Exhibit 32

to the Declaration of Cullen N. Pendleton in Support of Amgen's Opposition to Roche's Motion for Summary Judgment that Claim 7 of the '349 Patent is Invalid Under 35 USC §112 and is Not Infringed

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RADIOIMMUNOASSAY OF HUMAN ERYTHROPOIETIN*

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Abstract

RADIOIMMUNOASSAY OF HUMAN ERYTHROPOIETIN.

A radioimmunoassay for the glycoprotein hormone "erythropoietin" has been developed using a purified human urinary erythropoietin preparation and an anti-erythropoietin antiserum prepared in rabbits. The erythropoietin used for labelling with ^{125}I was freed from some contaminating protein by G-150 Sephadex gel filtration after first absorbing with an antiserum prepared against normal human urinary protein. The erythropoietin peak was then combined with an anti-erythropoietin antiserum and again placed on the Sephadex column. The labelled erythropoietin γ-globulin complex, thus separated, was dissociated by an acidification-heating procedure which freed the labelled erythropoietin. With the double antibody technique, this labelled erythropoietin was then used in a radioimmunoassay of human plasma erythropoietin with the first international reference preparation as a standard. This radioimmunoassay for human erythropoietin is more sensitive than the standard bioassay for human erythropoietin by a factor of approximately 10. With this radioimmunoassay scheme, plasmas from normal individuals always give low values with an average for a series of males of 4.9 mU/ml and females of 4.3 mU/ml. Plasmas from anaemic individuals always give higher values, although usually not as high as the bioassay for erythropoietin would indicate. Support for the validation of the radioimmunoassay for human plasma erythropoietin was obtained by the parallel relationship with the standard of halving dilutions of a high erythropoietin plasma. Further, bleeding of a normal individual increases the radioimmunoassayable erythropoietin level and return of the blood decreases it. Although this response in plasma is below the detectability of the bioassay for erythropoietin, the bioassay of concentrates of urine maintained the same relative picture as that seen by the radioimmunoassay of the plasma. The radioimmunoassay appears to be specific for human erythropoietin, with no competition afforded by several animal erythropoietins. Although removal of the sialic acid completely destroys the biological activity, it does not interfere with the radioimmunoassay of human erythropoietin. Further details of this radioimmunoassay, as well as its application to the variety of clinical material, are discussed.

INTRODUCTION

The maintenance of the volume of red blood cells in the normal individual is exquisitely controlled. If one loses blood, the marrow increases its production of red blood cells and, when the normal level is reached, the production rate returns to normal. On exposure to high altitude the body recognizes the need for more red blood cells in order to maintain its tissue oxygen requirements, and so red blood cell production is increased above normal, resulting in a polycythaemic state in the individual. After return to lower altitudes, the body recognizes that it has more red blood cells than it needs, and so red blood cell production is depressed. Although several mechanisms have been proposed, it is now generally accepted that the control imposed on the marrow production of red blood cells is mediated by a humoral factor [1]. This hormone has been given the name "erythropoietin". It can

* This work was done under the auspices of the United States Atomic Energy Commission.

be extracted from the plasma and urine of a variety of animal species including the human. In fact, the urine of severely anaemic humans has become one of the best sources of erythropoietin [2].

In assaying for erythropoietin the initial studies consisted of haematocrit, haemoglobin concentration, or reticulocyte studies [3]. Increases in total red cell mass have also been used in the assay of erythropoietin [2]. Later the incorporation of radioiron into red cells became the popular means of assay for this hormone. A variety of recipient animal preparations were used, two such were the hypophysectomized and fasted rats [4]. Both of these preparations have depressed erythropoiesis, and respond quantitatively to the administration of erythropoietin with increased radioiron incorporation into red blood cells. For the past 10 years the polycythaemic mouse has been almost exclusively used for the bioassay of erythropoietin [5]. Such an assay animal, with a haematocrit of approximately 70% will show essentially no radioiron incorporation into red cells. The injection of as little as 0.05 unit of erythropoietin will result in a significant increase in radioiron incorporation. While this bioassay is capable of measuring increased circulating levels of erythropoietin, it is still inadequate for the measurement of plasma levels in normal subjects. It has been used, however, to adequately measure erythropoietin in urinary concentrates of normal individuals [6].

