EXHIBIT 12

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Radioimmunoassay of Erythropoietin

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ABSTRACT. **A radioimmunoassay for erythropoietin has been developed using ^{125}I labeled pure human erythropoietin and an anti-erythropoietin antiserum produced in a rabbit immunized with human erythropoietin. Two techniques are presented for labeling erythropoietin, both resulting in an immunologically reactive labeled reactant. One method involves the use of lactoperoxidase and the other a reagent known as IODO-GEN. The second International Reference Preparation of human erythropoietin is used as a standard and a double antibody scheme is used for the separation of the free and antibody bound labeled hormone. The radioimmunoassay is sensitive to an absolute amount of erythropoietin equivalent to 0.4 milliunits. Bioassays for erythropoietin require approximately 100 times this amount. The use of pure erythropoietin as the labeled reactant has removed certain discrepancies seen in previous attempts to develop radioimmunoassays for this hormone. e.g., sera from patients without kidneys do not give the high values previously seen. Sera from anemic individuals not only give rise to high radioimmunoassay values but also show a parallel relationship with the erythropoietin standard when halving dilutions are analyzed. Desialated erythropoietin is also reactive with the same parallelism. Bleeding of a normal individual increases the serum erythropoietin level and transfusion decreases it. Erythropoietin from a variety of laboratory animals is also reactive in the radioimmunoassay, with very high values being observed in hypoxic animals.**

KEY WORDS: **Erythropoietin — Radioimmunoassay.**

INTRODUCTION

Since the ability to produce antibody which will neutralize the biologic activity of erythropoietin was first demonstrated [5], various investigators have been concerned with the application of immunologic techniques to the measurement of erythropoietin. Goudsmit et al. [7] used an agar double diffusion technique

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in an immunologic assay for human erythropoietin. Lange et al. [8] explored the use of the hemagglutination-inhibition technique with tanned red cells sensitized with relatively crude erythropoietin extracts. Several studies utilizing the radioimmunoassay approach in attempts to measure the erythropoietin in human plasma or serum [2. 3. 4. 9] have been reported. These studies have been hampered by the use of a partially purified preparation of human erythropoietin. Also. a radioimmunoassay for sheep plasma erythropoietin has been attempted by Cotes [1].

The essential requirements for the development of a radioimmunoassay for a hormone are: (a) the availability of the hormone in a pure form and its ability to accept a radioactive label: (b) the availability of specific antibody for the hormone: and (c) a technique for the separation of antibody-bound and non-antibody-bound (free) hormone after a given incubation period.

It is the first of these requirements. i.e.. that of the availability of erythropoietin in a pure form. that has heretofore frustrated attempts at developing a reliable radioimmunoassay for this hormone. Recently, Miyake et al. [10] succeeded in purifying human erythropoietin to apparent homogeneity, and it is the use of such material, as the labeled reactant in a radioimmunoassay. that is the subject of this report.

MATERIAL AND METHODS

Erythropoietin Iodination

The erythropoietin used for labeling was obtained in the pure form by extraction from urine from severely anemic humans. The method of extraction [10] resulted in a preparation consisting of approximately 50% carbohydrate. and with a specific activity of about 70,000 units per mg of protein and a molecular weight of about 40.000. The pure erythropoietin was stored at −20° C in 1–1.5 µg amounts lyophilized in the bottom of polyethylene microcentrifuge tubes. Two iodination methods were used: a modification of the lactoperoxidase method of Thorell and Johansson [13] and a method by Speck et al. [12] which utilizes 1. 3. 4. 6-Tetrachloro-3a.6a-diphenylglycouril (obtained under trade name IODO-GEN. Pierce Chemical Co.. Rockford. Ill.). The radioiodine was obtained from New England Nuclear (NEZ-33L) as carrier free Na ^{125}I. at a concentration of approximately 500 mCi/ml.

