# Expression, bioactivity, and clinical assessment of recombinant feline erythropoietin

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**Objective**—To determine the activity of recombinant feline erythropoietin (rfEPO) in murine bioassays and evaluate its efficacy and safety in cats with erythropoietin-dependent nonregenerative anemia.

**Animals**—26 cats (group 1, 19 cats with anemia attributed to chronic kidney disease [CKD]; group 2, 7 cats with CKD and recombinant human erythropoietin [rhEPO]-induced red cell aplasia [RCA]).

**Procedure**—The rfEPO was synthesized by use of Chinese hamster ovary (CHO) cells transfected with feline erythropoietin complementary DNA. Preclinical assessments of rfEPO included an erythroid cell proliferation assay and measurements of reticulocytosis in Balb/C mice. Clinical assessments of cats included hematologic, biochemical, and clinical examinations during 12 (group 1) or 6 (group 2) months of rfEPO treatment.

**Results**—Biological activity of rfEPO was broadly equivalent to rhEPO in preclinical murine bioassays. Median Hct and absolute reticulocyte count in cats increased significantly during the first 3 weeks of rfEPO treatment, and median Hct generally could be maintained within a target range of 30% to 40% with periodic adjustments of rfEPO doses. Unexpectedly, 5 cats in group 1 and 3 cats in group 2 that initially responded to rfEPO treatment again developed anemia that was refractory to additional rfEPO treatments, even at higher doses.

**Conclusions and Clinical Relevance**—Treatment with rfEPO can reestablish active erythropoiesis in most cats with CKD, even those with anemia attributable to rhEPO-induced RCA. Unfortunately, development of RCA during treatment with CHO cell-derived recombinant erythropoietin proteins was not eliminated as a serious safety concern, even for this feline-specific preparation. (*Am J Vet Res* 2004;65:1355–1366)

Erythropoietin **(EPO)** is a glycosylated protein produced by peritubular cells of the renal cortex.[1] It stimulates erythrocyte production in the bone marrow.[1-3] Erythropoietin deficiency resulting from the progressive loss of EPO-producing cells during **chronic kidney disease (CKD)** is believed to be the most important cause of nonregenerative anemia that accompanies uremia in cats, dogs, and humans.[2,4-6] This EPO-dependent anemia contributes substantially to the clinical signs of weakness, lethargy, and poor appetite encountered in these patients.[4,6] To treat people with anemia attributable to CKD, commercially available preparations of **recombinant human EPO (rhEPO)** are widely used to stimulate RBC production.[7,8] Similarly, rhEPO administered to cats and dogs with nonregenerative anemia secondary to CKD induces a rapid and substantial red cell response.[9-12] Unfortunately, in many treated animals, the effect is short-lived because of the development of antibodies against rhEPO.[10-12] Apparently, there is sufficient structural variation between rhEPO and **feline EPO (fEPO)** or **canine EPO (cEPO)** that the immune systems of cats and dogs can recognize rhEPO as a foreign substance and mount an immune response.[12,13] The resultant antibodies are believed to block bioactivity of rhEPO and may have the potential to cross-neutralize endogenous fEPO or cEPO, leading to the development of life-threatening **red cell aplasia (RCA)**.[12]

The concept of EPO replacement seems appropriate for treating companion animals with anemia attributable to CKD; the problem is the immunogenicity of rhEPO. In fact, when a species-specific EPO preparation for dogs **(recombinant cEPO [rcEPO])** was administered to clinically normal Beagles for a 6-month period and to privately owned dogs that had nonregenerative anemia secondary to CKD, it effectively stimulated erythrocyte production without causing the RCA encountered in rhEPO-treated dogs.[14-16]

The study reported here was designed to extend this same concept to cats by testing the hypothesis that **recombinant fEPO (rfEPO)** will be effective for the therapeutic management of cats with EPO-dependent nonregenerative anemia while simultaneously providing an improved safety profile by avoiding the immunogenicity problems of rhEPO. The 3 primary objectives defined were to develop a eukaryotic expression system for the production of rfEPO and compare its biological activity with that of rhEPO in preclinical

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murine bioassay systems, evaluate the efficacy and safety of rfEPO in cats with anemia attributable to CKD, and determine whether rfEPO can restore RBC production in cats with CKD that had developed rhEPO-induced RCA.

## Materials and Methods

**Animals**—Twenty-six client-owned cats with naturally developing CKD were enrolled in the study between January and December 2001. Criteria for inclusion included a diagnosis of CKD clearly documented by use of established standards, nonregenerative anemia (Hct ≤ 22% and absolute reticulocyte count < 40,000 cells/μL) with no evidence of hemorrhage or hemolysis, and systolic blood pressure ≤ 180 mm Hg. Baseline data for each cat were collected within the 7 days preceding initiation of rfEPO treatment (designated as week 0). One cat did not have an absolute reticulocyte count during the defined baseline interval (ie, week 0), but a count 1 week before the baseline interval was 23,220 cells/μL. In another cat, the absolute reticulocyte count for the baseline interval was 56,300 cells/μL despite a count of 8,000 cells/μL 2 weeks earlier. This cat's Hct continued to decrease during the 2 weeks preceding onset of rfEPO treatment with no evidence of hemorrhage or hemolysis; thus, the cat was enrolled in the study. Treatment of cats for CKD before inclusion in the study included use of fluids (administered SC; 22 cats), diets formulated for cats with renal disease (20), phosphate binders (17), $H_2$-receptor blockers (14), vitamin or mineral supplements (10), potassium supplements (6), appetite stimulants (5), antihypertensive agents (5), androgenic steroids (4), and antiemetics (2). Five cats were treated by use of antimicrobials, and 3 cats were receiving products to soften the feces.