Since the first demonstration of the ability to produce antibody, which will neutralize the biological activity of erythropoietin [7], investigators have been concerned with the applications of immunological techniques to the measurement of circulating levels of erythropoietin. Goudsmit et al. [8] have developed an immunological technique, utilizing an Ouchterlony-type double diffusion system for the assay of human plasma erythropoietin. Also, Lange et al. [9] have utilized a haemagglutination inhibition system for the measurement of circulating erythropoietin levels in normal individuals. Preliminary studies utilizing the more classical radioimmunoassay approach, as described by Berson and Yalow [10], have been presented for the measurement of plasma erythropoietin [11]. The present communication further extends these preliminary studies to a large series of normal sera, haematologic patient sera, column effluents and animal erythropoietins.

RADIOIMMUNOASSAY TECHNIQUE, MATERIALS AND METHODS

Basically the radioimmunoassay depends on the ability of unlabelled antigen to inhibit, by competition, the binding of labelled antigen to specific antibody. The determination of the concentration of the antigen in a sample is obtained by comparison of the degree to which it inhibits the binding of the labelled antigen by antibody with the degree of inhibition obtained by a series of standard antigen dilutions containing known amounts of the antigen. This technique has been utilized in the measurement of a wide variety of hormones and other materials in plasma.

The essential requirements for the development of a radioimmunoassay for a hormone are: (1) the availability of the hormone in a pure form and its ability to accept a radioactive label, (2) the ability to produce specific antibody to that hormone, and (3) a technique for the separation of antibody-bound and non-antibody-bound hormone after a given incubation period.

Labelled erythropoietin

Unfortunately, the state of the biochemistry of erythropoietin has not reached the point where amounts of pure erythropoietin are available for labelling for radioimmunoassay. It is only recently that small amounts of pure sheep plasma erythropoietin have been obtained by Goldwasser and Kung [12] for chemical characterization studies. This had an erythropoietic potency of approximately 9 000 units per mg protein with a calculated molecular weight of 46 000, and was characterized as a glycoprotein with 74% protein and 26% carbohydrate. The carbohydrate consisted of 10% sialic acid, 6% galactose, 4% mannose, 4% glucosamine and 2% glucose. Because of the suggestions that there may be species differences in erythropoietin molecules [11], human erythropoietin was used throughout this radioimmunoassay study.

A small quantity of highly purified human erythropoietin was supplied through the generosity of Dr. Joaquin Espada.[1] This was originally extracted from the urine of severely anaemic patients by either pressure filtration through a collodion membrane [2] or by a benzoic acid precipitation technique [13]. These relatively crude extracts, with specific activities of approximately 20 - 30 units per mg protein, were then purified to specific activities of approximately 8000 units per mg protein by a series of chromatographic steps involving DEAE, hydroxyapatite and Sephadex [14].

The purified human erythropoietin was labelled with ^{125}I, using the method of Greenwood et al. [15]. The ^{125}I was obtained from New England Nuclear Corp., and had specific activities in excess of 100 mCi/ml. Five to 6.5 μg of erythropoietin and 1 - 4 mCi of ^{125}I were used. This resulted in preparations of labelled erythropoietin which usually had specific activities greater than 100 mCi/μg. Any remaining free ^{125}I was separated from the labelled erythropoietin by gel filtration on a small G-50 Sephadex column. The labelled erythropoietin was further purified by G-150 Sephadex gel filtration after first absorbing it with an antiserum prepared against normal human urinary protein. This removed any erythropoietin damaged by the labelling process, and possibly some labelled protein contamination to which antibody was present in the antiserum against normal urinary extract. The labelled erythropoietin peak was then combined with an anti-erythropoietin antiserum and again placed on a G-150 Sephadex column. The labelled erythropoietin, now complexed to γ-globulin, was thus separated from labelled material which was not antigenic with either of the antisera used. The complexed labelled erythropoietin was then dissociated from γ-globulin by acidification to pH 5.0 and then heated in a boiling-water bath for 5 min. This "immunologically purified" labelled erythropoietin was used in the radioimmunoassay for erythropoietin. Details of this preparation are presented elsewhere [11].

Antibody

Antibody to erythropoietin was produced in rabbits immunized with human urinary erythropoietin, obtained by pressure filtration of the urine of severely anaemic patients. Ten milligrams containing approximately

[1] A gift, kindly supplied by Dr. Joaquin Espada, Catedra de Bioquimica, Facultad de Medicina, U.N.N.E., Corrientes, Argentina. The specific activity of this erythropoietin was stated to be about 8000 U/mg.

