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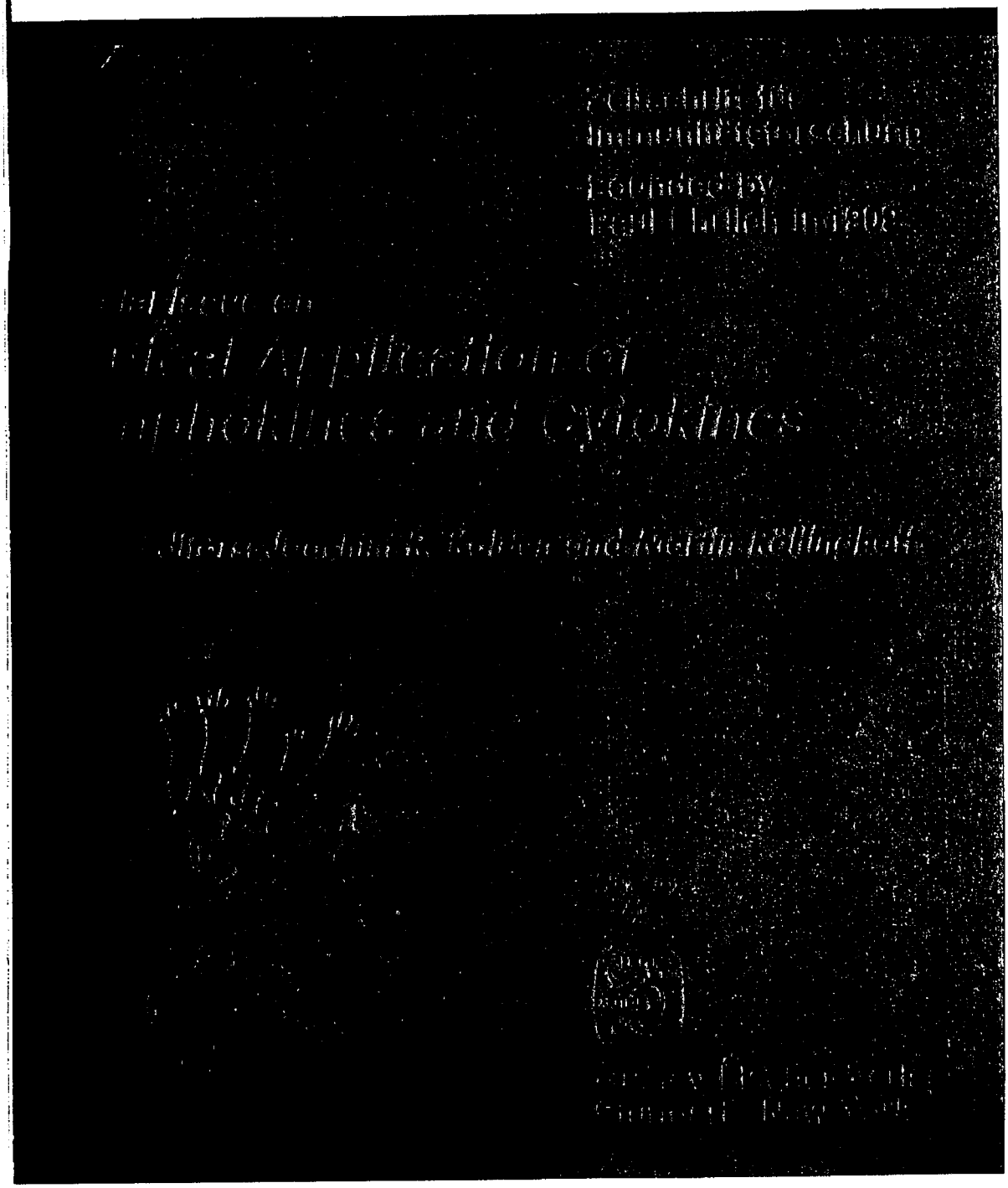
Immunobiol., Volume 172, No. 3/4/5
pp. 157–460. September 1986
ZIMMDO 172 (3/4/5) (1986)
ISSN 0171-2985

Immunobiology

Volume 172 · No. 3/4/5 · 1986



Immunobiol., vol. 172, pp. 213–224 (1986)

Amgen, Thousand Oaks, California, U.S.A.