Nineteen of the 26 cats had never been treated with rhEPO (group 1); the remaining 7 cats had developed RCA as a consequence of prior rhEPO treatment (group 2). A diagnosis of rhEPO-induced RCA was determined on the basis of an initial increase followed by a subsequent decrease in Hct, concurrent reticulocytopenia despite continued administration of rhEPO at the same or higher dosages, and profound erythroid hypoplasia confirmed during examination of a sample of bone marrow. Median interval between cessation of treatment with rhEPO and initiation of rfEPO treatment in these 7 cats was 3 weeks (range, 2 to 31 weeks).

Cats of group 1 (12 males and 7 females; all were neutered) ranged from 7 to 17 years of age (median, 12 years) and weighed between 2.5 and 5.0 kg (median, 3.6 kg). Cats of group 2 (4 males and 3 females; all were neutered) ranged from 4 to 12 years of age (median, 8 years) and weighed between 2.0 and 7.0 kg (median, 4.2 kg). Breeds represented were domestic shorthair (group 1, 12; group 2, 4), domestic longhair (group 1, 6), Himalayan (group 1, 1; group 2, 2), and Siamese (group 2, 1).

Three cats were treated at the Cornell University Hospital for Animals; the other 23 cats were treated at participating veterinary medical teaching hospitals or privately owned veterinary practices. An Internet-based computer program was developed to support collection and input of primary data from participating veterinary hospitals and to facilitate communication between study coordinators and collaborating veterinarians. The design of experiments involving animals and the use of rfEPO in client-owned cats was approved by the Institutional Animal Care and Use Committee of Cornell University. The FDA-Center for Veterinary Medicine sanctioned the use of rfEPO as an investigational new animal drug for the purpose of the study. Informed consent was provided by each owner.

**Expression of rfEPO**—Full-length fEPO **complementary DNA** (**cDNA**; GenBank accession No. U00685) was obtained.[a] Before construction of the expression vector, the base sequence was modified by use of a **polymerase chain reaction (PCR)** assay to change nucleotides immediately upstream of the translational start site in an effort to conform as much as possible with Kozak's consensus for optimal translation efficiency.[17] Two oligonucleotide primers were synthesized. The sense primer (5'–AAA TCT AGA CGC CGC CACC ATG GGG TCG TGC GAA–3') contained a region (ATG GGG TCG TGC GAA) that corresponded to the fEPO cDNA sequence. The antisense primer (5'–AAA GTC GAC ACC TGG TCA CCT GTC TCC TCT–3') also contained a region (ACC TGG TCA CCT GTC TCC TCT) that corresponded to the fEPO cDNA sequence. The nonannealing 5' end of the sense primer included an *Xba 1* linker (TCT AGA) and introduced nucleotides intended to optimize ribosomal assembly. The nonannealing 5' end of the antisense primer included a *Sal 1* linker (GTC GAC). These nucleotide changes did not alter the encoded amino acid sequence predicted for fEPO (GenBank accession No. U00685).

The expression system was created by cloning the modified fEPO cDNA into a commercial vector[b] by use of the engineered *Xba 1* and *Sal 1* linkers. Correct nucleotide identity and orientation of the insert in the expression construct were confirmed by use of bidirectional DNA sequencing. The resulting plasmid was then cotransfected with pSV2-**dihydrofolate reductase (dhfr)**[c] into dhfr-deficient **Chinese hamster ovary (CHO)** cells[d] by use of calcium phosphate coprecipitation.[18] Following transfection, G418[e] (400 μg/mL) was added to the culture medium to eliminate nontransformants.

Methotrexate-induced amplification of rfEPO expression was conducted in accordance with standard methods.[19] Four pools of G418-resistant dhfr-deficient CHO cell clones were plated initially in medium containing 0.08μM methotrexate. After adaptation to the methotrexate during multiple passages in culture, the resulting effect on rfEPO expression was evaluated at a transcriptional and translational level, as described elsewhere.[14] The pool of cells that had the highest amount of rfEPO expression was then plated into medium containing a 4-fold increase in methotrexate concentration, and the process was repeated.

After completion of methotrexate-induced amplification of rfEPO expression, bidirectional nucleotide sequencing by use of both of the aforementioned terminal primers and internal nested primers (sense primer AAT GTC CTG CCC TGC TGC TT from exon 2 and antisense primer TCC CAG TGC CCG AAG CA from exon 4) was used to confirm fEPO cDNA identity matching with the original sequence (GenBank accession No. U00685; data not shown). There was no evidence that any DNA mutations were introduced within the amino acid coding region during the cloning or amplification steps used in the generation of the CHO-fEPO cell line. The cells were then fully adapted to growth in serum-free conditions in defined medium.[f]

For the clinical assessments that involved client-owned cats, rfEPO was partially purified from conditioned culture medium by use of anion-exchange chromatography.[20,21] Conditioned culture medium was diluted 1:2 with 10mM Tris buffer (pH, 7.0) and loaded onto an anion-exchange column.[g] In this buffer, rfEPO binds tightly to the gel matrix because its isoelectric point is slightly less than 4.0. Other proteins in the conditioned CHO cell medium that bound to the column were partially removed by use of 3 sequential washes with buffer solutions prepared at pH values of 6.5, 5.5, and 4.5 and consisting of 5mM acetic acid, 1mM glycine, and 6M urea. The column was then equilibrated back to neutral pH by use of a solution of 55mM NaCl and 10mM Tris (pH, 7.0). Elution of rfEPO was achieved with a gradient of NaCl (140mM to 500mM) in 10mM Tris (pH, 7.0). The rfEPO and sodium (140mM) concentrations were adjusted



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by use of 10mM phosphate buffer (pH, 7.2), which was followed by supplementation with 0.25% (wt:vol) feline albumin.[h] The rfEPO solution was then sterilized by passage through a 0.22-µm filter, placed in sterile vials, and stored frozen until use.

