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Solid-Phase Binding Assay for Erythropoietin Antibodies[1] (41366)

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Abstract. The affinity of phytohemagglutinin-E (PHA-E) and wheat germ agglutinin (WGA) for erythropoietin was exploited to develop a solid-phase assay for erythropoietin antibodies. Purified PHA-E or WGA was covalently coupled to polystyrene tubes with the water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. Coupling was highly reproducible; approximately 8 μg of lectin was bound covalently per tube. For the assay, the lectin-derivatized tubes were loaded with erythropoietin, washed, and incubated with the immune serum to be tested. The presence of bound antibody was detected by adding ^{125}I-labeled staphylococcal protein A (SPA) and measuring residual ^{125}I-SPA after washing. In the absence of antigen, antibody was not bound in the lectin-derivatized tubes. Furthermore, in the presence of erythropoietin, nonimmune serum from a variety of species failed to bind to the tubes. The particular immune serum used in this study could be detected with as little as 5 μg of bound antigen. Since an impure preparation of human urine erythropoietin was used both as the antigen to produce the antierythropoietin immune serum and as the antigen for the assay, the specificity of the assay for erythropoietin antibodies could not be definitively established. Nevertheless, this solid-phase assay permits rapid screening (3.0 hr) of antisera for reactivity with human urine proteins and should be useful for identifying antibody-producing clones formed by somatic cell hybridomas when a partially purified urine erythropoietin preparation is used as the antigen.

Progress in erythropoietin research has been retarded by lack of adequate quantities of pure erythropoietin. Normally, only minute amounts of the hormone are excreted in the urine and a cumbersome bioassay is required for its detection and quantitation. Since the hormone has a molecular weight similar to that of 12 known urine glycoproteins and an isoelectric point similar to 7, the use of conventional techniques for separating proteins on the basis of size and charge is not an efficient method for isolating erythropoietin. Purification of erythropoietin using conventional techniques was first achieved by Miyake, *et al.* (1). This was a major contribution but their protocol required 2550 liters of erythropoietin-rich urine from severely anemic patients. Owing to current transfusion practice in this country, quantities of crude starting material of similar magnitude and specific activity are virtually unavailable. Since erythropoietin is a glycoprotein, affinity chromatography with immobilized PHA-E or WGA has been used as an alternative method for partial purification of erythropoietin from urine (2–4).

Immunoaffinity chromatography which has been successfully used to purify a variety of proteins present in minute quantities in body tissues or fluids (5–8) would be a logical approach for the purification of erythropoietin. However, lack of sufficient quantities of the purified hormone for use as antigen has prevented application of this technique to the purification of erythropoietin. The development by Kohler and Milstein (9, 10) of the hybridoma system for production of monospecific antibodies obviates the need for a pure antigen, if a suitable technique for rapidly screening antibody producing clones is available. Since the biological activity of the antibodies pro-

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duced is not predictable, screening assays which rely on a specific activity such as neutralization or cytotoxicity are unsuitable. Furthermore, the currently available bioassays for erythropoietin (11–14) are too time consuming, expensive, or insensitive to even be considered for routine screening purposes. To date, however, a solid-phase binding assay for the hormone or its antibodies has not been developed.

Based on our observations that erythropoietin can be separated from 90% of contaminating urine proteins by chromatography on immobilized lectins, we have developed a rapid solid-phase binding assay for erythropoietin antibodies. This solid-phase assay should facilitate the detection of erythropoietin antibodies produced by hybridoma technology and could also be employed to facilitate screening for antibodies against other glycoproteins for which a lectin-protein interaction can be established.

Materials and methods. *Reagents.* The E isolectin of PHA was purified from crude PHA (PHA-P, Difco, Detroit, Mich.) by affinity chromatography on agarose–thyroglobulin (15). Wheat germ agglutinin was affinity purified on *N*-acetylglucosamine–Sepharose 4B (16).

Staphylococcal protein A (SPA) was obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methane-*p*-toluenesulfonate were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Guanidine hydrochloride (ultrapure) was purchased from Schwartz/Mann, Orangeburg, New York; *N*-acetyl glucosamine from Pfanstiehl, Waukegan, Illinois; *N,N*-diacetyl chitobiose from Polysciences, Warrington, Pennsylvania; and human and bovine serum albumin (BSA) (Fraction V) from Sigma Chemical Corporation, St. Louis, Missouri. All other chemicals were reagent grade.