Although the method of lactoperoxidase iodination resulted in acceptably labeled erythropoietin for radioimmunoassay. variations will certainly be attempted and some may result in an improved labeled reactant. The reaction was carried out in a polyethylene microcentrifuge tube that contained 1–1.5 µg of lyophilized pure erythropoietin. It was dissolved by the addition of 5 µl 0.5 *M* PO_4 buffer at pH 7.0. This was followed by 10 µl of the same buffer at 0.05 *M*. One microliter of ^{125} iodide (0.5 mCi) was added and this was followed by 2 µl lactoperoxidase (1 mg/ml 0.05 *M* PO_4. pH 7.0) and 2 µl H_2O_2 (1:15.000 of 30% H_2O_2 in 0.05 *M* PO_4. pH 7.0). After an incubation period of 1 min the reaction was stopped by dilution with 400 µl of the 0.05 *M* PO_4 buffer. The total contents of the reaction tube were transferred immediately with a Pasteur pipette to a 1.5 × 5 cm Sephadex G-25 column (prepacked by Pharmacia) that had been previously equilibrated with bovine serum albumin to minimize adsorption of the labeled erythropoietin. and eluted with 0.05 *M* PO_4. pH 7.5. Approximately 1 ml fractions were collected in one dram glass vials containing 5 drops of 5% bovine serum albumin. Figure 1 shows such a fractionation. By careful monitoring of the eluant with a radiation survey meter. most of the first peak. containing the protein-bound ^{125}I. is collected in the 4th fraction. The unreacted ^{125}I appears later in fractions 8–11. The procedure is relatively rapid. the iodination. from the beginning of the reaction to the separation of the labeled erythropoietin. being accomplished in approximately 5 min. The total contents of

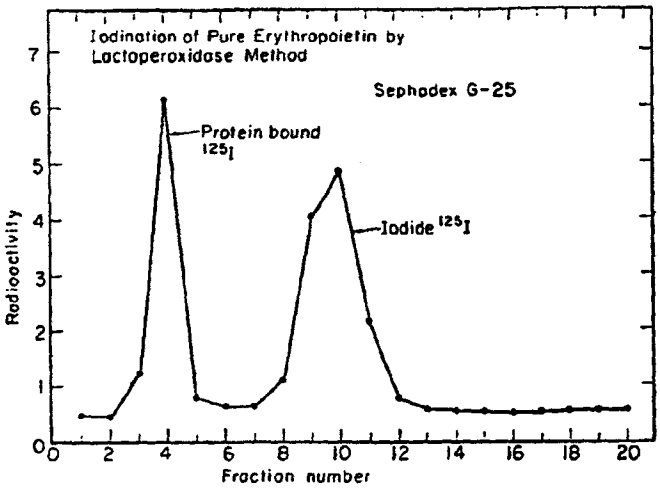

Fig. 1. Separation of erythropoietin, labeled by the lactoperoxidase technique, from unreacted iodide on a Sephadex G-25, column

fraction 4 were then transferred to a 1.5 × 30 cm Sephadex G-150 column (previously equilibrated with bovine serum albumin) and eluted in 20 drop fractions with 0.05 *M* PO_4, pH 7.5. This is shown in Figure 2. Experience with other hormone iodinations has suggested the value of such a step in that any damaged labeled hormone, in the form of aggregates, will appear at the void volume, while the undamaged labeled hormone appears as a second peak, retarded on such a column. This is where one finds the biologic activity of erythropoietin on such a column. It is material from this second peak that has been used in the radioimmunoassay described here.

Erythropoietin iodination with the IODO-GEN reagent was carried out in a conical bottomed reaction vial (REACTI-VIAL, Pierce Chemical Co., Rockford, Ill.). Fifty micrograms of IODO-GEN dissolved in 20 µl chloroform were allowed to dry in the bottom of the vial, thus plating the bottom portion of the cone. Lyophilized erythropoietin was dissolved in 10 µl distilled water and transferred to the reaction vial. Five microliters of 0.5 *M* PO_4, pH 7.0, were added, followed by 1 µl ^{125}iodide (0.5 mCi). An appropriate magnetic stirring bar was added and the reaction was allowed to proceed for 30 s, at which time the total contents of the vial were transferred to a second vial containing 200 µl potassium iodide (10 mg/ml in 0.5 *M* PO_4, pH 7.0) plus 5 µl 5% bovine serum albumin. This was then transferred to a 1.5 × 5 cm Sephadex G-25 column equilibrated with bovine serum albumin and eluted in the same way as for the lactoperoxidase iodinated erythropoietin. The labeled erythropoietin in the 4th fraction was also further processed on a Sephadex G-150 column, producing two peaks. The labeled material in the second peak was used in the radioimmunoassay scheme with no further manipulation. The fact that a greater amount of radioactivity was observed in the first peak with the IODO-GEN method is of no particular significance and probably varies with both iodination methods in each particular iodination.

The pure erythropoietin, labeled by the two methods described here, resulted in equally immunologically reactive erythropoietin. We have found that iodination of erythropoietin results in loss of biologic activity. In the radioimmunoassay technique, in which antibodies are used as binding agents, the possibility that the biologic activity has been lost as a result of the iodination is not critical as long as the immunologic activity remains intact. However, labeled pure erythropoietin being considered for use in studies involving cell membrane receptors as binding agents must have its biologic activity intact.