**Preclinical assays of bioactivity of rfEPO**—Biological activity of rfEPO was examined in vitro by use of splenic erythroid progenitor cells isolated from phenylhydrazine-treated mice.[22] For each assay, a single adult Balb/C mouse was treated with an intraperitoneal injection of phenylhydrazine (60 mg/kg) for 2 consecutive days to induce intravascular hemolysis. The resulting anemia stimulated extramedullary RBC production in the spleen. Three days later, the mouse was euthanatized, the spleen was removed and extruded through a sterile wire mesh, and a primary preparation of cells was isolated. Light microscopic examination of cells stained by use of Wright's-Giemsa stain confirmed that > 90% of these cells were of the erythroid lineage. Aliquots of $4 \times 10^6$ cells were transferred to separate microcentrifuge tubes, pelleted, and resuspended in 1 mL of culture medium that contained 14 dilutions of either rfEPO or rhEPO. Changes in DNA synthesis were used to assess the ability of EPO to stimulate cellular replication. Each EPO dilution (rfEPO and rhEPO) was evaluated in triplicate in a 96-well microtiter plate.[i] Cells were plated at a density of $8 \times 10^5$ cells in 0.2 mL of culture medium. After 22 hours in culture, the cells were labeled by incubation for 2 hours with 0.2 µCi $^3$H-thymidine.[j] Cells were harvested onto glass filter mats by use of a cell harvester,[k] and $^3$H-thymidine decay events were quantified in a liquid scintillation counter.[l] Medium without rfEPO or rhEPO and medium conditioned by nontransfected CHO cells were used as negative-control samples.

Bioactivity of rfEPO was assessed in vivo by direct quantitation of circulating reticulocytes in mice.[23] For 3 consecutive days, clinically normal adult Balb/C mice were administered SC injections of rfEPO or rhEPO at doses ranging from 0 to 30 U/mouse brought to a total volume of 200 µL with PBS solution. Control mice received injections of culture medium that had been conditioned by nontransfected CHO cells. One day after the third injection, an aliquot of blood was collected into EDTA-containing tubes. The percentage of reticulocytes in each blood sample was determined by flow cytometric analysis[m] of 10,000 cells stained with the fluorescent dye thiazole orange.[24,25,n]

**Dosage of rfEPO**—Commercial rhEPO[o-q] is supplied at defined concentrations[26] and was used as a control standard to estimate rfEPO concentrations through parallel preclinical bioactivity assays[22] and immunoblots.[14] The intended initial dosage of rfEPO (100 U/kg, SC, 3 times/wk) for cats of group 1 was consistent with established dosing recommendations for commercially available rhEPO in cats.[6,11] For cats of group 2, a higher dosage of rfEPO (400 U/kg, SC, 3 times/wk) was chosen because of the seemingly poor erythroid response in a study[16] of dogs with rhEPO-induced RCA that were treated at a dosage of 100 U of rcEPO/kg. Unexpectedly, erythropoietic responses in the first cats treated with rfEPO far exceeded values that would have been predicted by use of a comparable dose of rhEPO. As a result, activity estimates were reevaluated, which caused some variation in the initial amount of rfEPO administered to study participants. However, the dose of rfEPO for all cats was adjusted empirically at 1- to 4-week intervals in an attempt to achieve and then maintain the Hct in a target range between 30% and 40%. This target range was chosen to allow a substantial increase in Hct to ameliorate clinical signs of anemia while minimizing the possibility for the development of polycythemia. The first week of rfEPO treatment was designated as week 1.

**Clinical assessments of cats**—Body weight, clinical evaluations by participating veterinarians, and subjective impressions of owners regarding the cat's response to treatment were recorded at 7-day intervals (± 2 days) for the first 8 weeks after treatment with rfEPO, then at 30-day intervals (± 7 days) for 12 months (group 1) or 6 months (group 2). Indirect systolic blood pressure measurements obtained by use of automated oscillometry[r] or Doppler flow[s] were recorded at 30-day intervals (± 7 days).

**Laboratory assessments**—Hemograms, including percentage of aggregate reticulocytes, and serum iron variables (iron concentrations, total iron binding capacity, and percentage transferrin saturation) were performed immediately before initiation of rfEPO treatment and at monthly intervals thereafter. In addition, Hct, number of RBCs, and percentage of aggregate reticulocytes were determined weekly for the first 8 weeks after initiation of treatment. Absolute reticulocyte count was calculated by multiplying the percentage of aggregate reticulocytes by the number of RBCs. Serum biochemical analysis (sodium, potassium, chloride, BUN, creatinine, calcium, phosphate, total protein, albumin, glucose, total bilirubin, cholesterol, and bicarbonate concentrations and activities of alanine transaminase, aspartate transaminase, alkaline phosphatase, and γ-glutamyl transferase) was performed before starting rfEPO treatment and every 2 months thereafter. Multiple laboratories were used to analyze samples, except for serum iron variables, which were all performed at the College of Veterinary Medicine at Cornell University by use of automated procedures.[t] Reference ranges established at Cornell University by use of automated procedures[t,u] were used as guidelines for interpretation of data.

