Amgen Inc. v. F. Hoffmann-LaRoche LTD et al Doc. 679 Att. 4
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EXHIBIT 4
(PART 2 OF 2)

protein portion of r-hEPO, comprises 38 ± 1% of the total weight.

Human urinary EPO and CHO-cell-derived r-hEPO migrate identically in SDS-polyacrylamide gels, indicating that both molecules are glycosylated to a similar extent. The carbohydrate composition of r-hEPO was determined as described by Zanetta et al. (1972) and compared to literature values for human urinary EPO (Dordal et al. 1985). The carbohydrate composition of r-hEPO was essentially the same as that of urinary EPO (T.W. Strickland et al., in prep.). Trace amounts of *N*-acetylgalactosamine were found in r-hEPO, indicating the presence of O-linked glycosylation.

Shown in Figure 4 are the results of a deglycosylation experiment that indicates that both r-hEPO and urinary EPO contain both N-linked and O-linked carbohydrates in similar amounts. Both r-hEPO and urinary EPO were analyzed by Western blot analysis after sequential glycosylase digestion. Figure 4 (lanes 1 and 5) shows urinary EPO and r-hEPO, respectively, prior to treatment. After treatment with endoglycosidase F, which removes N-linked carbohydrate, the apparent molecular weight of both r-hEPO and urinary EPO is shifted to approximately 19,500 with a minor band at about 18,400 (lanes 2 and 6). Following further treatment, first with sialidase (lanes 3 and 7) and then by *O*-glycanase (lanes 4 and 8), which remove O-linked carbohydrate, both r-hEPO and urinary EPO migrated as a single band with an apparent molecular weight of 18,400. Although the presence of *N*-acetylgalactosamine had not been detected previously (Dordal et al. 1985), these results demonstrate that urinary EPO, as well as r-hEPO, contains O-linked carbohydrate. In addition, direct carbohydrate analysis of endoglycosidase-F-treated r-hEPO yields galactose, sialic acid, and *N*-acetyl galactosamine, confirming the presence of O-linked carbohydrate (T.W. Strickland et al., in prep.). As shown in Figure 4, the proportion of EPO containing O-linked carbohydrate is comparable in urinary EPO and r-hEPO.

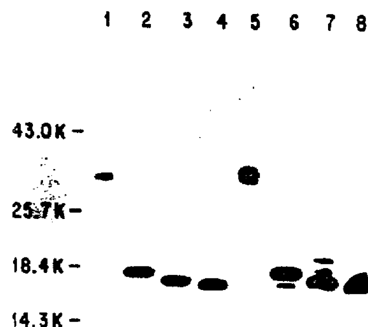

Figure 4. Western blot analysis of enzymatically deglycosylated urinary EPO (lanes *1–4*) and r-hEPO (lanes *5–8*). Samples were digested sequentially with glycosidases, and the products were separated on a 12.5% SDS-polyacrylamide gel under reducing conditions. An EPO-specific monoclonal antibody was used to visualize EPO after transfer to a nitrocellulose membrane. (*1,5*) No treatment; (*2,6*) endoglycosidase F; (*3,7*) endoglycosidase F plus neuraminidase; (*4,8*) endoglycosidase F, neuraminidase plus *O*-glycanase.

Samples of r-hEPO and urinary EPO taken over the course of endoglycosidase-F digestion were analyzed by Western blot analysis. Two clear partial digestion products, in addition to the final product, were revealed, indicating that all three potential N-linked glycosylation sites are utilized (data not shown).

The immunoreactivity of r-hEPO and urinary EPO were evaluated by comparing the dose-response curves of each preparation in a series of RIAs. The first RIA compared the ability of r-hEPO or urinary EPO to compete the binding of urinary ^{125}I-labeled EPO by a rabbit polyclonal antibody raised against a 1% pure preparation of urinary EPO. In this assay, identical dose-response curves were obtained with the recombinant and natural hormones (Egrie et al. 1985, 1986). Identical dose-response curves were also obtained for each source of hormone when ^{125}I-labeled r-hEPO was used as the tracer or when rabbit polyclonal sera to r-hEPO was used in combination with either tracer (data not shown; J.C. Egrie et al., in prep.). These experiments demonstrate that there are no epitopes present on one hormone preparation that are not present on the other.