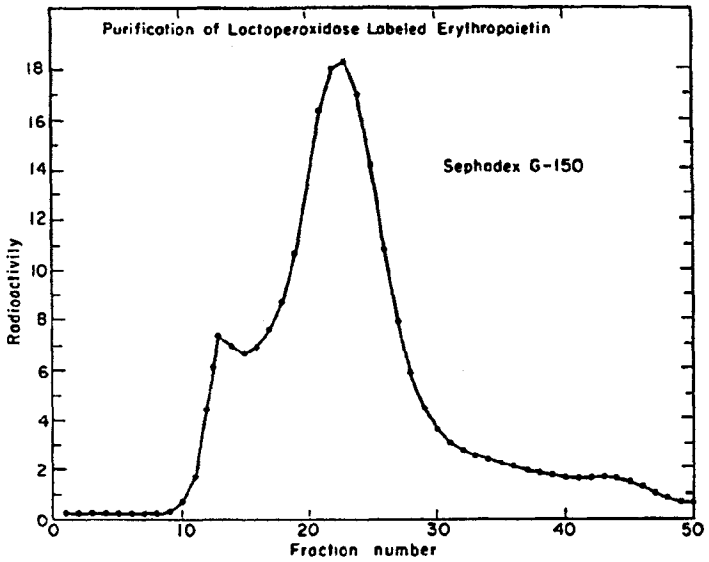

Fig. 2. Separation of labeled erythropoietin from erythropoietin damaged as a result of iodination

Anti-Erythropoietin Antibody

The selection of a proper antiserum is an important feature in the development of a successful radioimmunoassay. The production of antibody varies greatly from animal to animal, even with the same immunization schedule, and even in the animal itself at different times throughout the immunization. To obtain a good antibody for radioimmunoassay purposes a large number of animals may need to be immunized.

Besides specificity, there are two characteristics of antibody which come into consideration in the development of a radioimmunoassay. These are its binding capacity and its avidity. The binding capacity refers to the total amount of the antigen that can be bound by the antiserum. This is a function of antibody concentration and can be varied by dilution. The avidity is an intrinsic characteristic of a particular antibody and indicates how energetically an antibody binds the antigen. This is the important factor in determining the sensitivity of a radioimmunoassay. Each antiserum is then a mixture of antibodies, varying in both these aspects, and its effect must be considered as the sum of these characteristics.

For the development of a hormone radioimmunoassay, in practice, one finds the dilution of an antiserum which will result in the binding of a certain amount of labeled hormone (function of the binding capacity). Then one chooses the antiserum which gives the steepest slope when a small amount of unlabeled hormone is added (function of avidity). It is possible then to select an antiserum which may bind less hormone than another, but because of a higher avidity it would be the one of choice for a sensitive radioimmunoassay.

Antibody to erythropoietin was produced in rabbits immunized with a crude human urinary erythropoietin extract prepared by pressure filtration through a collodion membrane [14]. Ten milligrams of such an extract were dissolved in 1 ml distilled water and emulsified with 1 ml complete Freund's adjuvant and given in four subcutaneous sites at weekly intervals for 3–4 weeks. This procedure usually results in erythropoietin neutralizing aniserum in 1/3 to 1/2 of the rabbits immunized. Recently, of 12 rabbits immunized in this way, four developed neutralizing antibody

to erythropoietin. In addition to human erythropoietin, a variety of animal erythropoietins could be neutralized by such antisera [6], suggesting that portions of the erythropoietin molecule may be similar across species. However, one erythropoietin antiserum was produced which had the ability to neutralize human erythropoietin, whereas rabbit erythropoietin remained unaffected [2]. This suggests that portions of various species erythropoietin molecules may have dissimilarities. Rioux and Ersley [11] have suggested the possibility of differences between sheep and mouse erythropoietin. Although most of our antisera will neutralize 20–25 units of human erythropoietin per ml, they will usually neutralize in the order of 1/10 this amount of rat or sheep erythropoietin.

These differences can become important in considering the development of a radioimmunoassay since such extreme dilutions are used and finite incubation periods are imposed. With these constraints, only the antibodies present in the serum of the highest avidities (which may be against antigenic sites on the particular species erythropoietin used for immunization) may be allowed to significantly express themselves. In neutralization studies, large amounts of antisera are usually used, in which the presence of antibodies of lower avidities may more readily express themselves. It will not be surprising if the wider use of an erythropoietin radioimmunoassay results in differences in the degree of cross reactivity seen between various species erythropoietins with different antisera.