Characterization and Biological Effects of Recombinant Human Erythropoietin

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Abstract

Human recombinant erythropoietin (rHuEPO) has been purified to apparent homogeneity and compared to purified human urinary erythropoietin (EPO). Both the purified natural and recombinant EPO preparations were characterized in a competition radioimmunoassay (RIA), the exhypoxic polycythemic mouse bioassay, *in vitro* tissue culture bioassays using bone marrow cells, and by Western analysis. In the immunological and biological activity assays, the rHuEPO shows a dose response which parallels that of the natural hormone. By Western analysis, the recombinant and human urinary EPO migrate identically. Administration of rHuEPO increases the hematocrit of normal mice in a dose-dependent manner. Additionally, the rHuEPO is able to increase the hematocrit of rats made uremic as a result of subtotal nephrectomy. In summary, by all criteria examined, the rHuEPO is biologically active and equivalent to the natural hormone.

Introduction

Erythropoietin (EPO) is a sialylglycoprotein hormone which is the primary regulator of red blood cell formation in mammals (1–3). EPO is produced primarily in the kidneys of the adult and in the liver of the fetus (2). The synthesis of EPO is increased in response to lowered tissue oxygen tension and decreased under conditions of hyperoxia (1–3). In response to increased circulatory EPO levels, marrow erythropoiesis is stimulated, resulting in an increased production of red blood cells.

EPO has been purified to homogeneity from the urine of severe aplastic anemia patients (4). Although this has provided material for studies on some of the physical and chemical properties of the hormone, there has not been sufficient quantities to allow for more extensive structural characterization studies or use of the hormone as a therapeutic in the treatment of various anemias. More recently, in an effort to obtain larger quantities of the hormone, the human gene for EPO has been isolated (5–7), used to transform mammalian cells in culture, and the expressed rHuEPO has been purified. In this paper, we report on initial characterization studies to compare the purified rHuEPO to the natural hormone, and to test its biological efficacy in animals.

Abbreviations: rHuEPO = recombinant human EPO; *EPO* = erythropoietin; *RIA* = radioimmunoassay; *PBS* = phosphate-buffered saline; *EDTA* = ethylenediamine tetraacetic acid; *SDS* = sodium dodecyl sulfate.

Materials and Methods

Purification of natural EPO and rHuEPO

Natural EPO was purified to apparent homogeneity from the urine of a patient with aplastic anemia using the procedure of MIYAKE et al. (4). rHuEPO was purified from the cell conditioned media of Chinese hamster ovary cells stably transformed with the human EPO gene (5, 6) by sequential column chromatography.

EPO bioassays

In vivo biological activity was determined using the exhypoxic polycythemic mouse bioassay (8), and *in vitro* biological activity was demonstrated by determining the incorporation of ^{59}Fe into heme of cultured rat bone marrow cells after incubation with EPO (9).

EPO RIA

EPO sample or standard (CAT-1, provided by Dr. Eugene Goldwasser) was incubated at 37 °C for 2 h with rabbit antiserum raised against a crude EPO preparation in a total volume of 0.5 ml. Reaction diluent was phosphate-buffered saline (PBS), pH 7.4, containing 0.1 % bovine serum albumin. After the 2 h incubation, the reaction tubes were cooled on ice, ^{125}I-purified human urinary EPO (10) was added, and the incubation continued at 4 °C overnight. Antibody-bound ^{125}I-EPO was separated from free ^{125}I-EPO by the addition of Tachisorb (goat anti-rabbit IgG conjugated to *Staphylococcus aureus* cells, Calbiochem, La Jolla, CA, U.S.A.). The anti-crude EPO antiserum was generated by immunizing rabbits with a 1 % pure human urinary EPO preparation. The final concentration of antibody used was approximately 1:100 000 at which 10–20 % of the input ^{125}I-EPO is immunoprecipitated.

