EXHIBIT 14

A Radioimmunoassay for Erythropoietin

By Judith B. Sherwood and Eugene Goldwasser

We describe the development of a radioimmunoassay (RIA) for erythropoietin. Antisera were raised in rabbits with an impure human urinary erythropoietin preparation used as immunogen, but with pure human erythropoietin serving as the labeled antigen in the RIA and as a primary standard. The immunoreactivity of erythropoietin is not altered significantly by the mode of labeling with radioiodine, even though the biologic activity is lost. With this method, it is possible to detect 2–3 mU of erythropoietin in a volume of 0.1–0.3 ml. Therefore, the method can be used for detection of normal and subnormal serum titers as well as elevated titers. RIA for erythropoietin does not distinguish between the native (active in vivo) and the asialo form (inactive in vivo) and cannot yet be used for routine monitoring of crude fractions during purification.

THE NEED for a reliable, precise, highly sensitive and specific assay for erythropoietin (epo) has long been evident. Whole-animal assay methods, although specific, are costly, lack precision, and are relatively insensitive; with the plethoric mouse method, for example, one can usually detect a minimum of about 0.05 U[1] and with the fasted rat method, about 1 U.[2] This degree of sensitivity is sufficient for the detection of serum titers considerably above normal, but does not permit the simple determination of normal or subnormal levels of the hormone.

Several sensitive assay methods in which cell culture techniques are used have been described.[3-5] These methods are generally applicable to purified epo samples, but are often affected by nonspecific inhibitors present in crude materials.

There have been reports of immunologic assays for epo[6-9] in which hormone preparations were used that were not demonstrably pure and thus did not have the requisite specificity. In this article, we report on the development of a radioimmunoassay that is specific and sensitive enough to detect 0.002–0.003 U of human epo. With this method, normal and depressed levels of serum epo can be measured.

MATERIALS AND METHODS

Purification of Erythropoietin

The preparation of pure epo has been described by Miyake et al.[10] Fraction II (α-epo), eluted from a hydroxylapatite column with 1 m*M* phosphate buffer, had a potency of 70,400 U/mg protein, based on the activity of the International Reference Preparation.[11] Analysis of this material by polyacrylamide gel electrophoresis at pH 6 and 9, and at pH 7 in the presence of sodium dodecylsulfate, showed a single band. It also has a single, symmetrical band on gel filtration. This preparation was used for radiolabeling and as standard.

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Radioiodination

We followed the method described by Fraker and Speck[12] for protein iodination. Fifty micrograms of the water-insoluble oxidizing agent, 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril (Iodogen, Pierce Chemical Co., Rockford, Ill.), dissolved in 20 μl of chloroform, were plated onto the walls of a 0.3-ml "reactivial" (Pierce Chemical Co., Rockford, Ill.) by evaporation of the solvent. To the reactivial, 5 μl of deionized water, 5 μl of 0.5 *M* phosphate buffer, pH 7, 5 μl of epo (0.5–1.0 μg), and 5 μl of $Na^{125}I$ (500 μCi, Amersham Corp., Arlington Heights, Ill.) were added sequentially. The reaction was allowed to proceed for 30 sec, and the solution was then transferred to a tube containing 200 μl of KI (10 mg/ml in 0.5 *M* phosphate buffer) and 25 μl of a 10% bovine serum albumin solution. The ^{125}I-labeled epo was separated from unreacted iodide on a Sephadex G25 column (45 × 1.5 cm diameter) previously equilibrated with phosphate-buffered saline (0.15 *M* NaCl–0.01 *M* phosphate) containing 0.1% bovine serum albumin at pH 7.0. Labeled Ep was recovered in the void volume. The specific activity of ^{125}I-labeled Ep was 130–260 μCi/μg, with 40%–50% of the initial radioactivity being incorporated into protein.