Although pure hormone is absolutely necessary for the labeled reactant in the radioimmunoassay, it is not required for immunization purposes. Antibodies formed against contaminating molecules should not interfere in a radioimmunoassay since the only reaction of consequence is that between labeled hormone and its antibody.

The antiserum used in the radioimmunoassay described here was obtained after a booster immunization of partially purified human erythropoietin and has a neutralizing capacity in excess of 300 units of human erythropoietin per ml. It was used in the radioimmunoassay without manipulation: no absorptions were carried out with normal or polycythemic plasma, as has been done by others [8], because of the possibility that the small amount of erythropoietin present in such sera would remove the antibody with the highest avidity for erythropoietin.

Separation

The final step in the radioimmunoassay is the separation of the antibody-bound labeled hormone from the free labeled hormone. A variety of schemes have been used to accomplish this, including electrophoretic, chemical and immunologic methods. Electrophoresis and chromatoelectrophoresis on paper have been used. With these methods both the antibody-bound and the free labeled hormone are counted and the results are presented as the ratio of bound/free labeled hormone against the concentration of unlabeled hormone used as a standard. With these methods, both the antibody-bound and the free labeled hormone must be counted since the accuracy with which one can apply the relatively small amounts to the strip is variable. However, any scheme in which the total of the antibody-bound or free labeled hormone can be isolated and counted is sufficient for a curve to be developed.

Schemes for the isolation of the free labeled hormone have been developed utilizing adsorption to materials such as charcoal or talc. However, most commonly, the antibody-bound labeled hormone is isolated. This has been accomplished by precipitation with sodium or ammonium sulfate and with alcohol, or by precipitation with a second antibody directed against the gamma globulin of the animal species used to produce the anti-hormone antibody. This is the so-called 'double antibody technique,' and is the method we have used. For this we maintain goats immunized with rabbit gamma globulin.

Erythropoietin Radioimmunoassay

The radioimmunoassay presented here was designed to measure the plasma or serum concentration of erythropoietin. The second International Reference Preparation of human erythropoietin was used as a standard. This standard was dissolved in a diluent made up of 5% human serum albumin in 0.05 *M* phosphate buffer at pH 7.5. Halving concentrations of the erythropoietin standard from 500 mU/ml down to 1 mU/ml were made using the same 5% human serum albumin phosphate

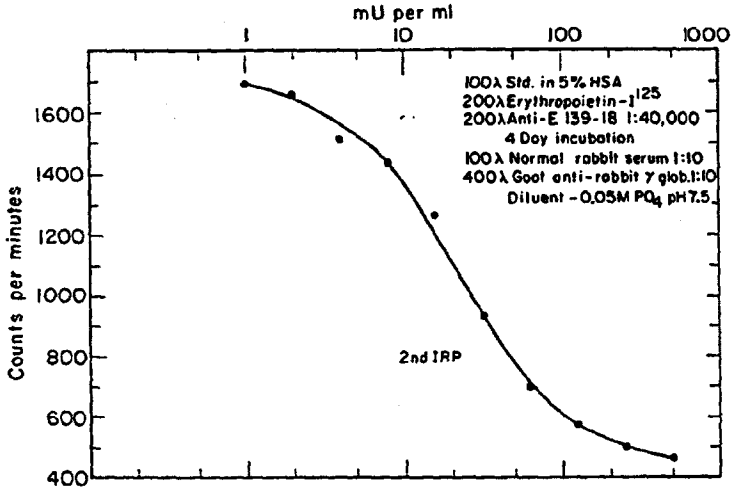

Fig. 3. A typical standard curve for erythropoietin radioimmunoassay. 2nd IRP, second International Reference Preparation

diluent. This was done in an attempt to keep this protein constituent in the erythropoietin standards similar to that in plasma or serum samples.