Western analysis

EPO was subjected to electrophoresis on a 12.5 % sodium dodecyl sulfate (SDS), polyacrylamide gel according to the method of LAEMMLI (11). The proteins were transferred to 0.2 µm nitrocellulose (SCHLEICHER and SCHUELL, Keene, NH, U.S.A.) using a Transblot apparatus (BioRad Laboratories, Richmond, CA, U.S.A.) at 60V for 4–6 h. The nitrocellulose was blocked for 1 h in PBS, pH 7.6, containing 10 % horse serum (Gibco Laboratories, Grand Island, NY, U.S.A.) prior to an overnight incubation with a mouse anti-EPO monoclonal antibody specific for the amino terminus of human EPO (12). The EPO-anti-EPO monoclonal antibody complex was visualized using horseradish peroxidase-conjugated horse anti-mouse IgG reagents (Vector Labs, Burlingame, CA, U.S.A.) and 4-chloro-1-naphthol color development reagent (BioRad Laboratories, Richmond, CA, U.S.A.).

N-glycanase digestion of EPO

Approximately 1 µg of either purified recombinant or natural EPO was incubated at 30 °C in 50 mM sodium phosphate, pH 8.6, containing 10 mM EDTA, 1 % NP-40 and 0.4 units of N-glycanase (Genzyme Corp., Boston, MA, U.S.A.). At times corresponding to 0, ½, 1, 2, 4 and 8 h post-incubation, samples equivalent to approximately 75 ng or 150 ng of the natural and recombinant EPO, respectively, were removed and boiled for 2 min in Laemmli sample buffer (11) to stop the enzyme reaction. For the last four h of incubation, the temperature was increased to 37 °C to insure complete removal of the carbohydrate chains.

Treatment of mice with rHuEPO

Eight-week-old female CD-1 mice (29–32 g), obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.), were injected intraperitoneally three times a week, for a total of ten injections, with 0.1 ml of either a control solution, PBS pH 7.4, containing 0.025 % mouse serum albumin, or with purified rHuEPO at a dose level of 50, 150, 450 or 1,500 U/kg formulated in the control solution. Blood for hematocrit determinations was

collected directly into hematocrit tubes from the retroorbital sinus. Baseline hematocrit determinations were performed prior to the first injection and twice weekly thereafter.

Subtotal nephrectomized rat model

Male Sprague Dawley rats weighing 200 g (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) underwent either a two-stage sham laparotomy (with handling of the kidneys), or a two-stage surgical procedure in which first one half of the left kidney was removed, followed one week later by complete excision of the right kidney. One week following the completion of the surgical procedures, the animals received a 0.1 ml intramuscular injection of either a control solution, 0.9 % sodium chloride, or 10 units of purified rHuEPO. Injections were repeated five times a week for a total of two weeks. Body weight, hematocrit and plasma urea nitrogen were determined before and at the completion of the EPO treatment period. Plasma urea nitrogen was determined by a colorimetric assay using a diagnostic kit (Sigma Chem. Co., St. Louis, MO, U.S.A.).

Results

Comparison of purified rHuEPO and human urinary EPO in biological and immunological assays

In order to characterize the rHuEPO and compare it to the natural human urinary hormone, the response of both EPO preparations was

