gen-antibody system. The possibility of using this agglutination test for HCG as a suitable test for pregnancy is being explored.

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Immunochemical Studies of Human Urinary Erythropoietin.* (27191)

JOHN C. SCHOOLEY AND JOSEPH F. GARCIA

Donner Laboratory and Lawrence Radiation Laboratory, University of California, Berkeley

The presence of substances capable of specifically stimulating erythropoiesis has been demonstrated in plasma and urine of anemic animals and of humans with various hematologic disorders. Ion exchange procedures for fractionation and purification of erythropoietin obtained from the plasma of anemic sheep have been described and studies of the biochemical and physical properties of the various fractions have been made(1,2). The available evidence suggests that the active molecule in human urinary concentrates is either a mucoprotein or is intimately associated with a mucoprotein(3). It is not known whether the erythropoietically active material found in human urine is identical to the active material of anemic animal plasma. In general, no species specificity has been found. Concentration of the active material in human urine has been accomplished by ultrafiltration(4) and adsorption on kaolin(5). The present paper concerns an immunochemical study of erythropoietically active concentrates of human urine.

Materials and methods. Erythropoietically active urine obtained from anemic patients was concentrated by positive pressure ultrafiltration through collodion membranes as described by Van Dyke, Garcia, and Lawrence(4). Similar concentrates hereafter termed N were made of non-erythropoietically active human urine. The concentrate from an anemic patient has been termed E^{BS}. Comparison in the starved rat assay of the erythropoietic activity of these urinary concentrates with purified sheep erythropoietin obtained from Armour (Lot No. K 103-124) indicated that E^{BS} contains approximately 16 cobalt units/mg.

Three rabbits were immunized against E^{BS} by monthly injection of 16.6 mg of the urinary concentrate dissolved in 1 ml saline. Blood was collected from the central artery of the ear 6 days after the fourth injection; the serum was separated, pooled, and frozen until used. This serum has been termed anti-E^{BS}. Three rabbits were immunized against N by weekly injection of 1 mg of the concentrate dissolved in 1 ml saline. Serum termed anti-N was taken from these rabbits 6 days after the ninth injection. The first injections of E^{BS} and N were given subcutaneously (0.25 ml in each leg) after mixing with an equal volume of complete Freund's adjuvant (Difco); all subsequent injections were given intravenously.

Qualitative comparisons of the antigens in the urinary concentrates and the precipitins produced in rabbits by injection of these concentrates were made, using the technic of double diffusion in 2 dimensions developed by Ouchterlony(6). A solution of 0.5 or 1.0% agar containing 1% sodium azide was poured into petri dishes to obtain a layer about 6 mm

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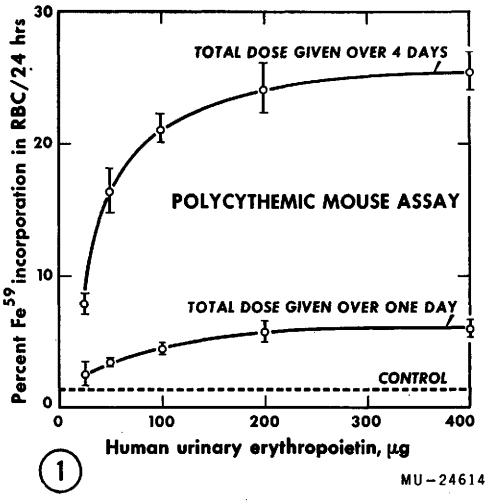
thick. The agar was made either with distilled water or saline. Various patterns of round wells were cut in the agar with a cork borer. The distances between the edges of adjacent wells was 1 cm. After the wells were filled the dishes were placed in a humid atmosphere at room temperature and examined daily for at least 9 days. The antigens were introduced into the wells at a concentration of 2 mg/ml and the immune sera were used undiluted. Identification of the antigens present in the urinary concentrates was attempted by comparing the precipitin lines produced against the concentrates with those lines produced against a variety of known human plasma proteins. The human plasma proteins used were alpha globulin Fraction IV-4, beta globulin Fraction III, alpha globulin Fraction IV-1, gamma globulin Fraction II, albumin (crystallized), globulins Fraction II and III, and alpha globulin Fraction IV (obtained from Pentex, Inc.).