Biological Effects of EPO

The biological activities of r-hEPO and urinary EPO were also indistinguishable, as measured by the dose-response curve of each preparation in both in vitro and in vivo biological assays (Egrie et al. 1986). The exhypoxic polycythemic mouse bioassay (Cotes and Bangham 1961) measures the incorporation of ^{59}Fe into red blood cells in mice made polycythemic by exposure to low-oxygen conditions. Due to the polycythemia, these animals have a reduced rate of red blood cell synthesis after return to normal atmospheric conditions and therefore have a low background rate of incorporation of iron into red blood cells. However, these animals will respond to exogenous administration of EPO, in a dose-dependent manner, by increasing the rate of red blood cell synthesis and incorporation of ^{59}Fe. The biological activity of EPO was also measured by the incorporation of ^{59}Fe into heme in primary cultures of rat bone-marrow cells. In each of these assays (Egrie et al. 1986), as well as in mouse BFU-e and CFU-e assays (S.B. Krantz, pers. comm.), r-hEPO and urinary EPO have indistinguishable dose-response curves. These results indicate that r-hEPO produced in CHO cells has all the biological and immunological properties of natural EPO that can be measured by these assays. In addition, r-hEPO has the same activity in the RIAs and in vitro and in vivo bioassays, indicating that it is fully biologically active (Egrie et al. 1986).

The biological and immunological activities of multiple lots of purified r-hEPO have been measured.

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These assays are standardized with respect to the second international reference preparation of EPO (IRP#2) (Annable et al. 1972). r-hEPO has a constant specific activity of 174,000 units/A_{280} ± 5%, which is approximately 2.5 times the reported specific activity of urinary EPO (Miyake et al. 1977). Perhaps the harsh conditions required for purification of EPO from concentrated urine are responsible for the difference in specific activity.

Because of earlier notions that EPO may not be the only factor involved in production of red blood cell mass, we determined whether EPO alone could stimulate red blood cell production in normal mice, rats, and dogs. In all three species, there was a dose-dependent increase in reticulocyte counts and an increase in red blood cells. The results in Figure 5 show the effect on reticulocyte and red blood cell counts in dogs treated three times per week for 3 weeks. No effects were observed in excipient-treated controls, but doses of 280 U/kg and 2800 U/kg gave dose-dependent increases in both reticulocyte and red blood cell counts. It is noteworthy that the reticulocyte counts began to drop before the end of treatment and then dropped rapidly after treatment ceased, whereas the red blood cell counts continued to rise and then showed a gradual decrease. This particular study involved very high doses of EPO and thus allowed assessment of potential toxicity effects of EPO; however, no adverse effects of any note were observed. Overall, EPO was well tolerated and showed no histological abnormalities. Demonstration of an increase in red blood cells with highly purified EPO in normal, intact experimental animals indicates, for the first time, that EPO alone is capable of this effect and that other factors are not limiting in stimulating red blood cell production.

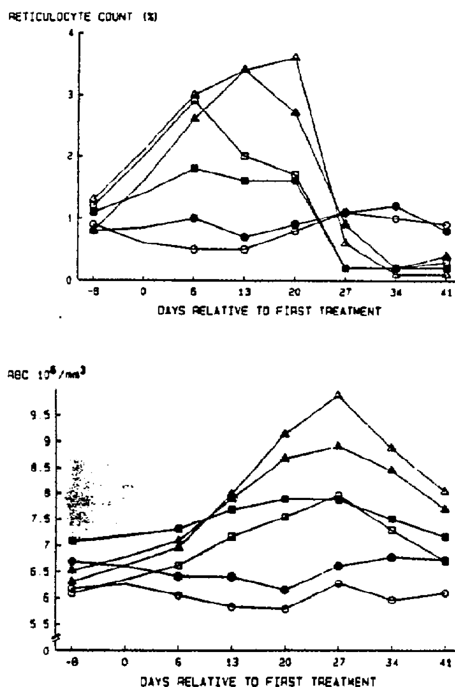

Figure 5. Effect of treatment with r-hEPO on the reticulocyte and red blood cell count of beagles. Young adult male (○, □, △) and female (●, ■, ▲) beagles were injected intravenously three times a week for 3 weeks with either a buffered saline control solution (○, ●) or a dose of r-hEPO at 280.0 U/kg (□, ■) or 2800 U/kg (△, ▲). The last treatment with r-hEPO was on day 20.