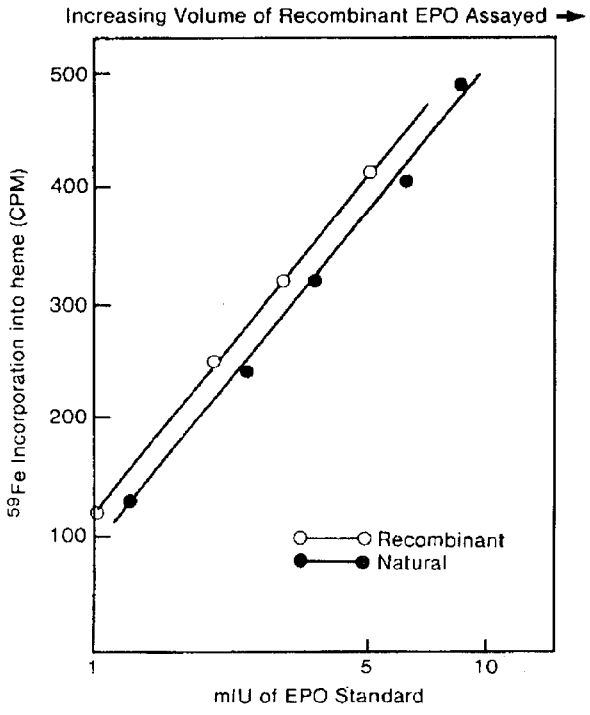

Fig. 1. Comparison of natural and rHuEPO in an *in vivo* bioassay. Increasing aliquots of purified rHuEPO (○–○) or a human urinary EPO standard (●–●) were assayed in the exhypoxic polycythemic mouse bioassay (8).

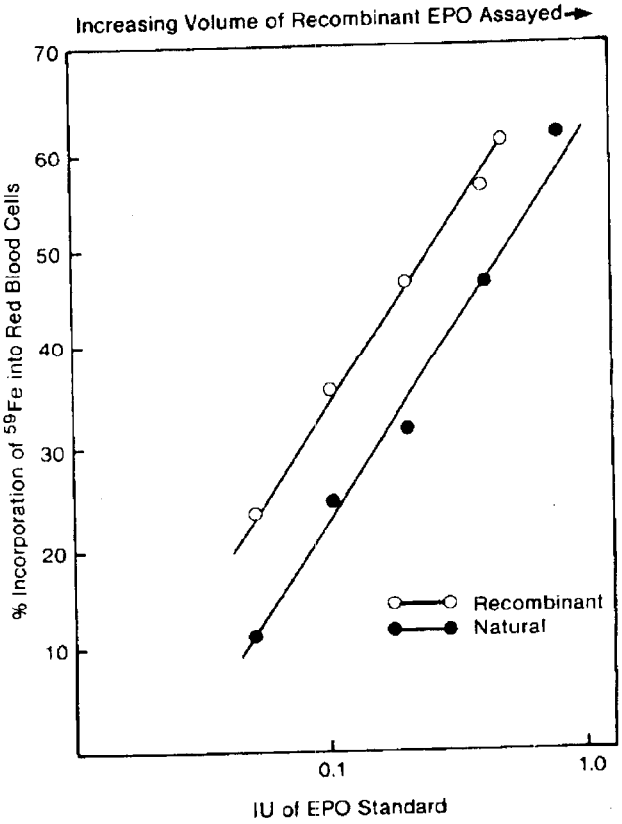

Fig. 2. Comparison of natural and rHuEPO in an *in vitro* bone marrow culture assay. Increasing aliquots of purified rHuEPO (○–○) or human urinary EPO standard (●–●) were assayed for their ability to increase the incorporation of ^{59}Fe into heme of cultured rat bone marrow cells (9).

examined in three different EPO assays, an *in vivo* and an *in vitro* bioassay and an EPO RIA. Figure 1 shows a comparison of the two preparations in the exhypoxic polycythemic mouse *in vivo* bioassay (8). This assay measures the ability of animals rendered polycythemic by exposure to low oxygen tension to respond to exogenous EPO after return to normal atmospheric conditions. The increase in erythropoiesis is measured by the incorporation of ^{59}Fe into circulating red blood cells. In the *in vivo* bioassay, increasing quantities purified rHuEPO exhibited a dose-response which was parallel to that of the human urinary EPO (Figure 1). Figure 2 illustrates that in an *in vitro* bioassay, which measures the incorporation of ^{59}Fe into heme of cultured rat bone marrow cells in response to EPO (9), the purified rHuEPO produced a dose-response curve which was parallel to that generated by purified human urinary EPO.