200 units of erythropoietin were dissolved in water and combined with complete Freund's adjuvant and given in four subcutaneous sites at weekly intervals for 3 - 4 weeks. This procedure usually resulted in erythropoietin neutralizing antiserum in greater than 50% of the rabbits so immunized [11]. Precipitin lines can be demonstrated when anti-erythropoietin antiserum is allowed to react in Ouchterlony plates with a wide variety of purified human plasma protein fractions. However, if the anti-erythropoietin antiserum is absorbed with these proteins, no reduction in the erythropoietin neutralizing ability of the antiserum is observed [16]. Thus, the anti-erythropoietin antiserum contains a mixture of antibodies against a variety of proteins as well as antibody specifically directed against erythropoietin. The anti-erythropoietin activity can be assessed by its effect in completely depressing the ^{59}Fe incorporation into red cells of normal mice. Also, as a corollary to this, the fact that erythropoiesis in normal mice can be depressed by the administration of anti-erythropoietin antiserum must mean that the normal production of red cells is mediated by erythropoietin – that erythropoietin is not just a hormone brought into action in severely anaemic animals or by exposure to extreme high altitudes, but plays a role in the maintenance of 'day-to-day' erythropoiesis. Erythropoietin antibody can also be assessed by combining it with a standard amount of erythropoietin, and assaying in the polycythaemic mouse for any remaining erythropoietin.

In one case, a rabbit which had been previously immunized, as above, with a relatively crude extract of human urinary erythropoietin, was given a single booster immunization of a very small amount (13 μg) of the highly purified human urinary erythropoietin. The antiserum obtained at 9 days after this immunization had a very high neutralizing ability for human erythropoietin. One millilitre of this antiserum will completely neutralize more than 300 units of human erythropoietin. Fractionation of this antiserum on G-200 Sephadex revealed that both the erythropoietin neutralizing ability and the ability to bind labelled erythropoietin resided in the 7-S γ-globulins [11]. This antiserum is used in the radioimmunoassay at a final incubation dilution of 1:1 000 000. No pre-absorption of the antiserum was carried out before its use in the radioimmunoassay.

Radioimmunoassay

Because of the successful use of the double antibody technique for the separation of free and antibody-bound hormone in other hormone radioimmunoassays in our laboratory, this separation scheme was extended to the erythropoietin studies. Goats immunized with rabbit γ-globulin were used as a source for the precipitating second antibody.

The procedure presently being used for the radioimmunoassay of human erythropoietin is as follows: 1 ml of plasma, serum or varying standard erythropoietin concentrations is pipetted into a 15-ml disposable plastic test tube. The erythropoietin standard, consisting of the 1st international reference preparation of human urinary erythropoietin, was diluted by halving concentrations from 100 mU/ml down to 0.78 mU/ml. The diluent for the erythropoietin standard consisted of 5% human serum albumin in 0.05M phosphate buffer at pH 7.5. The human serum albumin was used in an attempt to keep at least this protein constituent similar to that of plasma. This was followed by 2 ml of the immunologically purified labelled human erythropoietin. Approximately 5 000 - 10 000 counts/min containing 0.1 to

0.2 mU of erythropoietin was used. The labelled erythropoietin was followed by 2 ml of a 1:400 000 dilution of rabbit anti-erythropoietin. The diluent for both the labelled erythropoietin and the anti-erythropoietin consisted of 0.05M phosphate buffer at pH 7.5 with 1% bovine serum albumin added. At present an incubation period of 4 - 5 days at 4°C is being used. After the incubation period, 1 ml of a 1:10 dilution of normal rabbit serum was added as a source of carrier rabbit γ-globulin. This was followed by an amount of goat anti-rabbit γ-globulin serum which had previously been determined would maximally precipitate the rabbit γ-globulin in the test tube and any labelled erythropoietin which was antibody bound. After a 2-hour period, the samples were centrifuged at 700 × g in a refrigerated centrifuge for 30 min, and the supernatant decanted. The ^{125}I radioactivity in the precipitates was then counted in a Nuclear Chicago automatic scintillation well-type counter. Curves are plotted using semi-logarithmic paper with the erythropoietin concentrations on the logarithmic scale and the counts per minute of labelled erythropoietin which is bound to antibody on the linear scale. All samples of plasma or serum were maintained in the frozen state until they were used in an assay.