**Cytologic examination of bone marrow**—Bone marrow aspirates were collected, stained, and analyzed, as described elsewhere.[15,27] Slides from all bone marrow aspirates were reviewed by 1 investigator (TS) with board certification in veterinary clinical pathology. Bone marrow aspiration was performed before administration of rfEPO (1 cat of group 1 and all 7 cats of group 2) and at weeks 14 (2 cats of group 1) and 16 (1 cat of group 1 and 1 cat of group 2) after initiation of rfEPO treatment.

**Statistical analysis**—Data were analyzed in a manner similar to that reported for an evaluation of dogs treated by use of rcEPO.[16] Comparisons of continuous hematologic and biochemical values between the 2 groups of cats before initiation of treatment (ie, baseline) were made by use of the Wilcoxon rank-sum test. Median and ranges were estimated and compared in light of the relatively small sample sizes and lack of normal distributions for most of the variables evaluated. Comparisons of categoric variables (eg, proportion of cats responding to treatment) between groups were made by use of the $\chi^2$ test of independence.[28]

Median hematologic and biochemical values were plotted by week within each group to assess patterns over time. Then, clinically relevant changes from baseline values were evaluated to determine significant differences by use of the Wilcoxon signed-rank test for only those cats that had values at each time point. Statistical evaluations were not performed for time points that had 3 or fewer cats. Correlations between the values of continuous variables (eg, iron concentration and absolute reticulocyte count) were determined by use of the Spearman correlation coefficient.

Survival and time to rfEPO response for each group were estimated by use of Kaplan-Meier curves. Statistical comparison between the Kaplan-Meier curves was made by use of the log-rank test.[28]

## Results

**Expression and preclinical bioactivity assay of rfEPO**—The rfEPO was expressed as a broad band



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approximately 32 to 36 kd in size, consistent with results reported for rcEPO and commercial rhEPO.[14] Four sequential increases in methotrexate concentrations in culture medium (ie, 0.08 to 4.80μM) increased expression of rfEPO by more than 20-fold in transfected CHO cells (**Figure 1**). The expression system consistently achieved rfEPO concentrations > 12 μg/mL in conditioned culture medium even after the cells were adapted for growth in serum-free conditions. These yields were maintained in large-scale rfEPO preparative cultures completed without the addition of methotrexate and G418. Analytical SDS-PAGE followed by silver staining illustrated the accumulation of numerous proteins in the conditioned culture medium. Partial purification of rfEPO was achieved by use of anion-exchange column chromatography (**Figure 2**).

In preclinical assays, the overall pattern of rfEPO activity was broadly parallel to that of commercial rhEPO.[o] In vitro, the proliferation of splenic erythroid progenitor cells was stimulated in a dose-dependent manner by increasing amounts of rfEPO (**Figure 3**). No increase in $^3$H-thymidine incorporation was observed with defined culture medium[f] conditioned by use of nontransfected CHO cells. In mice, reticulocytosis was stimulated by rfEPO in a dose-dependent manner (**Figure 4**). Control mice receiving equivalent volumes of culture medium conditioned by nontransfected CHO cells had typical reticulocyte values for young adult mice of approximately 3%.

**Clinical assessment of cats—**After entry into the study, 3 additional cats were treated with antihypertensive agents, 2 with SC administration of fluids, 2 with products designed to soften feces, 2 with vitamin-mineral supplements, 2 with appetite stimulants, and 1 each with a phosphate-binding agent, an $H_2$-receptor blocker, and potassium supplements. Seven additional cats were treated with antimicrobials during the study.

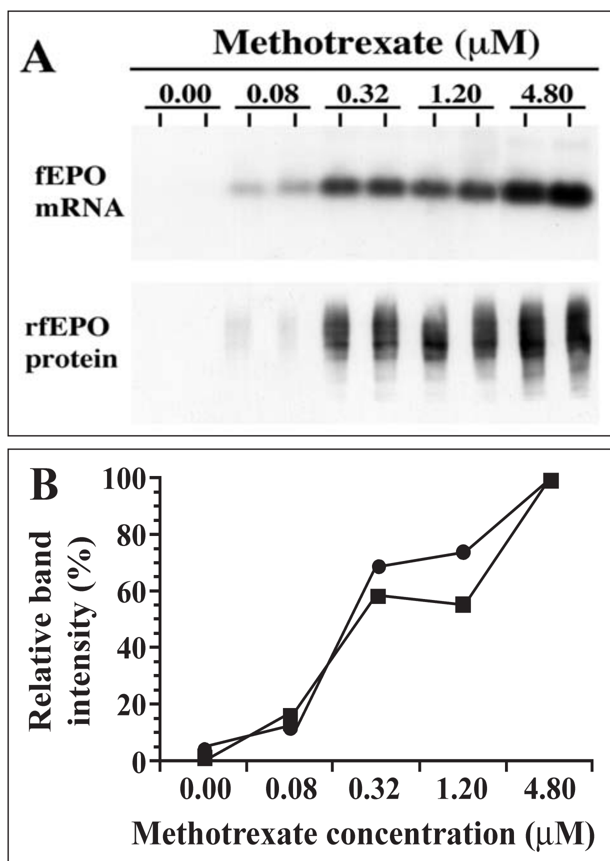


Figure 1—Northern blot of RNA data (top band) and western immunoblot of protein data (bottom band) for methotrexate-dependent amplification of feline erythropoietin (fEPO) expression (A) and graphic depiction of those same data (B). Dihydrofolate reductase-deficient Chinese hamster ovary (CHO) cells were cotransfected with expression vectors encoding dihydrofolate reductase and fEPO. Stepwise increases in selection pressure with methotrexate induced a 67-fold increase in steady-state fEPO mRNA concentrations and a 22-fold increase in concentrations of recombinant fEPO (rfEPO) protein in culture medium. For the graph, relative results for mRNA (squares) and protein (circles) values are reported, with the results for 4.80μM methotrexate defined as values of 100.