Immunization

Antibodies to epo were raised in 4 male New Zealand white rabbits; the antigen used was a preparation of crude human urinary epo with a specific activity of 640 U/mg. The solution was emulsified with an equal volume of Freund's adjuvant, either complete (primary injection) or incomplete (booster injections). Multiple intradermal injections of the material, totaling approximately 600 U of epo, were administered to each rabbit. The following schedule was used: rabbits were bled 2 wk after the primary injection, a booster injection was given 6 wk after the bleeding, and a second bleeding 2 wk after that; the last booster injection was given 8 wk after the second bleeding, and the third bleeding was 2 wk after that. Blood was obtained from the ear vein, the hematocrit was determined, and the blood was allowed to clot. The antisera were stored at −20°C.

Preparation of Samples

Human sera were obtained from the hematology and renal services of the Michael Reese and Billings Hospitals, University of Chicago, Chicago, Ill. Tissue extracts were prepared from kidneys of normal rabbits and from bovine fetal livers, according to the procedure recently described.[13]

Pure lung cell colony-stimulating factor was a gift of Dr. Anthony Burgess, Cancer Research Unit, Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia.

Asialoepo was prepared by incubation of pure, native epo in 0.1 *M* acetate, pH 5, for 30 min at 37°C with 0.8 ml (0.4 U of enzyme) of agarose-bound neuraminidase (Sigma Chemical Co., St. Louis, Mo.). The desialated hormone retained full biologic activity in the marrow cell assay, but was inactive in the in vivo starved rat assay, which was in agreement with previous findings.[14]

Pure human orosomucoid (α-1, acid glycoprotein) was a gift from Dr. Karl Schmid, Boston University School of Medicine, Boston, Mass.

Bioassay Methods

The level of biologically active epo present in the tissue extracts and serum samples was measured by the in vitro or in vivo assay. In the in vitro method, rat bone marrow cells in culture are incubated with standard amounts of epo, or with the sample, and the amount of hemoglobin synthesized is determined by measurement of the incorporation of ^{59}Fe.[3] The in vivo assays used were the fasted rat method[2] and the plethoric mouse method (animals are exposed to 0.5 atmosphere for 3 wk, followed by 3 days at ambient pressure[1]). The amount of ^{59}Fe incorporated in hemoglobin (in vitro bone marrow assay) or in circulating red cells (in vivo assay) is directly related to the amount of epo added. A unit of epo is defined as the activity contained in one-tenth of an ampule of the International Reference Preparation. The values presented are the means of 5–6 replicates ± the standard deviation of the mean.

Radioimmunoassay Technique

The ^{125}I-labeled epo and an epo standard or unknown sample were incubated together with antiserum for 4 days at 4°C in a diluent consisting of 0.025 *M* phosphate buffer, 0.01 *M* KCl, 0.01 *M* EDTA, with 1% bovine serum albumin, at pH 7.4. Each assay tube contained 500 μl of incubation mixture, consisting

of 100 μl of diluted antiserum, 100 μl of ^{125}I-labeled epo, and 100 μl of epo standard or 100–300 μl of unknown sample, with the phosphate-buffer diluent making up the remaining volume. The antiserum was used at a dilution that gave a bound-to-total ^{125}I ratio (B/T) between 0.10 and 0.20. The epo used for the standard curve was the purified preparation described above, diluted in the same phosphate buffer.

The bound and free ^{125}I-labeled epo were separated from each other by the double-antibody method.[15] After addition of goat anti-rabbit gamma-globulin and normal rabbit serum (Research Products International, Elk Grove Village, Ill.) as carrier, the mixture was incubated overnight at 4°C. Randomly sampled tubes (approximately 15% of the total) were counted in an automatic gamma counter (Model 1197, Searle) to determine the mean number of total cpm/tube. All of the tubes were then centrifuged at 4°C for 15 min at 1150 g; the pellets were washed with the phosphate buffer diluent, and the tubes were centrifuged again. The pellets were counted to determine the number of counts precipitated and the percent of bound tracer. The percentages were corrected for nonspecific precipitation of tracer by subtraction of the percent bound observed in the absence of antiserum to Ep, a value ranging from 0.2 to 2.0.

All epo standards and unknown samples were assayed in quadruplicate.