RESULTS AND DISCUSSION

A curve of a typical radioimmunoassay for human erythropoietin, using the procedure as outlined above, is presented in Fig. 1. On the ordinate is presented the per cent of the total labelled erythropoietin which is antibody bound for each dilution of the 1st international reference preparation of human erythropoietin on the abscissa. With the antibody concentration and the incubation period used, the percentage of labelled erythropoietin, which is bound, varied between 40 and 70% when no unlabelled erythropoietin was added. The range covered by this curve is better than a decade below the lowest erythropoietin concentration detectable by the most sensitive bioassay for erythropoietin. For this study the acceptable range was considered to be from 1 to 100 mU of the 1st international reference preparation of human erythropoietin per ml. An opportunity for comparison with the 2nd international reference preparation gave a similar curve. The separated 7-S γ-globulin of the antiserum can equally be used in the radioimmunoassay, and will result in a similar curve. Removal of the sialic acid, by use of the enzyme neuraminidase, does not appear to interfere either with the labelled erythropoietin or the unlabelled erythropoietin used for the development of the standard curve, although this treatment completely destroys the biological activity of erythropoietin. The curve for neuraminidase-treated erythropoietin was identical to that obtained with non-neuraminidase-treated erythropoietin.

This radioimmunoassay for erythropoietin has been used in some preliminary studies on selected clinical material. A summary of these results is presented in Table I. A large series of serum samples from normal individuals was obtained from a local health screening program. The average erythropoietin concentration measured in 457 females was 4.3 mU/ml as compared to 4.9 for 311 males. This small difference was significant with a p-value of < 0.02. Other previous small groups of normal subjects always have shown the same relative difference. Comparison of heparinized plasma and serum taken at the same time from normal individuals revealed no difference in assay results. However, although the

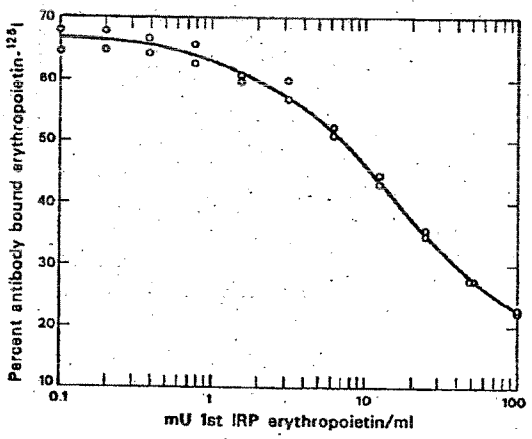

FIG. 1. A typical standard curve for the radioimmunoassay of human erythropoietin.

TABLE I. ERYTHROPOIETIN CONCENTRATION IN NORMAL AND SELECTED PATIENT SERA

	Number	Haemoglobin (g/100 ml)	Erythropoietin (mU/ml ± SE)	
Normal male	311	15.1	4.9	0.2
Normal female	457	13.6	4.3	0.2
Iron deficiency	37	9.0	32.3	5.4
Haemochromatosis	68	13.9	11.6	0.4
Polycythaemia vera	30	16.8	8.9	0.9
Secondary polycythaemia	13	17.9	8.5	0.7
Anephric	10	5.8	261	41
Kidney disease (on haemodialysis)	19	6.4	211	21

difference was small, serum samples taken in the morning from six normal subjects all gave higher erythropoietin values than serum samples taken in the afternoon on the same subjects.

Serum samples obtained from 37 patients with iron deficiency anaemia showed an increased erythropoietin level of 32.3 mU/ml. This was significantly different from the normal male and female levels, with a p-value of < 0.001. Samples of serum from a series of patients undergoing repeated phlebotomy as part of their treatment for polycythaemia or haemochromatosis

were assayed and the results are also presented in Table I. The erythropoietin levels in these patients, as measured by radioimmunoassay, are also significantly increased over that seen in the normal individuals with a p-value of < 0.001.