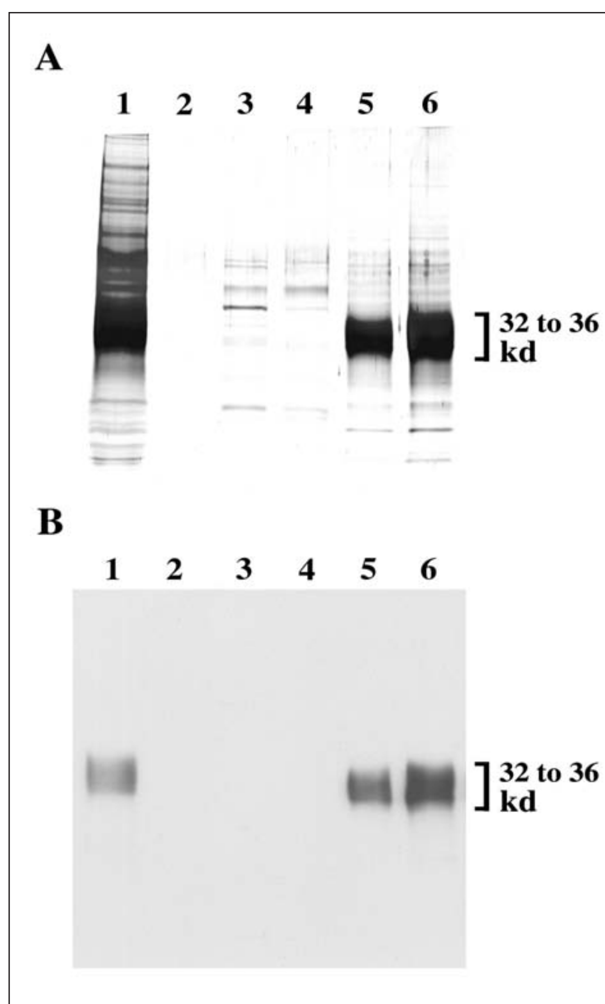


Figure 2—Partial purification of rfEPO from conditioned tissue culture medium by use of anion-exchange chromatography. Proteins were separated on the basis of size under reducing conditions by use of SDS-PAGE and developed with silver stain (A) or an immunoblot from a parallel gel by use of a primary antibody against erythropoietin (EPO) and chemiluminescence (B). The broad pattern of rfEPO (32 to 36 kd) reflects carbohydrate heterogeneity at glycosylation sites. Lanes were as follows: 1, conditioned medium from rfEPO-expressing CHO cells; 2 to 4, column wash solutions (highly diluted); and 5 and 6, rfEPO elution fractions.



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Twenty-two cats received daily oral iron supplements (median dose, 2 mg of elemental iron/kg; range, 0.7 to 5 mg/kg) starting no later than the onset of rfEPO treatment. Two cats received IM injections of iron (14 or 18 mg of elemental iron/kg); both cats received iron injections at the beginning of the study, and 1 cat received an additional injection at week 4. Two cats did not receive any iron supplements. Oral administration of iron supplements was discontinued in 3 cats and reduced in 1 cat because of adverse gastrointestinal effects.

**Hematologic tests**—For cats of group 1, median Hct steadily increased during the first 6 weeks after initiation of rfEPO treatment **(Figure 5; Table 1)** and was significantly ($P < 0.001$) different from the Hct for the baseline interval (week 0) after only 1 week of rfEPO treatment. Median Hct reached the target range of 30% to 40% by week 3 and was maintained at > 30% for the duration of the study. Intermittently during the study, the median Hct for cats of group 1 exceeded the target range but did not exceed 44%. For cats of group 2, median Hct was significantly ($P = 0.03$) increased by week 3, compared with the value for week 0, and reached the target range by week 5. After week 5, median Hct for > 3 cats of group 2 remained within the target range, except for week 7 (median Hct, 42.5%). Median absolute reticulocyte count was significantly increased from week 0 after only 1 week of rfEPO treatment in both groups (group 1, $P = 0.002$; group 2, $P = 0.04$); however, the initial median dose of rfEPO was 4-fold higher for group 2 than for group 1. In both groups, median absolute reticulocyte count decreased concomitant with the decrease in median dose of rfEPO. For the baseline interval, median Hct and absolute reticulocyte count were significantly ($P = 0.009$ and $P = 0.02$, respectively), and understandably, lower for the cats with rhEPO-induced RCA.

Cats in the study were classified as having an erythroid response to rfEPO treatment when their Hct increased by ≥ 50% above the baseline value without recent (within 2 weeks) administration of blood transfusions or physical or clinicopathologic evidence of profound dehydration. On the basis of this definition, 17 of 19 group 1 cats and 5 of 7 group 2 cats responded to rfEPO treatment. Using Kaplan-Meier estimates, more than half of the cats in each group responded by 2 to 3 weeks after initiation of rfEPO treatment. The 2 cats of group 1 classified as being nonresponsive to rfEPO on the basis of an increase in Hct of < 50% above the baseline value were treated for ≤ 3 weeks, so the lack of a substantial Hct response may have been attributable to an insufficient duration of treatment. Despite their abbreviated treatment with rfEPO, both of those cats had more than a 4-fold increase in absolute reticulocyte count. Although 3 cats (2 cats of group 1 and 1 cat of group 2) did respond to rfEPO on the basis of an increase of ≥ 50% above baseline Hct, their Hcts never reached the target range, and larger doses of rfEPO (600 to 1,200 U/kg/wk) were needed to maintain the number of RBCs or stimulate reticulocytosis.