Buffer. Phosphate-buffered saline (PBS), pH 7.4–7.5, was prepared by dissolving 8 g sodium chloride, 1.13 g Na_2HPO_4, and 0.2 g KH_2PO_4 in one liter of distilled water. For use in antibody reactions, the PBS contained 0.001 *M* $MgCl_2$ and 0.0015 *M* $CaCl_2$ and either 0.1% gelatin or 0.1% BSA.

Erythropoietin. Human urine erythropoietin (Lot H-29, specific activity 50 units/mg protein) was obtained from the Blood Disease Branch of the National Heart, Lung and Blood Institute. An erythropoietin preparation of low specific activity (Lot J.R., 0.05 unit/mg protein) was prepared from the urine of an anemic patient according to the method of Lowy (17). Sheep plasma erythropoietin (Lot 3023-1, specific activity 6.7 units/mg protein) was purchased from Connaught Laboratories, Ltd., Willowdale, Ontario, Canada.

Erythropoietin antiserum. Antibodies to erythropoietin were prepared by immunizing rabbits with a crude erythropoietin extract from human urine (specific activity 3.2 units/mg protein) according to the method of Schooley *et al.* (18). A second human urine erythropoietin preparation (210 units/mg protein) was used to boost antibody titers in rabbits producing antibodies to erythropoietin (19). One milliliter of unfractionated immune serum neutralized approximately 30 units of human urine erythropoietin as determined by the exhypoxic polycythemic mouse bioassay.

Assay for erythropoietin. The exhypoxic polycythemic mouse bioassay was employed to measure erythropoietin activity as previously described (20). Briefly, polycythemia was induced in 20- to 25-g, female Swiss–Webster mice (Buckberg, Tomkins Cove, N.Y.) by continuous exposure to reduced oxygen tension for 2 weeks in dimethyl silicone membrane enclosures (General Electric Corp., Schenectady, N.Y.). Five days after removal from the enclosures, mice with hematocrits greater than 0.62 were injected in groups of 5 with the test material or an erythropoietin standard. Two days later, the mice were injected with 2 μCi of ^{59}Fe as ferrous citrate (specific activity 2–4 μCi/g, New England Nuclear, Boston, Mass.) and after 2 days, blood was obtained for determination of ^{59}Fe incorporation into newly formed red cells from mice with hematocrits of 0.55 or greater. The lower limit of sensitivity of this

bioassay in our laboratory is 0.05 unit of erythropoietin. The erythropoietin activity present in test material was quantitated by comparison with the activity of a standard preparation of erythropoietin (International Reference Preparation).

Radiodination of proteins. PHA-E, WGA, and SPA were iodinated by the chloroglycoluril technique of Fraker and Speck (21). Approximately 100 μg of each protein was reacted for 5 min at 4° with 250 μCi of carrier-free Na[^{125}I]iodide (Amersham, Arlington Heights, Ill.) in a total volume of 100 μl of phosphate buffer, pH 7.0, in 12 × 75-mm glass tubes coated with a 10-μg film of 1,3,4,6-tetrachloro-3α, 6α-glycoluril (Pierce Chemical Co., Rockford, Ill.). The reaction mixture was transferred to a column of Sephadex G-25 (8 × 1 cm) and the protein fraction was separated from free [^{125}I]iodide by elution with phosphate buffer, pH 7.0. The percentage of [^{125}I]-iodide incorporated into TCA-insoluble material was 59% for WGA, 84% for PHA-E, and 92% for SPA. Assuming complete recovery of the SPA, its specific activity was 0.5 μCi/μg protein.

Preparation of assay tubes. Polystyrene tubes (12 × 75 mm, Falcon Plastics, Cockeysville, Md.) were derivatized with PHA-E or WGA according to the method of Turley and Roth (22). The tubes were treated for 1 hr with 1 ml of concentrated H_2SO_4 at 37°, washed extensively with distilled water, and treated with 1 ml of aqueous ammonium hydroxide (30%, w/v) for 24 hr at room temperature. After washing with distilled water, the tubes were incubated with 1 ml of an aqueous solution containing 25 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and varying quantities of either PHA-E or WGA for 48 hr at 37°. After incubation, the tubes were washed three times with PBS, capped, and stored in 2 ml of PBS at 4°. The lectin-derivatized tubes could be stored for at least 10 days without loss of activity.