The most strikingly increased levels of immunoreactive erythropoietin observed were in severely anaemic kidney patients, either anephric patients or patients with kidney disease undergoing haemodialysis. Although a few of the patients with kidney disease did show detectable levels of erythropoietin by bioassay, none of the anephric patients ever showed detectable erythropoietin levels in the polycythaemic mouse assay. These results are thus similar to that obtained with neuraminidase-treated erythropoietin, where, although the immunoreactive erythropoietin activity is retained, the biological activity in the polycythaemic mouse assay is completely lost. No other clinical material so far examined by the radioimmunoassay for erythropoietin has given such high results as that observed in patients with kidney disease with the exception of three out of four patients diagnosed as having chronic lymphatic leukemia, who also showed similarly high values.

Generally, plasma or serum from anaemic individuals give higher immunoreactive erythropoietin levels than that observed in normal subjects. Although, as yet, no strict correlation has been obtained with sera which fall within the bioassayable range. In these samples the erythropoietin level, as determined by the radioimmunoassay, is not as high as the bioassay would indicate. In some cases this difference may be greater than a factor of 10. In this respect, attempts so far to validate the radioimmunoassay with the existing bioassay for erythropoietin have been disappointing. Not only are there examples of high immunoreactive erythropoietin with no bioassayable erythropoietin, as seen in the anephric patient, but many of the anaemic sera are not nearly as high in the radioimmunoassay as the bioassay would indicate. Perhaps the work of certain investigators concerning the existence in plasma and serum of inhibiting or enhancing factors may have a bearing on this point [17]. In the bioassay for erythropoietin such factors could express themselves by modifying the biological activity of erythropoietin molecules; whereas, a radioimmunoassay for erythropoietin would be correlated only with the number of erythropoietin molecules present in the sample. The possible existence of various erythropoietin molecules or various forms of one erythropoietin molecule by aggregation or fragmentation must also be considered. In this respect, Dukes et al. [18] have observed great differences in the biological activity of various erythropoietin preparations when compared in the polycythaemic mouse assay with an in-vitro system measuring ^{14}C-glucosamine incorporation into rat bone marrow cells, and an in-vitro system measuring the incorporation of ^{59}Fe into haem of rat bone marrow cells.

Evidence supporting the identity of that immunoreactive material in plasma or serum with the erythropoietin standard can be obtained by showing that the slopes of the radioimmunoassay curves obtained by both materials are parallel, indicating that both materials react with the antibody with the same affinity. In Fig. 2 are presented the results of halving dilutions of high immunoreactive erythropoietin serum samples obtained from anephric patients or patients with iron deficiency anaemia. Some of these sera have been treated using the acidification-heating procedure described in the method for the preparation of the labelled erythropoietin. The curves generally show a parallel relationship with the 1st international reference preparation of

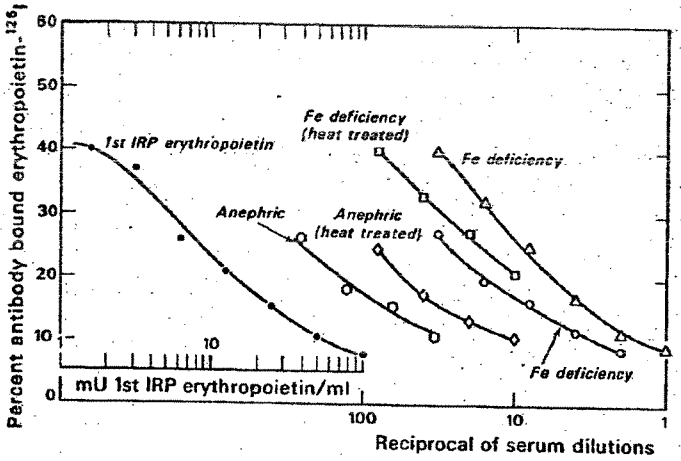

FIG. 2. Dilution-response curves of anephric and iron-deficient sera compared with the 1st international reference preparation of human erythropoietin.

human erythropoietin. This is interpreted as evidence supporting the hypothesis that the material in these sera which inhibits the binding of labelled erythropoietin to its antibody is the erythropoietin molecule itself. Although not presented in the figure, the addition of rabbit or sheep erythropoietin up to concentrations of 1000 mU/ml did not result in any inhibition of the binding of labelled human erythropoietin to the antibody, thus supporting the existence of some molecular differences in the erythropoietin molecules of these species from human erythropoietin.