Previous studies of the pharmacokinetic features of EPO in experimental animals have involved use of biologically inactive, iodinated materials (Emmanouel et al. 1984). Production of EPO in a defined and controllable system has allowed labeling with ^{35}S-labeled amino acids during manufacture. This material has been found to be fully biologically active and its clearance is comparable to that of iodinated EPO, in terms of the rate of distribution and elimination. There was a modest reduction in clearance characteristics in functionally nephrectomized rats (A.C. Cohen et al., in prep.). These observations are encouraging in terms of the potential clinical use of EPO in patients with end-stage renal disease.

In view of the potential utility of EPO in patients with end-stage renal disease, we examined the responsiveness of nephrectomized rats to r-hEPO. Control animals were subjected to laparotomy without ligation of the kidneys. These animals showed no changes in blood urea nitrogen or hematocrit. Subtotal nephrectomized rats showed, as expected, increased blood urea nitrogen. Frank anemia was not observed in these animals presumably because insufficient renal mass had been removed. Both control (sham operated) and subtotal nephrectomized rats were responsive to EPO treatment in terms of increased hematocrits, as indicated by the results in Table 1. Because this occurred without significant changes in body weight, we conclude that the hematocrit increase is due to an increase in red blood cells and not simply contraction of intravascular fluid space.

Anemias associated with inflammatory diseases, such as rheumatoid arthritis, provide other possible indications for an agent such as EPO. In this case, abnormalities of iron turnover and compartmentalization might override the effects of EPO. Thus, we examined the effect of EPO on the anemia of adjuvant-linked inflammation in rats. Data (Table 2) show that this experimental anemia is corrected by treatment with EPO. Specifically, this type of anemia is a consequence of impaired recirculation of iron from the liver to the marrow. This was evidenced by the decrease in plasma iron and hemoglobin concentration in the adjuvant-treated rats. EPO corrected this situation by stimulating the production of erythrocytes. In the process, plasma iron was decreased further as it diverted into hemoglobin synthesis, as evidenced by a return of

Table 1. Treatment of Subtotally Nephrectomized Rats with r-hEPO

	Pretreatment		Posttreatment			
	sham	nephx	sham + 0 EPO	sham + 10 units EPO	nephx + 0 EPO	nephx + 10 units EPO
Body weight (g)	210 ± 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[a]	41.9 ± 3.8	48.2 ± 3.2[a]
Blood urea nitrogen (mg/dl)	24.1 ± 4.6	40.1 ± 8.0[b]	29.5 ± 4.0	26.0 ± 5.4	45.5 ± 2.9[b]	47.0 ± 0.4[b]

[a] $p < 0.05$ vs. respective treatment control (0 EPO).
[b] $p < 0.05$ vs. sham control.

hemoglobin concentrations to normal values, with a concomitant decrease in the percentage of saturation. Total iron-binding capacity (TIBC) was unaffected by EPO.

DISCUSSION

Paucity of structural information, tissue of origin, and specific assays rendered the cloning of human EPO particularly difficult and required refinement of methods to allow identification of a single-gene copy in the genome using multiple DNA probes. The structural heterogeneity of urinary EPO, as exemplified by its disperse nature on SDS-PAGE, was ambiguous in terms of whether there was a family of related proteins and/or variable secondary modifications of the primary protein structure. The successful cloning and expression of the protein, as well as characterization of natural urinary EPO, has indicated that there is a single unique gene for this protein. The cloning approach used was risky in that it relied on considerable homology between human EPO and monkey EPO in the sequences used to construct probes. An alternative strategy involving use only of a human fetal cDNA library (Jacobs et al. 1985) was confounded by the possibility of a distinct fetal EPO gene. Current data indicate that there is only one gene for human EPO, and if there is also a distinct fetal gene, it is only distantly related in structure to the EPO gene described here. Although it is probable that EPO can be secreted by tissues other than the kidney (anepheric individuals retain the ability to produce low amounts of EPO), the results of the screening of monkey tissue mRNA clearly indicate that the kidney responds to anemia by increasing the rate of synthesis of EPO.