Antibody assay. One milligram of crude urine erythropoietin (0.05–50 units/mg protein) in 1 ml of PBS was added to lectin-derivatized tubes after removal of the storage buffer. The tubes were capped and incubated at 30° in a shaking water bath for 20 min. After incubation, the erythropoietin was decanted and the tubes were washed three times with PBS. To each tube was added 1 ml of PBS containing 50 μl of a heterologous rabbit antiserum prepared against human urine erythropoietin. The tubes were capped and incubated at 37° for 60 min with constant shaking. After the incubation period, the solution was decanted and the tubes washed three times with PBS. To each tube 1 ml of PBS containing 0.5 μg of ^{125}I-labeled SPA was added, and the tubes were capped and incubated at 30° for 45 min in a shaking water bath. The incubation was terminated by decanting the solution and washing the tubes twice with PBS. Measurements of residual radioactivity in the tubes indicated that two washes were sufficient to remove unbound radioactive material. The quantity of ^{125}I-SPA bound in each tube was determined by counting the tubes directly in a gamma spectrometer and is expressed as a percentage of the total amount of ^{125}I-SPA initially added to the tube.

Results. *Derivatization of polystyrene tubes.* The quantity of lectin coupled to the treated polystyrene tubes in the presence of the carbodiimide was determined by adding a trace amount of ^{125}I-labeled lectin. As shown in Table I for PHA-E, increasing the quantity of lectin in the presence of a fixed amount of carbodiimide resulted in a modest increase in the amount of lectin bound up to a concentration of 300 μg. However, increasing the quantity of carbodiimide up to sixfold did not increase the amount of lectin coupled. Similar results were observed with WGA (data not shown).

The amount of lectin covalently coupled to the tubes was determined by addition of 2 ml of a solution of 8 *M* urea and 10% sodium dodecyl sulfate to the tubes and boiling for 20 min (20). After two washes with PBS, the residual radioactivity was determined. On average, 80% of the PHA-E bound to the tubes was covalently coupled (Table I). For PHA-E, this amounted to 7.8 μg per tube; for WGA 7.3 μg per tube. In the absence of the carbodiimide, lectin was not covalently bound. When 1-cyclohexyl-

TABLE I. COVALENT COUPLING OF PHA-E TO POLYSTYRENE TUBES

μg PHA-E added	μg Bound	μg Covalently coupled
50	7.4	5.9
100	8.3	7.0
200	9.6	7.5
300	11.5	9.5
400	9.9	7.9
500	11.5	9.1

Note. Coupling was performed as described under Methods in a reaction volume of 1 ml and 25 mg of 1 - (3 - dimethylaminopropyl) - 3 - ethylcarbodiimide. There was no covalent coupling of PHA-E in the absence of the carbodiimide.

3-(-2-morpholinoethyl)carbodiimide methane-*p*-toluenesulfonate was substituted for 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, the quantity of PHA-E or WGA covalently coupled to the tubes was negligible. If the tubes were not pretreated with H_2SO_4 and NH_4OH, the efficiency of covalent coupling was reduced by approximately 50%. In the studies to be described, 500 μg of lectin per tube was used for coupling. With the particular batches of PHA-E and WGA used, uniform coupling was achieved in 80 of 81 tubes. When used in the solid-phase binding assay, the coefficient of variation for replicate assays on the same sample was 1.0%.

Requirements of the solid-phase binding assay. Preliminary experiments using PHA-E and WGA covalently coupled to agarose beads revealed that neither lectin bound appreciable quantities of rabbit immunoglobulin or SPA. As shown in Table II, significant quantities of ^{125}I-SPA were bound to the lectin-derivatized tubes only in the presence of an antigen–antibody complex. Blank tubes, polysulfonamide tubes, and tubes treated with carbodiimide alone bound negligible quantities (1–2%) of ^{125}I-SPA.

To determine the degree to which ^{125}I-SPA binding was due to nonspecific binding, we tested the reactivity of nonimmune serum from a variety of species with crude human urine erythropoietin as the antigen. When rabbit, human, horse, fetal calf, newborn calf, goat or mouse serum was used in place of the specific immune rabbit serum, no appreciable binding of ^{125}I-SPA occurred (data not shown).