Figure 3 compares the immunoreactivity of the recombinant and natural EPO preparations in an RIA. This assay used an antibody raised against the natural hormone and measured the ability of urinary EPO standard or

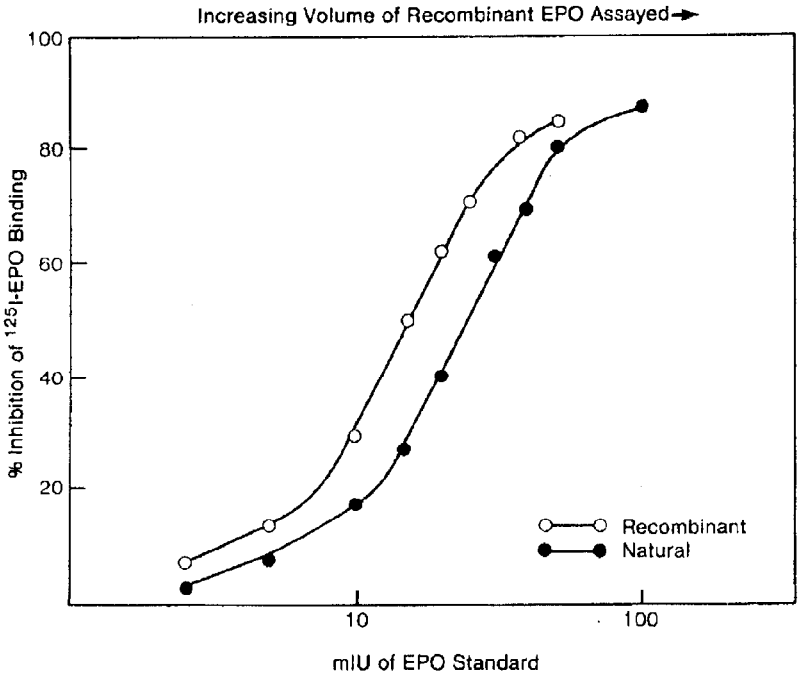

Fig. 3. Comparison of natural and purified rHuEPO by RIA. Increasing aliquots of purified rHuEPO (○–○) and a human urinary EPO standard (●–●) were assayed in an EPO RIA.

rHuEPO to compete the binding of ^{125}I-purified human urinary EPO to the antibody. Increasing aliquots of the purified rHuEPO exhibited an inhibition curve which was identical to that of the urinary-derived hormone suggesting an immunological identity of the recombinant and natural EPO. Identical results were obtained in assays using antisera raised against the purified recombinant EPO and ^{125}I-purified rHuEPO as the tracer, indicating there are no epitopes present on the recombinant hormone which cannot be competed by the natural hormone (data not shown).

These experiments indicate that the human urinary and recombinant EPO are indistinguishable in the parameters of biological and immunologi-

Table 1. Comparison of RIA and *in vitro* and *in vivo* bioassays for purified rHuEPO

Units of EPO/ml		
RIA	Bioassay	
	Bone marrow culture *in vitro* bioassay	Polycythemic mouse *in vivo* bioassay
6,025 ± 125	6,161 ± 547	6,136 ± 828

Purified rHuEPO was assayed by RIA and *in vitro* (9) and *in vivo* (8) bioassays. Values reported are the average ± SD of at least four independent determinations.

cal reactivity that can be measured by these assay systems. In addition, as illustrated in Table 1, the purified rHuEPO preparation has equivalent activity in the RIA and bioassays, indicating that the purified hormone is fully biologically active.

Western analysis of EPO

In order to determine whether the purified rHuEPO was the same size as the human urinary EPO, both preparations were characterized by Western analysis. After electrophoresis on a 12.5 % SDS polyacrylamide gel, the protein bands were transferred to nitrocellulose and the Western was developed using a monoclonal antibody specific for the amino terminus of EPO. As seen in Figure 4, purified rHuEPO migrates identically to human urinary EPO with an apparent molecular weight of approximately 36,000 daltons, suggesting that both molecules are glycosylated to the same extent.

Purified human urinary EPO has been characterized as a glycoprotein containing approximately 30 % sugar moieties by weight and having a carbohydrate composition consistent with the presence of several complex-type N-linked-oligosaccharide chains (4, 13). The amino acid sequence of the mature EPO protein identifies three potential N-linked glycosylation sites based on the consensus glycosylation site Asn-X-Ser/Thr (5, 14).