One hundred microliters of the various erythropoietin standard dilutions were pipetted into 12 × 75 mm plastic test tubes. Other tubes were set up with 100 µl of the plasma or serum to be assayed. Two hundred microliters of labeled erythropoietin containing approximately 10,000 cpm and 200 µl of a 1:40,000 dilution of rabbit anti-erythropoietin antiserum were added to each tube. The diluent for both the labeled erythropoietin and the anti-erythropoietin consisted of 0.05 *M* PO_4 buffer at a pH of 7.5 with 0.05% bovine serum albumin added. The tubes were then incubated at 4° C for 4 days. After the incubation period, 100 µl of a 1:10 dilution of normal rabbit serum and 400 µl of a 1:10 dilution of goat anti-rabbit gamma globulin serum were added. After 2 h at 4° C the tubes were centrifuged at 700 × *g* in a refrigerated centrifuge for 30 min, and the supernatant decanted. The precipitate held together as a readily visible pellet in the bottom of the test tube on decanting. The radioiodine in the precipitate was then counted in a well-type scintillation counter. Curves were plotted on semilogarithmic paper with the standard erythropoietin concentrations on the logarithmic scale and the radioactivity of the labeled erythropoietin bound to antibody on the linear scale. Such a curve is presented on Figure 3. This curve shows that an appreciable competition exists with an erythropoietin concentration of 4 mU/ml. Since only 100 µl of sample is used in this assay, this is equivalent to an absolute amount of erythropoietin equivalent to 0.4 mU. The polycythemic mouse bioassay for erythropoietin requires approximately 100 times this amount.

In Figure 4 results are presented showing that dilutions of the pure erythropoietin (70,000 U/mg of protein) and a crude extract of human urinary erythropoietin (HUE 20 U/mg) both show a parallel relationship with the second International Reference Preparation of human erythropoietin. The diluent in all cases was made up of 5% human serum albumin in 0.05 *M* PO_4 buffer at pH 7.5. This parallelism is evidence that all three of these preparations of widely differing specific activities are reacting with antierythropoietin with the same avidity. This can be taken as evidence supporting the identity of the erythropoietin molecules in these preparations. Thus pure erythropoietin is not required as the standard reference preparation. Indeed, a pool of well titered serum with a high erythropoietin concentration should serve as an ideal standard preparation, since the greatest use of the radioimmunoassay will be in its application to the measurement of plasma and serum concentration of this hormone.

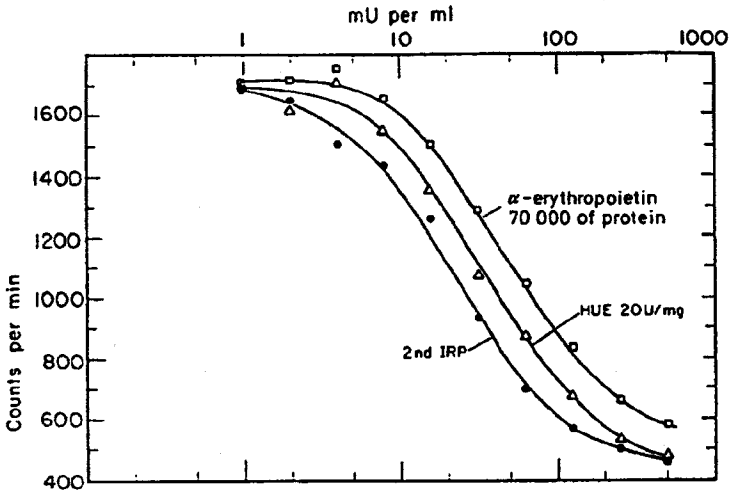

Fig. 4. Dilution curves for pure erythropoietin (70,400 U/mg) and a crude human urinary erythropoietin extract (HUE 20 U/mg) in comparison with the second International Reference Preparation (2nd IRP) for Human Erythropoietin

We have also noted that desialated erythropoietin reacts with anti-erythropoietin with the same avidity as does intact erythropoietin. This observation was also true in an earlier radioimmunoassay attempt [2].

RESULTS

Preliminary Radioimmunoassay Results

Only preliminary results are presented for the radioimmunoassay described here used on selected plasma and serum samples. In some cases, these samples were picked, in particular, because of critical discrepancies seen in a previous radioimmunoassay attempt for erythropoietin [3]. These discrepancies will be pointed out in this discussion.

In the present radioimmunoassay a sample from a pool of normal male serum gave a value of 23 mU/ml, whereas a sample from a pool of normal female serum gave 21 mU/ml. These numbers are some 4–5 times greater than those seen previously [3].

A sample from a pool of serum from patients without kidneys, assayed by the present method, gave a value of approximately 20 mU/ml instead of the very high values seen previously by radioimmunoassay [3]. This is certainly one of the gross discrepancies found with the previous radioimmunoassay, since no bioassayable erythropoietin could be detected in the serum of such anephric patients.

Table 1.