Cats in both groups characteristically had a rapid and substantial erythrocyte response to rfEPO treatment (Figure 5). Values for many cats (11/19 cats of group 1 and 3/7 cats of group 2) exceeded the target range for Hct, even with close supervision and reductions in the rfEPO dose **(Figure 6)**. Although preclinical in vitro and in vivo assays in mice revealed equivalent activities of rhEPO and rfEPO, rfEPO seemingly was more potent in cats. Not surprisingly, the magnitude of the reticulocyte response for all cats was significantly ($P < 0.001$) greater when the initial rfEPO dose was 1,200 U/kg/wk, compared with the response for an initial dose of 300 U/kg/wk.

Unexpectedly, 5 of 17 group 1 cats and 3 of 5 group 2 cats that initially responded to rfEPO subsequently redeveloped anemia (> 50% decrease in Hct from highest value) associated with profound reticulocytopenia (absolute reticulocyte count,

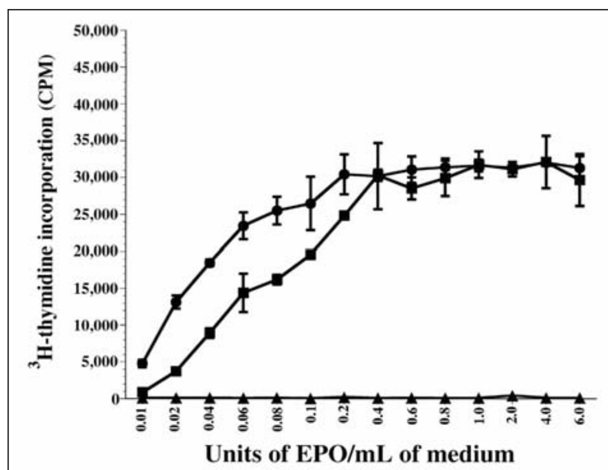


Figure 3—Mean ± SD results for stimulation of cell division of erythroid progenitor cells by rfEPO (circles) and recombinant human EPO (rhEPO; squares). Each concentration was analyzed in triplicate. Medium conditioned by nontransfected CHO cells (triangles) was used as a negative-control sample. CPM = Counts per minute.

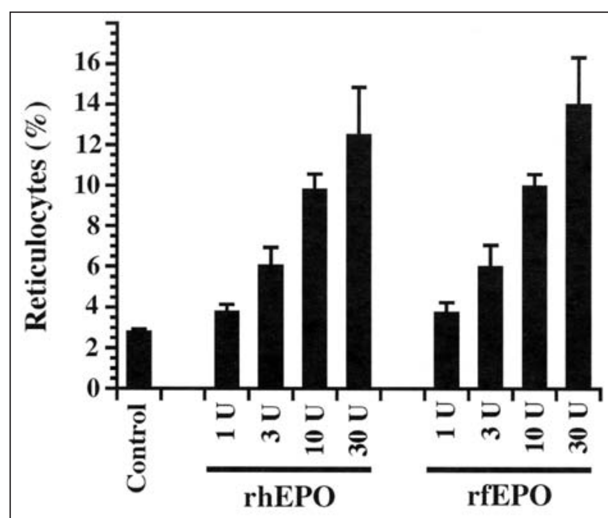


Figure 4—Mean ± SD results for stimulation of reticulocytes in mice by administration of various concentrations of rhEPO and rfEPO. Values reported represent data for each dose administered to 4 mice (2 females and 2 males). Control mice received injections of culture medium conditioned by nontransfected CHO cells.



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< 15,000 cells/μL) that was refractory to additional rfEPO treatment, even at higher doses. Median time of onset of this rfEPO-refractory anemia was 14.5 weeks (range, 12 to 38 weeks). The resultant anemia in 7 of these 8 cats was more profound than the anemia at the time of entry in the study (Figure 6b and 6d). There was no significant difference in the incidence of rfEPO-refractory anemia between cats receiving lower (300 U/kg/wk) or higher (1,200 U/kg/wk) initial doses of rfEPO. A single affected cat of group 1 appeared to recover spontaneously approximately 8 weeks after cessation of rfEPO treatment.

Median WBC counts for weeks with data available for > 3 cats were between $6.2 \times 10^3$ cells/μL and $13.2 \times 10^3$ cells/μL throughout the study for both groups and were not significantly different between the 2 groups for week 0. At most time points, certain cats (but not always the same cats) had WBC counts < $5.3 \times 10^3$ cells/μL (ie, below the lower limit of published reference ranges[29]). However, differential leukocyte counts only indicated neutropenia (defined as < 2,500 neutrophils/μL[29]) intermittently in 5 cats (4 cats of group 1 and 1 cat of group 2). In 4 of these cats, neutrophil counts returned to the reference range despite continued rfEPO administration. Median platelet counts for weeks with data available for > 3 cats were between $225 \times 10^3$ cells/μL and $425 \times 10^3$ cells/μL for group 1 cats throughout the study. None of the cats in either group had platelet numbers < $175 \times 10^3$ cells/μL, excluding those cats that had platelet clumps evident on examination of blood smears.