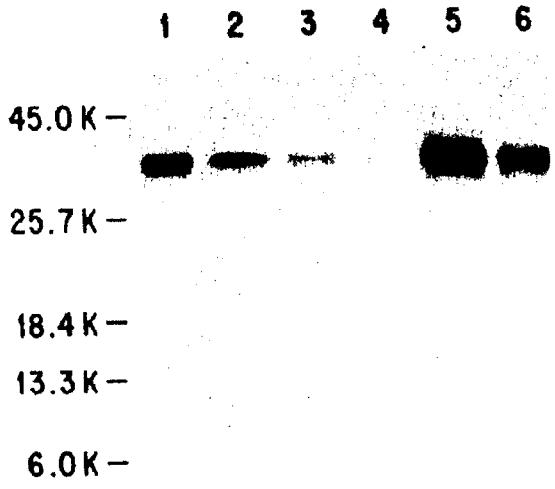

Fig. 4. Western analysis of purified recombinant and human urinary EPO. Purfied human urinary EPO, 175, 90, 45 and 15 ng, (lanes 1–4), respectively, and purified rHuEPO, 400, and 150 ng (lanes 5 and 6), respectively, were electrophoresed on a 12.5 % SDS polyacrylamide gel and subjected to Western analysis using a monoclonal antibody specific for the amino-terminus of EPO.

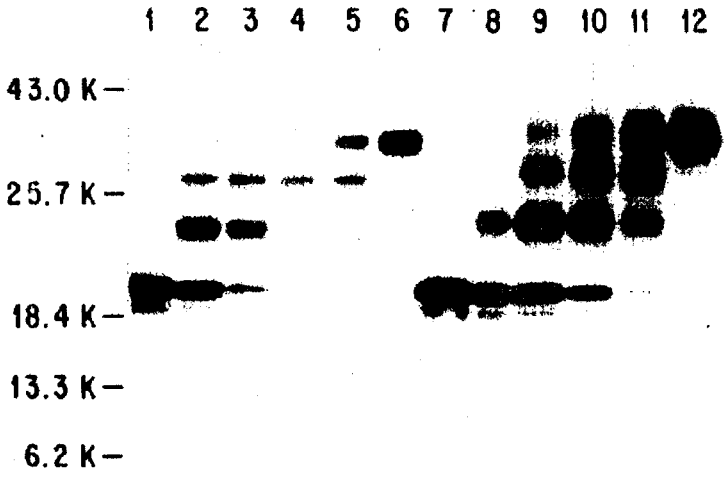

Fig. 5. Time course of treatment of human urinary and recombinant EPO with N-glycanase. Purified human urinary EPO (lanes 1–6) and purified rHuEPO (lanes 7–12) were treated with N-glycanase as described in the «Materials and Methods». At times corresponding to 0 h (lanes 6 and 12), ½ h (lanes 5 and 11), 1 h (lanes 4 and 10), 2 h (lanes 3 and 9), 4 h (lanes 2 and 8), and 8 h (lanes 1 and 7) after the addition of enzyme, the equivalent of approximately 75 ng or 150 ng of human urinary or rHuEPO, respectively, were removed from the incubation tube and boiled for 2 min in Laemmli sample buffer (11). After the 8 h incubation, the samples were electrophoresed on a 12.5 % SDS-polyacrylamide gel and subjected to Western analysis.

In order to gain further insight into the carbohydrate structure of both natural and rHuEPO, both hormone preparations were treated with N-glycanase, an enzyme which specifically cleaves oligosaccharides linked to asparagine residues. As seen in Figure 5, Western analysis of samples taken at different times during the N-glycanase treatment indicates that both recombinant and urinary-derived EPO contain three N-linked carbohydrate chains. Enzyme digestion for 8 h yields a darkly staining and a lighter staining band with apparent molecular weights of approximately 19,500 and 18,400 daltons, respectively. Sequential treatment of recombinant or urinary EPO with N-glycanase followed by sialidase and O-glycanase, an enzyme which specifically removes O-linked oligosaccharide chains from glycoproteins, causes the EPO to migrate as a single band with an apparent molecular weight of approximately 18,400 daltons (data not shown), the calculated molecular weight of the mature EPO protein. These experiments indicate that both recombinant and urinary EPO contain O-linked as well as three N-linked oligosaccharide chains.