	Hemoglobin (g/100 ml)	Erythropoietin (mU/ml)
Paroxysmal nocturnal hemoglobinuria (Female)	5.8	2.750
	6.1	3.750
	6.5	4.000
Fanconi's anemia (Female)	6.5	4.600
	6.7	5.000
	7.1	5.200
	7.8	2.500
	8.6	2.000
Diamond-Blackfan syndrome (Male)	4.7	17.500
	6.0	9.000
	6.4	10.000
	7.7	4.100

In Table 1 are presented results on the plasma of three severely anemic patients. Very high erythropoietin values are seen in all three patients. Another gross discrepancy found with the previous radioimmunoassay lay with the value observed in the Fanconi's anemia listed in Table 1. Plasma from this patient always resulted in radioimmunoassay values within the normal range and yet the bioassay values always indicated very high erythropoietin levels, similar to those seen in this table.

In passing, it should be pointed out that the erythropoietin level observed in the serum of anephric patients (20 mU/ml) should be compared with the values seen in Table 1, since the anephric patients have an anemia of similar magnitude.

In Figure 5 are presented the results, in a normal individual, of a serial bleeding over a 3-day period with the blood returned on the 4th day. The serum erythropoietin concentration increases from 20 mU/ml to a peak value of 68 mU/ml and then returns to normal values after the return of the blood. These values are greater than those observed with the previous erythropoietin radioimmunoassay by a factor of approximately five [3]; however, the same relative changes were observed. Also, although it is not presented here, bioassay of urine concentrates throughout this study revealed the same relative changes as those seen by radioimmunoassay of the serum.

In the previous radioimmunoassay it was not possible to show any competition with any animal erythropoietin, either in the form of serum or as an extracted preparation. This included the sera from primates with known high bioassay values. We observed, however, that an extract of sheep and rabbit erythropoietin results in appreciable competition in the present assay. Dilutions of sera from rats and mice also compete in the present erythropoietin radioimmunoassay. The normal mouse serum corresponds to approximately 40 mU/ml and the normal rat serum to approximately 25 mU/ml. The erythropoietin concentration in the serum from a rat exposed to a simulated altitude

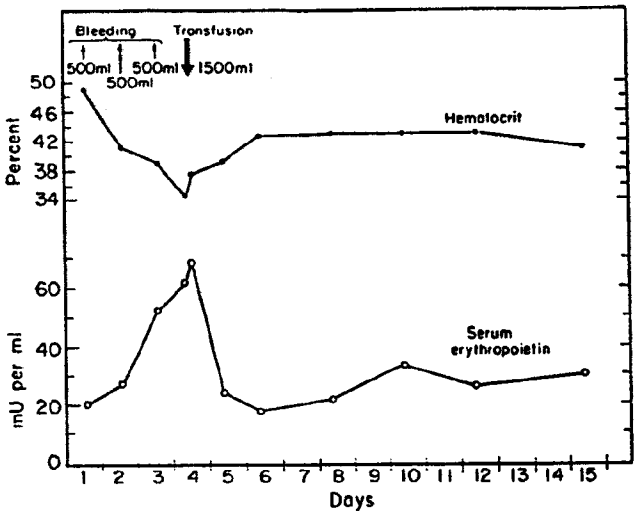

Fig. 5. Radioimmunoassay serum values in a normal individual during bleeding and transfusion

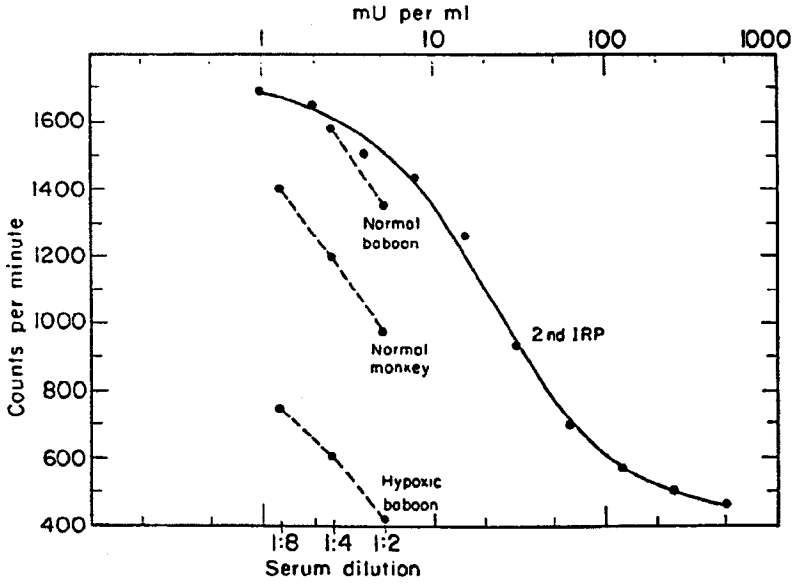

Fig. 6. Dilutions of monkey serum and baboon plasma in comparison with a standard curve for human erythropoietin. 2nd IRP. second International Reference Preparation