The EPO gene seems to be highly conserved in mammals. The amino acid sequences of the primary translation products in human and cynomolgus monkey EPO differ at 15 positions and the monkey protein is one residue shorter: The lysine at position 116 in human EPO is missing. Mature monkey EPO and human EPO also differ at the site of cleavage of the signal peptide. There are 33 positions different between mouse EPO and human EPO (McDonald et al. 1986). Mouse EPO has only three cysteine residues (position 33 is not a cysteine), so that the small loop maintained by a disulfide in human EPO (see Fig. 3) would not seem to be essential for activity. Estimates of evolution rates have been made from the three known mammalian EPO sequences and these indicate 1.3×10^{-9} amino acid substitutions per site per year. This is comparable (although slightly lower) to the rate of evolution of rapidly evolving proteins, such as the fibrinopeptides (9×10^{-9} substitutions/site/year), and much more rapid than the most slowly evolving proteins, such as histones (0.006×10^{-9} substitutions/site/year) (McDonald et al. 1986).

It is noteworthy that the N-linked glycosylation sites are conserved in human, monkey, and mouse EPO sequences. This is distinct from cases in which glycosylation sites in closely related proteins, even in the same species, are not conserved. For example, some subtypes of the human interferon-α family of proteins are glycosylated, but the sites are nonhomologous and distinct also from the glycosylation sites in human interferon-β, which is closely related in amino acid sequence to interferon-α (Stebbing 1986). A remarkable feature of human EPO produced in CHO cells is the similarity in the carbohydrate modifications that occur. The same broad spread of material on SDS-PAGE occurs with urinary EPO and CHO cell-derived EPO, and deglycosylation experiments yielded the same ratio of the N- and O-linked species. Furthermore, the car-

Table 2. Effect of r-hEPO on the Anemia of Adjuvant-induced Inflammation in Rats

	Untreated		Adjuvant-treated	
	saline	EPO	saline	EPO
PCV (%)	43.6 ± 0.8	54.9 ± 1.3	43.2 ± 0.6	52.9 ± 0.8
RBCV (ml/100 g)	2.02 ± 0.02	2.75 ± 0.06	2.10 ± 0.07	2.72 ± 0.04
Hemoglobin (g/dl)	13.89 ± 0.36	14.87 ± 0.69	11.62 ± 0.31	13.60 ± 1.05
Plasma iron (μg/dl)	145.9 ± 12.8	50.7 ± 26.7	124.4 ± 9.5	31.0 ± 8.3
TIBC (μg/dl)	505.6 ± 18.1	476.3 ± 16.1	491.4 ± 23.3	522.0 ± 29.4
% Saturation	28.9 ± 2.3	10.4 ± 5.4	25.3 ± 1.6	5.8 ± 1.4

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bohydrate composition of r-hEPO and urinary EPO is very similar. The mechanisms controlling these secondary modifications remain unclear. Recombinant EPO produced in *Escherichia coli*, and therefore lacking glycosylation, or r-hEPO deglycosylated enzymatically has greatly decreased in vivo activity, although in vitro, its biological activity is preserved. The reasons why glycosylation is important for biological activity are unclear.

A clinical use of EPO in end-stage renal disease is obvious from the biology reviewed here. Treatment with EPO in end-stage renal disease constitutes replacement therapy. As such, clinical schedules and doses should prove easier to establish than for other recombinant-DNA-derived materials, such as the various lymphokines now in clinical trials. The extent to which EPO may be useful in other forms of anemia remains to be established, but the preclinical studies carried out so far are promising with regard to anemia of cancers and inflammatory diseases.

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