Biological efficacy of rHuEPO

Female CD-1 mice were used as a model system to examine the effect of administration of purified rHuEPO on the hematocrit of normal animals.

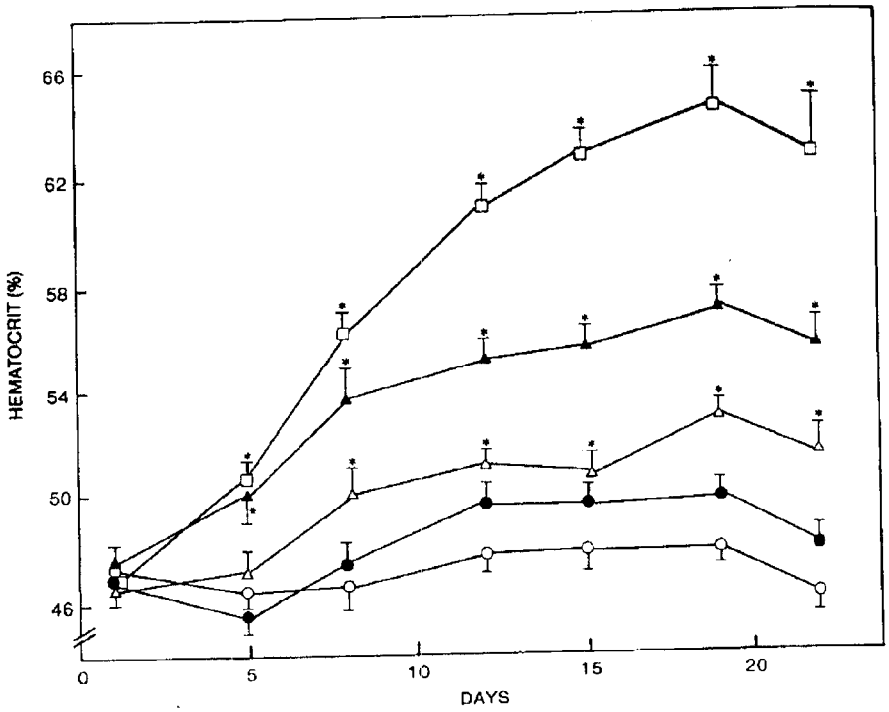

Fig. 6. Effect of purified rHuEPO on the hematocrit of normal mice. CD-1 female mice were injected intraperitoneally with 0.025 % mouse serum albumin (control solution, ○–○), or 50 (●–●), 150 (△–△), 450 (▲–▲), or 1500 (□–□) U/kg of purified rHuEPO formulated in the control solution. rHuEPO was administered three times a week for a total of 10 injections. Hematocrit determinations were performed before the start of treatment and twice weekly thereafter. All groups contained 15 mice. * $p < 0.01$ vs. control group.

Animals were injected intraperitoneally three times a week with either a control solution or with dose levels of recombinant EPO equivalent to 50, 150, 450 or 1,500 units/kg. As shown in Figure 6, in contrast to the control group whose hematocrit did not change with time, all groups receiving EPO injections displayed an increase in the group average hematocrit. This increase was EPO dose-dependent, with the higher dose levels of EPO producing the greater increase in hematocrit. Following withdrawal of EPO administration, the hematocrit of all groups returned to baseline (data not shown).

Because of the kidney's important role in erythropoietin production, anemia is a major complication of chronic renal failure (14). In order to determine the effectiveness of rHuEPO in uremic animals, the sub-totally nephrectomized rat was used as a model to simulate end stage renal disease. As shown in Table 2, there was no significant difference in body weight or hematocrit between the sham-operated and subtotally nephrectomized animals prior to EPO treatment. In response to the subtotal nephrectomy, the rats became uremic as indicated in Table 2 by the increase in plasma urea nitrogen as compared to the sham-operated group. This effect persisted

Table 2. Treatment of sham-operated and sub-totally nephrectomized rats with rHuEPO