The reaction between the immune rabbit serum and crude urine erythropoietin was analyzed in several ways. First, the amount of ^{125}I-SPA bound was determined following interaction of the immune serum with two preparations of crude erythropoietin which differed approximately 1000-fold in specific activity. In two separate experiments, binding of ^{125}I-SPA, was identical for both preparations with the rabbit immune serum, irrespective of the lectin employed (PHA-E or WGA) (Table II).

Second, after binding the crude urine erythropoietin to the lectin-derivatized tubes, we used either 4 *M* guanidine–HCl (pH 7.4) for PHA-E or *N,N*-diacetylchitobiose for WGA, to elute bound proteins before exposure to the rabbit immune serum. Exposure to guanidine–HCl re-

TABLE II. SOLID-PHASE BINDING ASSAY FOR RABBIT ANTISERUM TO HUMAN URINE ERYTHROPOIETIN

Additions	Percentage binding of ^{125}I-SPA	
	PHA-E	WGA
Erythropoietin (H-29) plus antiserum	68	72
Antiserum alone	5	11
Erythropoietin (J.R.) plus antiserum	63	71
Antiserum alone	1	4

Note. Binding assays were performed as described under Methods. The quantity of reactants in each tube was 1 mg of the particular erythropoietin preparation, 50 μl of undiluted rabbit antiserum, and 5 μl of ^{125}I-SPA.

TABLE III. EFFECT OF SHEEP PLASMA ERYTHROPOIETIN ON BINDING OF ^{125}I-SPA

Antigen	Percentage ^{125}I-SPA binding	
	PHA-E	WGA
Sheep plasma erythropoietin (6.7 μ/mg)	3.0	4.0
Bovine serum albumin	5.0	8.0
Human urine erythropoietin (H-29)	45.0	44.0

Note. The binding assay was performed as described under Methods.

moved sufficient antigen from the PHA–agarose to reduce the binding of ^{125}I-SPA from 73 to 18%. By contrast, *N,N*-diacetylchitobiose failed to remove sufficient antigen from the WGA–agarose to substantially impair the binding of the immune serum and subsequently ^{125}I-SPA. This was a reproducible but unexpected and unexplained observation since *N,N*-diacetylchitobiose at a concentration of 0.01 *M* quantitatively elutes erythropoietin from WGA immobilized on agarose beads (4).

To exclude the possibility that the urine erythropoietin was binding directly to the tube and not to the immobilized WGA, the following experiment was performed. Treated polystyrene tubes were each derivatized with 1 mg of bovine serum albumin, a protein which does not bind to WGA. The derivatized tubes were incubated with 1 mg of human urine erythropoietin for 20 min at 30°, washed, and incubated with 100 μg of ^{125}I-labeled WGA for 20 min at 30°. The tubes were washed and counted to determine the residual radioactivity. Only 0.1% of the labeled WGA (0.1 μg) was bound indicating that the quantity of urine protein adhering directly to the tube was negligible.

Third, we examined antibody binding using sheep plasma erythropoietin (Step III, 6.7 units/mg protein) as the antigen. As shown in Table III, there was no binding of the rabbit antiserum erythropoietin antibody when the sheep erythropoietin preparation was used as the antigen.

Finally, we determined the amount of erythropoietin bound to lectin-derivatized tubes. Human urine erythropoietin (Lot H-29, 50 units/mg protein) was incubated in a PHA-E derivatized tube for 20 min at 30°. After washing, the hormone bound to the PHA-E was eluted with 4 *M* guanidine–HCl, dialyzed against PBS, and assayed in exhypoxic polycythemic mice. Incorporation of ^{59}Fe in control mice was 0.9 ± 0.5% while incorporation of ^{59}Fe in mice injected with the erythropoietin eluate was 5.9 ± 0.8% ($P < 0.005$), indicating that a minimum of 0.05 unit of erythropoietin was bound per tube by the immobilized PHA-E. (Values for the erythropoietin standard were 0.06 unit, 4.6 ± 0.45%; 0.13 unit, 10.6 ± 1.8%; 0.26 unit, 13.4 ± 1.2%.)

Sensitivity of the solid-phase binding assay. To determine the sensitivity of the binding assay, we varied independently the quantity of antigen employed and the quantity of rabbit immune serum. As shown in Table IV, the assay could detect as little as 2 μg of the crude human urine erythropoietin preparation. The particular rabbit immune serum could be employed at a dilution of 1:32 to 1:64 depending on the lectin used.