RESULTS

Immunization

All four rabbits produced antibody to the human urinary epo preparation, as determined by the capacity of the rabbit serum to bind the iodinated human epo. Antibody titers in different rabbits after the primary immunization and the first booster dose were low; the percent bound at a final dilution of 1:1000 ranged from a high of 15.5, obtained with antiserum A-1, to a low of 4.0. The titer rose dramatically after the third injection of Ep (second bleeding), with antiserum A-2 at a final dilution of 1:1000 giving a percent bound of 56 (Fig. 1). After the fourth injection (third bleeding), the final dilution, giving a percent bound of 30, ranged from 1:5100 for antiserum 6692A to 1:52,000 for antiserum A-3 (Fig. 2). No correlation between the titer of anti-epo in the serum and the hematocrit of the donor rabbit was observed (Table 1).

Effect of the Method of Labeling on Immunoreactivity of Erythropoietin

The epo was routinely iodinated according to the Iodogen method, both because of the ease of working with this method and because of the relatively mild

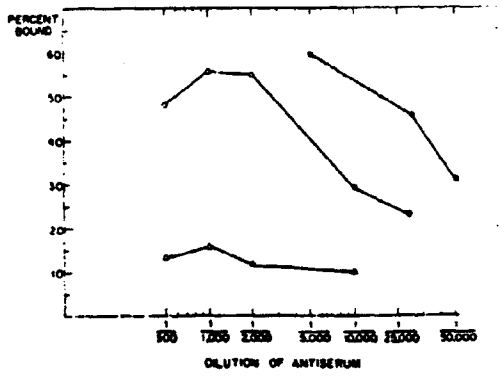

Fig. 1. Anti-erythropoietin titers as a function of immunization schedule. The sample A-1 was obtained 2 wk after the primary injection (-▲-). A-2 (-○-) was obtained 2 wk after the first booster injection that was given 6 wk after the first bleeding. A-3 (-●-) was obtained 2 wk after the second booster injection that was given 8 wk after the second bleeding.

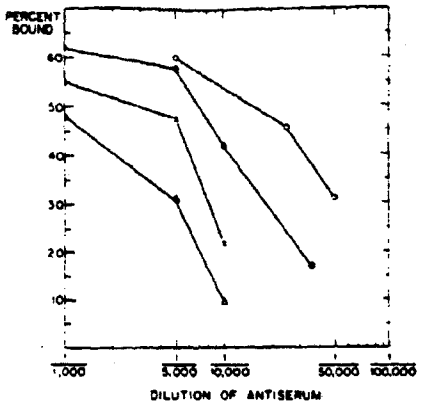

Fig. 2. Comparison of antierythropoietin titers in four rabbits. All of the rabbits were immunized according to the same schedule and the titers illustrated are those at the third bleeding. Rabbit A, hematocrit 26 (-○-), rabbit 6789, hematocrit 38 (-●-), rabbit 6692, hematocrit 42 (-▲-), and rabbit 6692A, hematocrit 28 (-△-).

conditions. Because the oxidant is in solid form, coating the wall of the vial, the reaction is readily terminated by pipetting off of the aqueous phase that contains the epo.

The effect of iodination on the immunoreactivity of epo was studied by comparison of displacement curves with epo labeled by the Iodogen method and by the Bolton-Hunter reagent[16] serving as tracers. In the latter method, the protein was reacted with iodinated 3-(4-hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester (New England Nuclear, Boston, Mass.), which acylates amino groups of the protein with the ^{125}I-*p*-hydroxyphenylpropionic residue.

The standard curves were prepared simultaneously; the pure epo standard and antiserum A-2 at a 1:10,000 dilution were used. The slopes of the two curves were equal, the linear portion extending from 1 to 200 mU of epo (Fig. 3). The competitive binding curves showed 50% displacement of the Iodogen-labeled tracer with 14 mU of epo standard and an equal displacement of the Bolton-Hunter-labeled tracer with 13.5 mU.