of 20,000 ft for 24 h is increased to approximately 500 mU/ml. Dilutions of monkey serum and baboon plasma also show competition in the erythropoietin radioimmunoassay presented here, as seen in Figure 6. This monkey serum corresponds to approximately 56 mU/ml. The normal baboon plasma corresponds 20 mU/ml, whereas the hypoxic baboon plasma corresponds to an erythropoietin concentration of approximately 450 mU/ml.

The removal of all of the problems that existed with the previous erythropoietin radioimmunoassay has been accomplished with only one essential change, i.e., the use of *pure erythropoietin* for the labeled reactant. The anti-erythropoietin antiserum used throughout the work with both radioimmunoassays has remained unchanged. One cannot leave these data, however, without speculating on the reasons for the differences observed. The high values previously seen in the serum from anephric patients were probably due to certain non-erythropoietin materials which exist in high concentration and to the presence of similarly labeled contaminants which had not been removed by the procedure employed. The lower radioimmunoassay values previously seen in the Fanconi's anemia in the face of high biologic activity are more difficult to explain. However, the answer may lie in the relatively drastic technique used for the dissociation of the labeled erythropoietin-anti-erythropoietin complex. This acidification-heat treatment may have altered the steric configuration of the labeled erythropoietin molecule in such a way as to make some of the antigenic sites unavailable to the antibody. This possibility may also be applicable to the previous inability of non-human erythropoietin to compete.

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Discussion

MILLER: With your earlier labeled erythropoietin preparation, you found that both polycythemia vera and secondary polycythemia had the same serum erythropoietin concentration. Have you had an opportunity to repeat those two groups?

GARCIA: No. I haven't really processed enough samples with the present assay, but I certainly have an indication that the polycythemia vera sera will be less than normal and secondary polycythemia will be more than normal by quite a bit.

MILLER: Have you had the opportunity to compare your bioassay with radioimmunoassay (RIA) in very anemic patients who obviously have high levels by bioassay? Is there the same amount of erythropoietin by RIA in such patients?

GARCIA: I haven't done that tight a comparison with the present RIA, but that patient with Fanconi's anemia (Table 1), did show levels by RIA in the range of the bioassay results.

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FLIEDNER: In one table you showed that in anephric patients you had an erythropoietin level of 261. How is that possible?

GARCIA: I did see that result with my earlier RIA attempt. However, using Dr. GOLDWASSER's pure erythropoietin as a labeled reactant, I do not see high levels in anephric patients. One of the possibilities that had occurred to me, in trying to explain the high values seen earlier in anephric patients, was that proerythropoietin could be biologically inactive but immunologically reactive, similar to the situation I've seen with desialated erythropoietin, which has no biological activity in the polycythemic mouse but in the immunoassay shows its full reactivity.

FLIEDNER: But can I discard that old data?

GARCIA: Yes.

EAVES: Have you had a chance to look at fetal sera?

GARCIA: No.

MOORE: I'm puzzled. Dr. GOLDWASSER told us at the Cold Spring Harbor Meeting last year that you could not radioiodinate erythropoietin *without* its losing its biologic activity, particularly with lactoperoxidase. Was there anything that you did other than the very conventional lactoperoxidase technique?

GARCIA: No, but I have not been concerned about biologic activity of erythropoietin. All I'm concerned with is that it retains its immunologic activity. It's true, Dr. GOLDWASSER has iodinated erythropoietin with a variety of techniques and feels he has lost the biologic activity of erythropoietin with all the techniques so far, and this is, perhaps, pertinent to the remark that I think Dr. ROBINSON made this morning about doing kinetic studies with labeled erythropoietin. One would want to be assured that the labeled material retained its biologic activity, and this is also true for anyone considering receptor-type work. In my case, I'm not concerned that it has its biologic activity as long as it has retained its immunologic activity.

PRICE: Unless, I misunderstood your scheme for immunologic purification, the antisera that you were using left behind a biologically active erythropoietin-like material. If that's correct, then did the sera not recognize all that may be biologically recognized as erythropoietin?