In spite of the evidence presented supporting the identity with erythropoietin of that material measured in plasma or serum by the radioimmunoassay procedure, it was hoped that further evidence would be gained if it were possible to show that physiological manipulation of the normal individual would produce the expected changes in immunoreactive erythropoietin. In Fig. 3 are presented erythropoietin values throughout a bleeding and transfusion study in a normal individual. The withdrawal of three units of blood over a three-day period reduced the haematocrit from 49 to 34.5%. The radioimmunoassayable serum erythropoietin level increased from 3.7 to 8.9 mU/ml following the bleeding, and then decreased following the return of the blood. Bioassay of these serum samples in polycythaemic mice did not result in any significant increase in radioiron incorporation in red cells. However, as shown in the lower frame of Fig. 3, the bioassay of 24-hour concentrates of the urine resulted in a mirroring in the urine, by bioassay, of the same relative picture as observed by the radioimmunoassay of the serum.

Finally, the radioimmunoassay has been used to monitor column effluents. In Fig. 4, 8 mg of a crude human urinary erythropoietin extract, with a tracer amount of the immunologically purified human erythropoietin

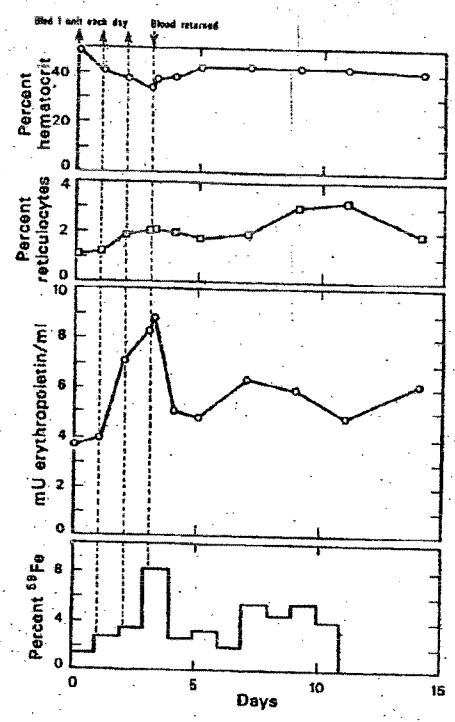

FIG. 3. Plasma erythropoietin values in a normal individual during and following bleeding and transfusion. The bar graph in the lower frame indicates the percentage of ^{59}Fe incorporation in polycythaemic mice following the injection of a 0.5-ml urine concentrate per mouse (equivalent to 8.5-hour collection of urine).

added, was placed on a G-200 Sephadex column. In the lower frame (b) it will be noted that, whereas most of the protein occurs at the void volume, the labelled erythropoietin forms a symmetrical peak at approximately 1.8 times the void volume. In the upper frame (a) both the biological activity, as measured by the radioiron incorporation into red cells of polycythaemic mice and the immunological activity, as measured by the radioimmunoassay, occur in the same area of the elution pattern as did the tracer amount of labelled erythropoietin.

One of the most disturbing and, at the same time intriguing observations presented here, is the extremely high radioimmunoassayable erythropoietin concentration observed in the serum of severely anaemic anephric patients. At the same time no bioassayable erythropoietin is present in this serum. Although not conclusive, the best evidence is that the kidney normally is the main source of erythropoietin. At the same time extraction of kidney tissue has been disappointing in supporting this conclusion.

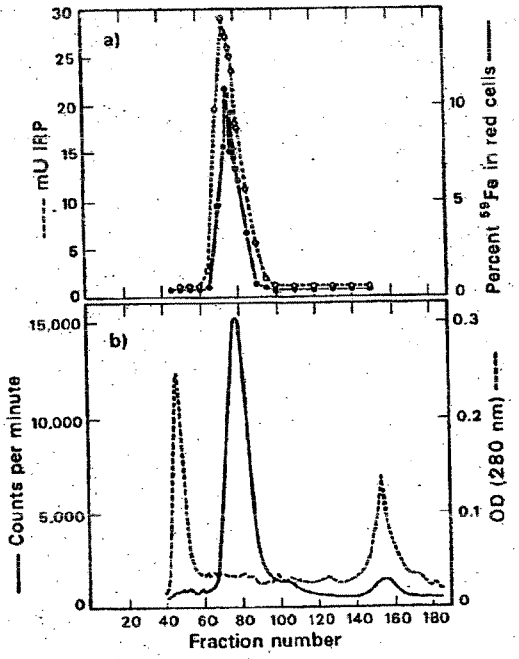

FIG. 4. Human urinary erythropoietin with a tracer amount of labelled erythropoietin added and placed on a 90 cm × 2.5 cm G-200 Sephadex column and eluted in 3-ml fractions.