Radioimmunoassay Sensitivity and Reproducibility

In the following studies, antiserum obtained from the second bleeding of rabbit A was used at a 1:10,000 dilution (antiserum A-2). The linear portion of the standard displacement curves for this antiserum was consistently located between 1 mU (approximately 12 pg of protein) and 100–200 U of epo. Fifty percent of the tracer was displaced by the addition of 10–15 mU of epo. The standard curves have been quite reproducible, even though different tracer preparations were used; for

Table 1. Binding of Erythropoietin to Antisera From Third Bleeding

Rabbit No	Dilution of Antiserum* Giving 30% Binding of ^{125}I-Ep	Hematocrit of Donor Rabbit
A	1:52,000	26
6789	1:18,000	38
6692	1: 8,000	42
6692A	1: 5,100	28

* Antisera from third bleeding of rabbits after four injections of Ep.

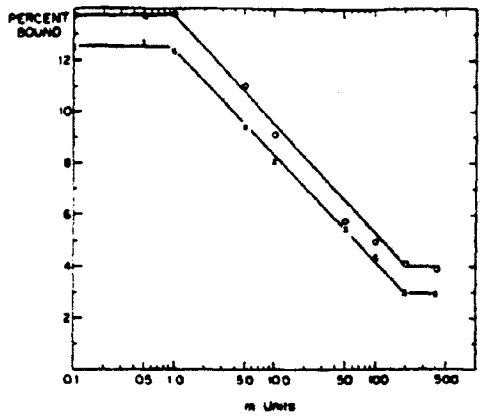

Fig. 3. Effect of labeling method on immunoreactivity of erythropoietin. Erythropoietin was labeled by the Iodogen method (-o-) or by the Bolton-Hunter method (-x-).

example, the 50% displacement point has varied over a 5-mo period from 9.8 to 15 mU, with a coefficient of variation of 18.2%.

Specificity of Assay for Erythropoietin

We tested the specificity of the assay with two glycoproteins, human serum orosomucoid and pure mouse lung cell colony-stimulating factor (CSF).[17] Orosomucoid was assayed at 0.1, 1.0, and 10 μg, and CSF at 10, 100 and 1000 pg. Neither competed with the binding of ^{125}I-epo to antiserum A-2 (Fig. 4).

When increasing amounts of human serum samples were assayed, the displacement curves obtained were parallel to the curve produced by the pure epo standard, suggesting that the material in the serum samples behaved immunologically like human epo (Fig. 5). By using antibody A-2 and iodinated native α-epo, we found that the displacement curves for α-Ep,[10] the β form of the hormone,[10] and asialoepo were parallel (Fig. 6).

Examples of Assay Results

Several human serum samples were tested in the radioimmunoassay, and where feasible, a comparison between RIA and the conventional plethoric mouse bioassay

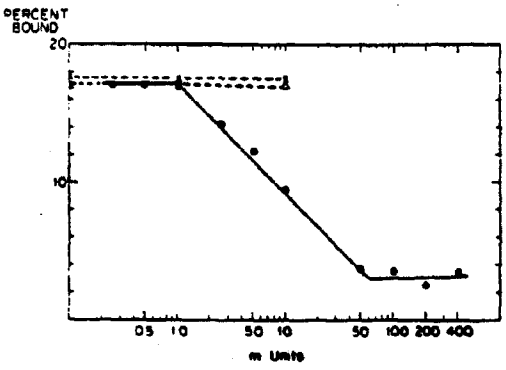

Fig. 4. Lack of cross-reaction by other glycoproteins. The standard erythropoietin (-•-) was contrasted with mouse lung cell colony-stimulating factor (-x-) at 10, 100 and 1000 pg and with human orosomucoid (-Δ-) at 0.1, 1.0 and 10 μg.

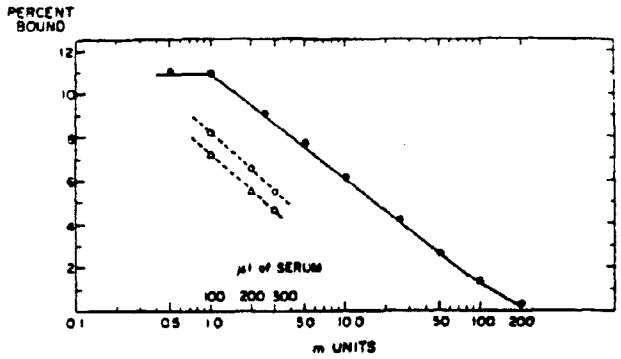

Fig. 5. Radioimmunoassay of human serum. Two sera (-○- and -△-) from anemic patients were compared with standard human erythropoietin (-●-).