Median **mean corpuscular volume (MCV)** for cats of group 1 was significantly less at week 4, compared with the median MCV for week 0 ($P < 0.001$), and smaller still by week 8 ($P = 0.003$). Median MCV for group 2 cats did not change significantly with rfEPO treatment. However, median MCV for week 0 for group 2 cats was significantly ($P = 0.02$) less than that of group 1 cats, perhaps reflecting preexisting iron deficiency from prior rhEPO-stimulated erythropoiesis. Compared with the median **mean corpuscular hemoglobin concentration (MCHC)** for week 0, MCHC was not significantly less at week 4 for group 1 but was significantly ($P = 0.03$) less for group 2. However, the biological importance of the change in MCHC and difference in MCV at week 0 between groups is questionable because values remained within the reference ranges.

**Serum biochemical analysis**—Results for serum biochemical analysis were analyzed for all cats because pretreatment values were not significantly different between the 2 groups and differing patterns in test results could not be appreciated. Furthermore, data were only available for ≤ 3 cats of group 2 for weeks 16 and 24. As expected, median serum BUN and creatinine concentrations were high at week 0 (**Table 2**). However, compared with pretreatment values, no significant changes were detected in biochemical values during rfEPO administration. An increase in median activity of γ-glutamyl transferase at several time points (including week 0), compared with the reference range established at Cornell University, was most likely attributable to the higher reference range for γ-glutamyl transferase activity used by other laboratories.

Median serum iron concentrations had a pattern of decreasing concentrations, reaching concentrations less than the reference range during the first 8 weeks after initiation of rfEPO administration in group 1 cats, which coincided with decreased median MCV values (**Table 3**). These changes supported a contribution of rfEPO treatment to the development of iron deficiency, although the decrease in serum iron values may also have reflected concurrent chronic disease. Median serum iron concentrations for group 2 cats were greater than or at the upper limit of the reference range during that same time period. Serum iron concentrations for week 0 were higher but not significantly different for group 2 cats, compared with concentrations for group 1 cats, with no overlap in their ranges of values. The higher initial serum iron concentrations in group 2 cats may have been attributable to a combination of decreased iron utilization secondary to RCA and blood transfusions received by 5 of the 6 cats before enrollment in the study. Initial hyperferremia in

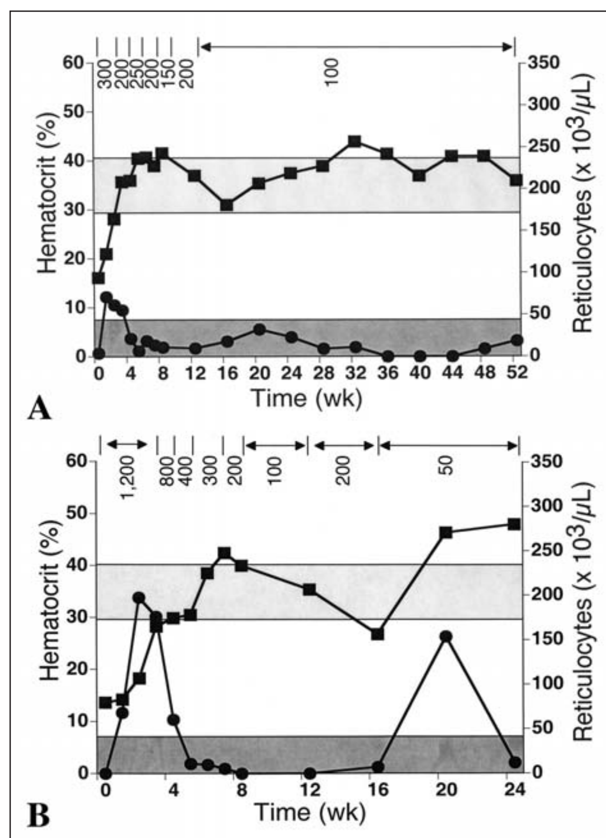


Figure 5—Median values for hematocrit (squares) and absolute reticulocyte count (circles) in 26 cats (19 cats with anemia attributable to chronic kidney disease [group 1; A] and 7 cats with chronic kidney disease and rhEPO-induced red cell aplasia [group 2; B]) during treatment with rfEPO. Values at the top of the graph represent the median rfEPO doses (U/kg/wk) for each group. Notice the target range for hematocrit (light gray band) and the reference range for absolute reticulocyte count (dark gray band). Week 0 represents baseline data points (before rfEPO administration), whereas week 1 indicates the first week of rfEPO administration. The number of cats in group 1 from which samples were obtained (panel A) decreased at weeks 2 (18 cats), 3 (17), 4 (15), 5 to 6 (14), 7 (13), 8 (12), 12 (11), 16 (9), 20 to 24 (6), 28 to 40 (5), and 44 to 52 (3) and in group 2 (panel B) decreased at weeks 6 to 7 (6), 8 (5), 12 (4), 16 (3), and 20 to 24 (1).



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Table 1—Results of hematologic tests in cats with anemia attributable to chronic kidney disease (group 1) and cats with chronic kidney disease and recombinant human erythropoietin (rhEPO)-induced red cell aplasia (group 2) during treatment with recombinant feline erythropoetin (rfEPO).