It would seem consistent with the above observation that biologically inactive, but immunologically reactive, erythropoietin may be produced elsewhere in the body, and that the role of the kidney may be one of activating this material. This activation could occur by an enzymatic addition of sialic acid to a biologically inactive asialoerythropoietin or possibly by an enzymatic cleavage of a biologically inactive pro-erythropoietin, both of which could be immunologically active in the radioimmunoassay. Such an enzymatic mechanism may be depressed in the polycythaemic mouse used for the bioassay of erythropoietin.

SUMMARY

A radioimmunoassay for human erythropoietin has been developed using a purified human urinary erythropoietin preparation and an anti-erythropoietin antiserum prepared in rabbits. The erythropoietin used for labelling with ^{125}I was freed from some contaminating protein by G-150 Sephadex gel filtration after first absorbing with an antiserum prepared against normal human urinary protein. The erythropoietin peak was then combined with an anti-erythropoietin antiserum and again placed on the Sephadex column.

The labelled erythropoietin γ-globulin complex, thus separated, was dissociated by an acidification-heating procedure which freed the labelled erythropoietin. By using the double antibody technique, this labelled erythropoietin was then used in a radioimmunoassay of human erythropoietin with the 1st international reference preparation as a standard. This radioimmunoassay for human erythropoietin is more sensitive than the standard bioassay for human erythropoietin by a factor of approximately 10. With this radioimmunoassay scheme, sera from normal individuals always give low values with an average for a large series of males of 4.9 mU/ml and females of 4.3 mU/ml. Sera from anaemic individuals always give higher values, although usually not as high as the bioassay would indicate. Sera from anephric patients gave very high erythropoietin levels by the radioimmunoassay; whereas, those same sera show no activity in the bioassay for erythropoietin. The significance of the radioimmunoassay in its application to this clinical material is discussed.

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DISCUSSION

P. Mary COTES: I would like to congratulate you, Dr. Garcia, on your excellent use of the highly purified erythropoietin from Dr. Espada. It is relatively easy to suggest explanations for the high radioimmunoassay estimates obtained with sera from anephric subjects and those with renal disease, both of which groups have low levels of serum erythropoietin (< 50 mIU/ml) undetectable by bioassay. I am disconcerted by the low radioimmunoassay estimates (32 mU/ml) obtained with sera from patients with iron-deficiency anaemia, who presumably showed relatively high levels of serum erythropoietin by bioassay.

Also, I wonder how one explains the finding, by radioimmunoassay, of essentially identical levels of serum erythropoietin in patients with polycythaemia rubra vera and those with secondary polycythaemia. We might expect to find higher levels in the latter group.

Finally, I would like to ask how much radioiodinated erythropoietin suitable for use in assays you obtained after purification of each 5 μg portion (40 IU) taken for radioiodination.

J.F. GARCIA: I, too, am disappointed in the low radioimmunoassay values which I have generally observed in anaemic individuals. However, as a group, the iron-deficiency anaemias are perhaps closer in radioimmunoassay activity to their biological activity than most.

Regarding the polycythaemic patients, one would certainly expect the polycythaemia rubra vera patients to have lower values than the secondary polycythaemia patients. In fact, the first two polycythaemia rubra vera patients I examined were in the low normal range. Additional studies, however, brought the average up to the figure that I have shown. I should point out that all the polycythaemic patients were at various stages of treatment by phlebotomy.

Finally, as regards the immunological purification of the labelled erythropoietin the amount of labelled material recovered in the final step is in the neighbourhood of 5 - 10%.

Rosalyn S. YALOW: The values in the anephric subjects are so high that it should be possible to fractionate on some system, perhaps Sephadex gel, in order to determine the immunoactivity in regions other than that known for intact hormone.

J.F. GARCIA: Yes, I agree, and I have in fact already begun such studies using Sephadex. However, not all the fractions have been assayed as yet, so I am unable to comment further.

L.E.M. MILES (Chairman): Urinary colony-stimulating factor is apparently physico-chemically similar to erythropoietin. I am wondering whether it contaminates or cross-reacts in your very elegant purification and assay procedures, do you know by any chance?

J.F. GARCIA: No, I am sorry I do not.