was made. The results (Table 2) showed that (A) the epo titer of normal serum can be measured by RIA; (B) serum from patient J2, whose diagnosis was polycythemia vera, had detectable epo in the normal range; (C) among the samples so far assayed, serum from patient M2, who was anephric, had the lowest titer measured; (D) in six cases (M2, H1, W1, W2, J4, and R), there was a reasonable correspondence between titers obtained by RIA and by bioassay; and (E) in two patients with chronic renal disease (J3 and B1), the titer obtained by RIA was very much higher than that by bioassay.

In Table 3, we present selected data showing that the RIA method is not yet suitable for assays of crude extracts or of partially purified epo preparations. The activities measured by RIA agree fairly well with those determined by the marrow cell assay, except for the fraction called "alcohol 2"; they do not agree, however

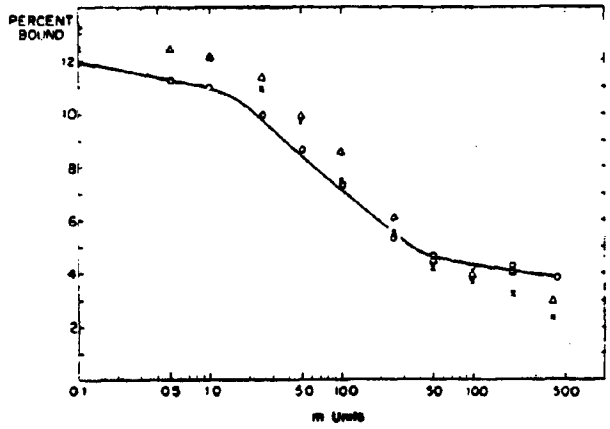

Fig. 6. Comparison of three forms of human erythropoietin. Pure α-erythropoietin (-○-) was compared with the β form (-▫-) and with acidic erythropoietin (-△-).

Table 2. Assay of Human Serum Samples

Serum	Diagnosis	Units of Ep/ml	
		RIA	Bioassay
Pool	Normal	0.020	—
J1	Normal	0.020	—
M1	Normal	0.036	—
J2	Polycythemia vera	0.028	—
M2	Anephric	0.010	0.02 ± 0.006*
H1	Erythrocytosis secondary to hypoxia	0.088	0.054 ± 0.015
J3	Chronic renal disease	0.240	0.085 ± 0.02
B1	Chronic renal disease	0.160	0.02 ± 0.008
W1	Atypical myeloproliferative disease	2.16	2.46 ± 0.5
W2	Atypical myeloproliferative disease	0.68	0.58 ± 0.1
R	Hypoplastic anemia	0.16	0.16 ± 0.02
J4	Iron-deficiency anemia	0.39	0.57 ± 0.1

*In this particular bioassay run using the plethoric mouse method, the control iron-uptake was 1.6%, 0.05 U yielded 8.8%, and 0.10 U, 13.4%. The sensitivity of the assay was sufficiently high to permit an appropriate estimate of the titers of the 2 1.0-ml serum samples, using a log-log dose–response curve. Sample M2 showed 4.8% iron-uptake and sample B1, 5.9%. If the curve is linear below the 0.05-U level, these estimates are reasonable; if the curve flattens at the low doses, these titers are overestimates.

(except in one case—"alcohol 3"), with the activity found by the fasted rat method. In general, both the RIA and marrow culture methods show higher titers than the in vivo assay. In the case of the fetal liver extract, however, we found considerably less activity by RIA than by bioassay. This finding is similar to that reported by Leung et al.[18] for prolactin.