Discussion. To develop a solid-phase assay for human urine erythropoietin antibodies, it was necessary to devise a method for immobilizing erythropoietin. Erythropoietin is a glycoprotein rich in sialic acid, galactose, and *N*-acetylglucosamine (23), and the affinity of PHA and WGA for these monosaccharides can be employed to separate erythropoietin in urine or plasma from 90% of the contaminating urine proteins (2, 3). Furthermore, erythropoietin bound to immobilized PHA-E can only be released by 4 *M* guanidine hydrochloride (4). These observations suggested that immobilized lectins, particularly PHA-E, could be used to develop a solid-phase binding assay for

TABLE IV. INFLUENCE OF ANTIGEN CONCENTRATION ON THE SOLID-PHASE BINDING ASSAY

Human urine erythropoietin (H-29) (μg)	Percentage ^{125}I-SPA binding	
	PHA-E	WGA
0	5	8
2	—	15
5	38	28
10	42	31
25	—	34
50	46	33

Note. The binding assay was performed as described under Methods. The quantity of antigen was varied as indicated for each assay.

erythropoietin antibodies. The data obtained in the present study support this contention.

Substantial quantities of PHA-E and WGA could be covalently coupled to polysulfonamidated polystyrene tubes in the presence of a water-soluble carbodiimide. Importantly, the immobilized lectins retained their ability to bind glycoproteins. From the data presented in Table IV, at least 5 μg of erythropoietin-rich human urine protein could be bound per lectin-derivatized tube. Coupling efficiency was uniform from tube to tube and the binding activity of the immobilized lectins was stable for at least 10 days when stored in PBS at 4°. In direct contrast to agarose-bound WGA, it was of interest that urine glycoproteins could not be efficiently eluted by *N,N*-diacetylchitobiose from the WGA immobilized in the polystyrene tubes. The reason for this is unknown but there was no evidence that the urinary proteins were binding directly to the tube walls rather than to the WGA.

Immobilized PHA-E and WGA did not directly bind immunoglobins, SPA, or human or bovine serum albumin. Furthermore, the lectins failed to interact significantly with nonimmune serum from a variety of species. These characteristics ensured a low background and the ability to assay for specific antibodies in the presence of serum-containing tissue culture media.

The solid-phase binding assay was easy to perform and could be completed within 3 hr. It was also sensitive. Fifty microliters of our antibody diluted in 1 ml of the reaction mixture could recognize as little as 2 to 5 μg of protein. Furthermore, the particular immune serum employed in this study could be detected at a dilution of 1:64 with PHA and 1:32 with WGA.

Although agarose-bound PHA-E binds erythropoietin more tightly than does agarose-bound WGA, the reactivity of both lectins in our solid-phase binding assay with the specimens tested was similar. Since in other studies both lectins appear to bind comparable amounts of human urine erythropoietin (4), the choice of lectin as the ligand in the solid-phase binding assay is only a matter of convenience.

The behavior of the binding assay with respect to the antigen and immune serum employed was defined by several experiments. First, in the absence of antigen, the quantity of ^{125}I-SPA bound was negligible. Second, in the presence of antigen, only the specific immune rabbit serum supported the binding of ^{125}I-SPA; nonimmune rabbit serum or sera from a variety of species failed to cause significant ^{125}I-SPA binding. Third, removal of the antigen from the lectin-derivatized tubes with guanidine–HCl markedly reduced the binding of ^{125}I-SPA. Fourth, this particular rabbit antibody which has a very low affinity for sheep erythropoietin failed to bind when a sheep erythropoietin preparation was employed as the antigen.

While it was possible to demonstrate erythropoietin binding in these tubes by bioassay, it was not possible to demonstrate that the immune serum was interacting solely with the hormone in the solid-

phase assay. Thus, ^{125}I-SPA binding was similar when the specific activities of the erythropoietin preparations varied by 1000-fold. This is not surprising because the antiserum was raised against a crude urine concentrate in which erythropoietin was only a minor constituent. This should not, however, diminish the value of the solid-phase binding assay. Once antibody-producing clones are identified, the specificity of the antibodies for erythropoietin can be confirmed by assays designed to examine biological activity.

Although this binding assay was designed to solve a problem related to erythropoietin research, it is obvious that it can be applied to any glycoprotein available only in small quantities and in an impure state, for which there is an established lectin–protein interaction.

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