	Pre-Treatment		Post-Treatment			
	Sham	Nephx	Sham + O EPO	Sham + 10 U EPO	Nephx + O EPO	Nephx + 10 U EPO
Body weight (g)	219 ± 15	199 ± 16	234 ± 20	236 ± 11	218 ± 23	229 ± 12
Hematocrit	42.4 ± 2.2	40.8 ± 2.0	39.7 ± 1.1	46.8 ± 4.4*	41.9 ± 3.8	48.2 ± 3.2*
Plasma Urea Nitrogen (mg/dl)	24.1 ± 4.6	40.1 ± 8.0^{+}	29.5 ± 4.0	26.0 ± 5.4	45.5 ± 2.9^{+}	47.0 ± 0.4^{+}

Rats were either sham-operated or partially nephrectomized using a two-stage surgical procedure. One week post-surgery, the animals received saline or 10 U of rHuEPO by intramuscular injection five times a week for two weeks. Each group contained 3–4 animals.

*$p < 0.05$ vs. respective treatment control (O EPO)

$^{+}p < 0.05$ vs. sham-operated control

throughout the experiment. After the two week-treatment with saline or rHuEPO, a significant increase in hematocrit was observed in both sham-operated and subtotally nephrectomized animals treated with rHuEPO as compared to the saline-treated groups. The observation that there was no significant difference in body weight between the saline control and the rHuEPO-treated groups suggests that the increased hematocrit was due to an increase in red cell mass and not due to a contraction of the intravascular fluid space. These experiments indicate that rHuEPO can increase the hematocrit of uremic animals to the same extent as control, non-uremic animals.

Discussion

Anemia is one of the most important problems in patients with chronic renal failure and can be a major factor preventing favorable rehabilitation of the patients. Since the primary cause of the anemia appears to be a deficiency of EPO due to the progressive destruction of the kidney mass (15, 16), replacement therapy with the hormone would appear to be the most desirable form of therapy. Although a limited number of animal studies have been performed using either EPO-rich plasma (17), or partially purified EPO preparations (18), use of EPO as a therapeutic could not be addressed before adequate quantities of the purified hormone were obtained. EPO has been purified from the urine of aplastic anemia patients (4); however, due to the restricted availability of the starting material, only very limited quantities of the purified hormone have been available. The recent cloning and expression of the human EPO gene (5–7) has introduced an alternate source for purification of the hormone.

The human EPO gene encodes a mature protein of 166 amino acids whose predicted sequence is identical to that of human urinary-derived EPO (14). The expressed and purified rHuEPO is biologically active and reacts in a manner that is indistinguishable from the natural hormone when assayed in both biological and immunological assays. The carbohydrate structure of EPO has been shown to be important for its biological activity *in vivo*. Enzymatic removal of either the terminal sialic acid moieties or the N-linked oligosaccharide chains results in an almost total loss of *in vivo* activity with no loss in *in vitro* biological activity (13, 19, unpublished observations). These results suggest that the carbohydrate structure of EPO has little effect on its interaction with receptors on target cells, but plays an important role *in vivo* possibly preventing rapid clearance of the hormone from the bloodstream. The amino acid sequence of the mature hormone predicts three potential N-linked glycosylation sites. As shown here, a time course of digestion of both purified native and rHuEPO indicates that both EPO preparations contain three asparagine-linked carbohydrate chains. Additional experiments using specific glycosidases have indicated that both

preparations also contain previously undetected O-linked glycosylation site(s). In addition, the carbohydrate composition of purified rHuEPO (Watson, E., unpublished observations) is very similar to that determined for natural EPO (13), indicating both the carbohydrate composition and number of carbohydrate chains of the EPO produced by the two different cell types (human kidney and Chinese hamster ovary) are very similar.

The purified rHuEPO is able to increase the hematocrit of both normal and uremic animals in a dose-dependent fashion. These experiments indicate that no other factors in addition to EPO are required for this effect, a possibility that could not have been eliminated in earlier animal studies using very crude or partially purified EPO preparations (17, 18). The fact that rHuEPO was capable of elevating the hematocrit of subtotally nephrectomized animals exhibiting significant azotemia suggests that EPO should be efficacious in the treatment of the anemia of chronic renal failure.

Acknowledgements

We gratefully acknowledge Dr. PETER DUKES for performing in exhypoxic polycythemic mouse bioassays.

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