GARCIA: I may have misled you. I've not done any biologic activity studies on material that was carried through that scheme. I have done other studies where erythropoietin was combined with antibody to erythropoietin, completely neutralizing its biologic activity, and then if that material is treated by the Borsook procedure, which is an acidification to pH 5.5, then heating in a

boiling water bath for 5–10 min, one can completely recover the biologic activity of erythropoietin by such a scheme.

BRECHER: Did I understand, Dr. GARCIA, that now you can measure normal erythropoietin levels in normal serum?

GARCIA: Not only normal, but we can also measure erythropoietin in less than normal concentrations.

FEINENDEGEN: Since you can measure the erythropoietin with such a degree of sensitivity, I wonder whether you have measured the amount of extraction of erythropoietin from your serum by a certain cell population? For example, a given bone marrow fraction may extract a larger amount of erythropoietin than another cell population which does not have receptors for erythropoietin.

GARCIA: Yes, theoretically, one could reassay the medium after interaction. We have not done that, perhaps we should! Are you saying that membrane receptors do not require the intact biologic activity of a hormone?

FEINENDEGEN: What else? The molecule which is responsible for membrane binding does not necessarily have to be the same site that exerts the biologic activity. We don't know that.

BRECHER: Did you say that you now do not find any erythropoietin in anephric patients?

GARCIA: No. I've just done this one pool of serum and that read, as I said, "20 mU/ml." I would want to do a lot more before saying that it's in the normal range or below normal. But one thing that did occur to me in preparing for this presentation concerns the anemia in such patients. They are really quite anemic and correspond to anemias that have erythropoietin levels in units/ml. Perhaps they should be better compared with those rather than with normal ones.

BRECHER: The anephric patients certainly still make red cells.

GARCIA: I don't know anything about the care of such patients, whether they're continually transfused.

CRONKITE: When they're adequately dialyzed, as we've seen in a clinic here on Long Island, and kept in good health, their transfusion requirements terminate because they make perfectly normal red cell masses in spite of the absence of kidneys.

GARCIA: Are you saying, Dr. CRONKITE, that they're not *anemic* then?

CRONKITE: Not when they're adequately dialyzed.

MILLER: Are you saying that they have a normal hematocrit?

CRONKITE: Yes, in the Nassau County Medical Center, they come up with practically normal hematocrits when they're very carefully and adequately dialyzed.

FLIEDNER: Yes, but in these circumstances you might also remove a lot of inhibitory factors in the low molecular range with the dialysis.

CRONKITE: It's a possibility. Certainly, the kidney is not the sole source of erythropoietin.

BRECHER: Is there red cell regulation without erythropoietin?

CRONKITE: Maybe there is! Has anyone yet found a condition in which there exists no erythropoietin? Or an abnormal erythropoietin? That disease is still being sought, I believe.

FLIEDNER: Thymus aplasia, pure red cell aplasia that we associate with anti-erythropoietin activity.

CRONKITE: That would be a very fascinating case to study.

SHADDUCK: My understanding is that virtually every red cell aplasia is associated with an IgG inhibitor and that the IgG inhibitor is directed against erythroid progenitor cells in varying degrees of development. To my knowledge, there are no case reports in which the antibody appears to be directed against the hormone.

GARCIA: I have to say that I have not seen any convincing evidence for the presence of an antibody to erythropoietin in any clinical situation.

FLIEDNER: We talked this morning about where erythropoietin affects erythropoiesis, at the level of the stem cell, at the level of the differentiating cells, etc. Would you visualize that a very purified erythropoietin would finally end up doing only *one* thing, and that what we have studied so far as erythropoietin response is really a mixture of various erythropoietins acting at different levels of differentiation?

GARCIA: I don't know how to answer. I've only thought of *one* erythropoietin, not various erythropoietins.

LAJTHA: You have really created a new definition of erythropoietin when you define it as that which dilutes your possibly inactivated labeled erythropoietin binding to the antibody.

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BURLINGTON: What is different in your new radioimmunoassay procedure from your earlier one?

GARCIA: The two materials that I used to label: The earlier material was stated to have a specific activity of 8,000 U/mg. The recent material of Dr. GOLDWASSER's has a specific activity of 70,000 U/mg. The antiserum we use is the same. the unlabeled erythropoietin used in the dilutions to construct the standard curves is the same. It is the use of the GOLDWASSER preparation as the competitive. labeled antigen which is new. and the radioimmunoassay is only as good as the antigen used for labeling.

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