Absorption of the immune sera anti-E^{BS} and anti-N was performed by addition of 3-5 ml of the immune sera to 12 mg of E^{BS} dissolved in 0.4 ml of saline. After each addition the solutions were incubated at 37 C for 1 hour, then refrigerated overnight. The precipitates were removed by centrifugation and the immune sera readded until no visible precipitate was formed. Complete absorption of E^{BS} with anti-N required 42 ml of sera and although complete absorption of E^{BS} with anti-E^{BS} required much less sera, a total volume of 42 ml was added. Other observations indicated that anti-E^{BS} was much more active than anti-N. During all absorptions a control received an identical treatment of E^{BS} with normal rabbit serum. The precipitates from all absorptions were saved and resuspended in a volume of saline equivalent to that of the immune sera used for absorption.

The erythropoietic activity of the various urinary concentrates and absorbed sera was assayed, using the transfusion-induced polycythemic mouse. The assay required 8 days. Male Swiss mice weighing about 25 g were hypertransfused by tail vein injections of 0.7 ml of 70% homologous red blood cells on 2 successive days. The red blood cells were obtained from the aorta of donor animals, washed 3 times with heparinized saline and the buffy coat removed. The material to be assayed was fractionated into 4 equal doses which were injected subcutaneously in the polycythemic mice on the next 4 days. On the 7th day, 1 μc of Fe^{59} was injected intraperitoneally and 24 hours later the mice were anesthetized with ether and approximately 0.8 ml of blood was drawn from the vena cava. An aliquot of this blood was pipetted (0.5 ml), and the radioactivity counted. Using the value of 5% of body weight to estimate the blood volume, Fe^{59} uptake in the total calculated blood volume was determined. Hematocrits done on the same sample of blood were always above 60% with an average of 69%. Reticulocytes of the peripheral blood were determined by incubating equal volumes of whole blood with one percent brilliant cresyl blue in saline for 20 minutes and then preparing smears. At least 1000 cells were randomly counted in the smears.

Results and discussion. The transfusion-induced polycythemic mouse is as sensitive a test for erythropoietic activity as the more commonly used starved or hypophysectomized animal, and has the advantage that other physiological parameters are less drastically altered. One particular advantage of this assay in the work to be described is that the effect of toxic agents, such as foreign proteins, which may produce a non-specific release of reticulocytes from the marrow is minimized since reticulocyte content of the marrow of these polycythemic mice is essentially zero at the beginning of assay. In Fig. 1 is compared the dose response of human urinary erythropoietin injected all on one day as opposed to the fractionation of the same total dose over 4 days. Using this assay a total dose of 25 μg of erythropoietin when injected fractionally over 4 days produced a response in Fe^{59} uptake in the peripheral red cells and in reticulocytes which was highly significant when compared to the saline injected controls ($P<0.001$). The significance of the dose responses, especially at low doses of erythropoietin, following fractional injection provides a sensitive repro-



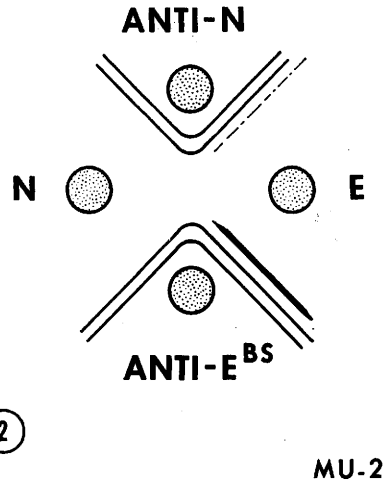


FIG. 1. Dose response of human urinary erythropoietin in the polycythemic mouse. Dose of erythropoietin given on abscissa corresponds to total dose received.

FIG. 2. Schematic representation of precipitin lines formed by the ninth day in the Ouchterlony plates. Solutions placed in each well are indicated.

ducible assay for erythropoietin and has been utilized in the subsequent experiments.

The various urinary concentrates contained a number of different antigens which produced good precipitin formation in the rabbit. Presumably many of these precipitins are against proteins found in normal urine (7). Indeed, the immune sera contained antibodies which formed visible precipitates against all the human plasma proteins tested.