DISCUSSION

The availability of pure human epo has permitted us to develop a radioimmunoassay that is sensitive and specific. Although investigators had previously[6–9] attempted to develop radioimmunoassays for human epo, the methods were not specific, because impure erythropoietin served both as the immunogen and as the labeled material. Garcia[7] purified an epo preparation by immunologic methods and used it as the tracer; the resulting material was considerably enriched in epo but was not shown to be homogeneous. As he reported more recently,[19] the ambiguities

Table 3. Comparison of RIA, Marrow Cell Culture Assay, and In Vivo Assay for Erythropoietin

Fraction	Ep U/ml Found by		
	RIA	Marrow Cell Culture	In Vivo Assay
PHA-2	66.7	69.8	19.6
PHA-3	34.0	35.4	12.8
PHA-5	240.0	202.0	118.0
PHA-6	74.0	75.1	33.8
Alcohol 2	340.0	190.0	192.0
Alcohol 3	2,200.0	2,900.0	2,040.0
Rabbit kidney cortex extract	2.9	2.7	—
Beef fetal liver extract	0.11	—	0.25

The in vivo assays were done by the fasted rat method. The samples labeled "PHA" were derived from an experiment using phytohemagglutinin coupled to sepharose as an affinity chromatographic medium. Those labeled "alcohol" were derived from the ethanol fractionation already described.[18]

that he had found with his previous method have been eliminated by the use of pure epo as the tracer.

With several antisera to epo that were raised in response to crude preparations, we found no correlation between the capacity of the antiserum to bind tracer and the hematocrit of the donor rabbit. For example, antisera A-3, which had a high binding titer, and 6692A, which had a low titer, came from rabbits with similar hematocrits (Table 1). The antiserum used in the displacement studies reported here (A-2) did not neutralize the activity of epo when tested by the in vitro marrow culture method.[3] This suggests that these particular antibodies may not be directed against the active site of epo required for its interaction with a target cell.

Intact biologic activity of epo is not necessary for its immunologic activity. Substitution of iodine on the tyrosine residues of epo by means of the Iodogen method, or on the free amino groups by the Bolton-Hunter method, results in loss of biologic activity (unpublished data); the displacement curves found with these two differently labeled epo preparations, however, are parallel and show essentially identical 50% displacement points (Fig. 3).

Similarly, removal of sialic acid residues does not alter the immunoreactivity of epo. This was shown by Garcia[7] and by our finding that the displacement curve for asialoepo is parallel to that found for the native hormone (Fig. 6).

Erythropoietin from both human and nonhuman sources reacts with antiserum A-2 and displaces the human tracer. The antibody thus appears to recognize an antigenic site that is common to several species. However, there may be a significant difference between the human and bovine samples with regard to reactivity with the antibody. This is suggested by the discrepancy between the amounts of epo found by bioassay and by radioimmunoassay (Table 3). Preliminary studies in our laboratory on binding by antibody confirm a report of Schooley and Mahlmann,[20] who showed that neutralization of sheep epo was not as effective as neutralization of human epo when an antiserum to human epo was used.

The following evidence supports the interpretation that the substance detected in human serum by radioimmunoassay is indeed epo: (A) Increasing amounts of human serum produced displacement curves parallel to that found with pure human epo (Fig. 5). (B) For some samples, there was reasonably good agreement between biologic activity, as determined in plethoric mice, and the amount of immunoreactive epo (Table 2). (C) There was a correlation between the clinical disorder and the titer of epo found by radioimmunoassay. A patient with anemia secondary to hypoxia had an epo concentration significantly greater than that found in sera from normal persons, and an anephric patient had a lower concentration. In patients J3 and B1, the titer determined by RIA was very much larger than that found by the conventional bioassay. This finding may be due to the presence, in the serum, of immunoreactive substances that have little or no biologic activity; more information is needed, however, before such a suggestion can be verified. In preliminary experiments, we have found that sample J3 contained a smaller immunoreactive component separable from epo by gel permeation.

The data presented in Table 3 show that the RIA, although it can be used for measurements of serum titers of epo, cannot be applied in its present state for quantitative assays of crude fractions. We tentatively interpret the higher titers found by RIA and by the marrow cell culture method as indicating that biologically

active native epo is substantially contaminated with biologically inactive asialo epo, which is both immunoreactive and active in cells in vitro. Until a method can be found for distinguishing between these two forms, neither in vitro method will serve the purpose of monitoring the fractionation of epo. We are currently seeking such a method.

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