse in vivo bioassay. As seen in Table I, for the five samples tested, there was no significant difference between EPO titers obtained by the in vivo bioassay and by each of the four variants of RIA method *a*.

Discussion

The studies reported here show that both recombinant and natural human EPO can be estimated in RIAs using reagents derived from the recombinant hormone in place of the natural hormone. Test samples studied were selected to be representative of a range of materials likely to be encountered in both research and clinical investi-

gations. They included sera from normal subjects, and an anephric subject (extrarenal EPO), and from patients with aplastic anemia, primary and secondary polycythemias and polycythemia of unknown etiology. Other test samples consisted of extracts of human urinary EPO and a partially purified preparation of R-HuEPO. In all four variants of the two RIA methods studied, samples tested gave log dose-response lines with slopes which, for the dilutions tested, were not significantly different from that of the EPO preparation used as the standard in the assay. In addition, potency estimates for all samples were independent of the assay variant used and where relevant they were appropriate to the clinical state (Table I). The data suggest that assays based upon R-HuEPO-derived reagents have the required specificity for estimation of the natural hormone. For each of the five samples examined both by RIA and in vivo bioassay, there was good agreement between the EPO concentration estimated by the different methods suggesting that the RIA is measuring biologically active EPO.

The two RIA methods which formed the starting point for our study used very different protocols and different antisera each raised against EPO from human urine. In both of those systems, replacement of ^{125}I-U-HuEPO with ^{125}I-R-HuEPO tracer antigen gave similar estimates of potency. Furthermore, when the two anti-U-HuEPO antisera were replaced by a common anti-R-HuEPO antiserum, irrespective of whether ^{125}I-U-HuEPO or ^{125}I-R-HuEPO was used as a tracer, potency estimates still showed remarkable consistency. Thus, we have shown that it is possible to use the R-HuEPO-derived reagents, or a combination of R- and U-HuEPO-derived reagents, to develop assays which can be optimized for convenient use and can assay EPO in a wide range of different types of samples.

In addition to providing research and diagnostic reagents, the availability of large quantities of R-HuEPO has opened up the possibility of its use in the treatment of certain anemias. Assays for the hormone may be useful in identifying patients who could benefit from therapy and in monitoring treatment. Estimates by RIA of EPO in clinical samples have already added to our understanding of the normal control of erythropoiesis and its pathology. Now the availability of EPO RIAs based on recombinant EPO-derived reagents permits their routine use in the investigation of hematological disorders.

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