A schematic representation of the precipitin patterns produced in the Ouchterlony plates with anti-E^{BS}, anti-N, E^{BS} and N is shown in Fig. 2. These patterns indicated that an antigen was present in E^{BS} which was not present in N in sufficient concentration adequate to give a visible precipitin line. The fact that after several days' diffusion a similar although very weak line was observed between anti-N and E^{BS} suggests that small amounts of this antigen must be present in N. The increased concentration of this antigen in the erythropoietically active urinary concentrates suggested the possibility that the antigen might be associated with erythropoietic activity. This was investigated by comparing the erythropoietic activity of active urinary concentrates after various absorptions with different immune sera.

E^{BS} was absorbed as previously described and the resultant solutions diluted with saline so that each solution contained the equivalent of 143 μg of E^{BS} per ml. The precipitates from each absorption were similarly diluted. Erythropoietic activity of these diluted sera was assayed as outlined above. A total of one milliliter of each solution was injected into each polycythemic mouse (0.25 ml per day). The results are shown in Table I. Absorption of E^{BS} with anti-E^{BS} completely neutralized the erythropoietic activity. Indeed, the response in these animals was significantly lower ($P<0.025$) than in the saline injected controls, suggesting that the excess anti-E^{BS} in the injected solution affected the already markedly decreased Fe^{59} uptake normally seen in polycythemic mice. The absence of stimulation after injection of

TABLE I. Effect of Various Immune Absorptions on Biological Activity of Human Erythropoietin in Polycythemic Mice.

Treatment	24-hr Fe^{59} uptake in total calculated blood vol (%)	Reticulocytes (%)
E^{BS} + anti-E^{BS}	.56 ± .04*	.00
E^{BS} + anti-N	13.24 ± .45	2.7
E^{BS} + normal sera	18.06 ± .85	3.3
Precipitate E^{BS} anti-E^{BS}	2.65 ± .33	.12
Precipitate E^{BS} anti-N	2.47 ± .21	.07
Saline	2.21 ± .42	.11

* Stand. error of mean.

Six animals/group, avg wt 25.3 g.

TABLE II. Depression of Erythropoiesis in Normal Mice Following Injection of Sera Obtained from Rabbits Immunized with an Erythropoietically Active Human Urinary Concentrate.

Treatment	24-hr Fe^{59} uptake in total calculated blood vol (%)	Reticulocytes (%)
Anti-E^{BS}	8.73 ± 1.90*	1.3
Anti-N	22.84 ± 1.93	3.8
Saline	21.00 ± 1.02	3.3

* Stand. error of mean.

Ten animals/group, avg wt 26.4 g.

the precipitate from this absorption would indicate that removal of the erythropoietically active material is not simply the result of non-specific adsorption on other antigen-antibody complexes. A very potent erythropoietic stimulation occurred upon injection of anti-N plus E^{BS}. However, this response was significantly less ($P<0.001$) than that observed after injection of E^{BS} plus normal sera. The precipitates from the absorptions were inactive.

The above experiment suggested that some neutralization of the animals' endogenous erythropoietin might have occurred. The effect of injections of anti-E^{BS} on erythropoiesis in normal mice was therefore investigated. Groups of 10 mice were injected with 0.25 ml of anti-E^{BS}, anti-N and saline for 4 days. Fe^{59} was given on the fifth day and 24 hours later Fe^{59} uptake in the total calculated blood volume was determined. Reticulocyte counts of the peripheral blood were also measured. The results are shown in Table II. The significant depression ($P< 0.001$) of Fe^{59} uptake and reticulocytes after injection of anti-E^{BS} supports the concept that erythropoietin is involved in the normal physiological control of erythropoiesis. Such immune sera may have some therapeutic value in treatment of various hematologic diseases.

We conclude that the neutralization of erythropoietic activity is the result of an antigen-antibody reaction. The antibody involved may be specifically directed either against the erythropoietically active molecule or against the class of proteins to which the active molecule belongs. It cannot be assumed, until further experiment, that this antigen-antibody reaction is of the precipitin type, *i.e.*, the strong precipitin line observed in the Ouchterlony plates when anti-E^{BS} is reacted against E^{BS} is not necessarily related to the erythropoietically active component of the urinary concentrates.

Summary. An immunochemical study of human urinary erythropoietin has been presented. The experiments indicate that the erythropoietic activity as assayed in the polycythemic mouse of erythropoietically active human urinary concentrates can be neutralized *in vitro* by addition of sera obtained from rabbits previously immunized with such concentrates. A significant depression of erythropoiesis was observed after injection of such sera into normal mice. It is concluded that neutralization is the result of an antigen-antibody reaction.

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