| Group | Week | No. of cats | Dose of rfEPO (U/kg/wk) | Hct (%) | n | Reticulocytes (X $10^3$ cells/μL) | n | RBC count (X $10^6$ cells/μL) | n | MCV (fL) | n | MCHC (g/dL) | n | WBC count (X $10^3$ cells/μL) | n | Platelet count (X $10^3$ cells/μL) | n |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | | | | | | | |
| | 0 | 19 | — | 16.1 (10.6–22.0) | 19 | 4.1 (0.0–56.3) | 18 | 3.5 (2.1–4.9) | 19 | 46 (40–62) | 19 | 32.4 (28.0–37.7) | 19 | 11.3 (5.0–17.9) | 19 | 425 (250–601) | 5 |
| | 1 | 19 | 300 (300–1,200) | 21.0 (16.1–36.0) | 19 | 71.2 (4.9–390.5) | 18 | 4.7 (3.0–6.8) | 18 | ND | — | ND | — | ND | — | ND | — |
| | 2 | 18 | 300 (300–1,200) | 28.1 (15.9–42.0) | 18 | 61.6 (5.2–862.2) | 17 | 6.2 (3.7–8.6) | 17 | ND | — | ND | — | ND | — | ND | — |
| | 3 | 17 | 300 (100–1,200) | 35.7 (21.9–51.0) | 17 | 55.5 (0.0–357.2) | 16 | 8.1 (4.6–11.3) | 16 | ND | — | ND | — | ND | — | ND | — |
| | 4 | 15 | 200 (100–800) | 36.0 (19.0–57.0) | 15 | 21.5 (5.7–474.7) | 14 | 9.5 (4.4–11.6) | 14 | 42 (35–49) | 15 | 32.0 (29.9–38.0) | 15 | 10.0 (4.8–24.5) | 15 | 350 (236–600) | 4 |
| | 5 | 14 | 250 (50–800) | 40.5 (23.0–53.0) | 14 | 6.6 (0.0–51.0) | 13 | 9.5 (5.2–12.2) | 13 | ND | — | ND | — | ND | — | ND | — |
| | 6 | 14 | 200 (50–800) | 40.8 (24.9–57.0) | 14 | 18.7 (0.0–798.0) | 13 | 10.1 (5.0–14.9) | 13 | ND | — | ND | — | ND | — | ND | — |
| | 7 | 13 | 200 (50–400) | 39.0 (24.5–62.0) | 13 | 13.7 (0.0–171.6) | 13 | 10.7 (5.2–15.6) | 13 | ND | — | ND | — | ND | — | ND | — |
| | 8 | 12 | 150 (0–400) | 41.6 (18.0–53.0) | 12 | 11.2 (0.0–46.0) | 12 | 10.9 (4.6–16.2) | 12 | 38 (30–42) | 12 | 32.9 (30.4–33.7) | 12 | 8.5 (3.6–22.5) | 12 | 325 (299–902) | 3 |
| | 12 | 11 | 200 (0–600) | 37.0 (17.1–49.0) | 11 | 10.0 (0.0–28.8) | 11 | 9.3 (4.9–14.2) | 11 | 39 (32–49) | 11 | 33.0 (29.0–35.5) | 11 | 6.8 (2.1–25.9) | 11 | 332 | 1 |
| | 16 | 9 | 100 (0–600) | 31.0 (14.1–47.7) | 9 | 18.2 (0.0–236.3) | 9 | 7.1 (3.5–13.9) | 9 | 36 (33–47) | 9 | 32.7 (30.0–36.2) | 9 | 8.5 (5.1–26.0) | 9 | 245 (90–430) | 5 |
| | 20 | 6 | 100 (50–600) | 35.4 (21.0–45.4) | 6 | 32.3 (10.1–117.6) | 6 | 8.6 (4.9–12.3) | 6 | 40 (36–47) | 6 | 32.9 (29.0–35.7) | 6 | 9.7 (6.0–27.0) | 6 | 243 (143–706) | 4 |
| | 24 | 6 | 100 (50–1050) | 37.5 (17.0–47.9) | 6 | 23.0 (9.6–330.1) | 6 | 9.7 (3.7–12.0) | 6 | 42 (38–48) | 6 | 33.0 (30.0–35.2) | 6 | 13.2 (6.7–26.8) | 6 | 281 (256–325) | 5 |
| | 28 | 5 | 100 (25–200) | 39.0 (32.1–45.0) | 5 | 9.3 (0.0–14.2) | 5 | 10.2 (7.1–10.8) | 5 | 40 (38–45) | 5 | 33.8 (31.0–35.2) | 5 | 8.0 (4.9–14.9) | 5 | 225 (177–265) | 4 |
| | 32 | 5 | 100 (25–350) | 44.0 (31.7–47.9) | 5 | 11.1 (0.0–25.8) | 5 | 11.1 (7.3–12.9) | 5 | 42 (37–44) | 5 | 33.0 (30.0–35.0) | 5 | 6.9 (6.5–19.7) | 5 | 253 (200–269) | 4 |
| | 36 | 5 | 100 (25–400) | 41.5 (27.3–53.0) | 5 | 0.0 (0.0–11.2) | 5 | 10.4 (6.2–15.4) | 5 | 42 (34–46) | 5 | 33.0 (30.0–34.9) | 5 | 8.6 (6.3–14.1) | 5 | 215 (129–250) | 3 |
| | 40 | 5 | 100 (0–114) | 37.0 (10.8–47.4) | 5 | 0.0 (0.0–12.1) | 5 | 10.4 (2.5–12.1) | 5 | 39 (31–43) | 5 | 34.8 (34.0–36.0) | 5 | 6.2 (5.5–13.4) | 5 | 312 (225–349) | 3 |
| | 44 | 3 | 100 (25–100) | 41.0 (36.2–48.3) | 3 | 0.0 (0.0–9.9) | 3 | 9.9 (9.4–12.4) | 3 | 39 (39–41) | 3 | 34.0 (33.9–35.9) | 3 | 6.0 (6.0–16.6) | 3 | 505 | 1 |
| | 48 | 3 | 100 (25–100) | 41.0 (36.7–47.1) | 3 | 8.9 (0.0–29.1) | 3 | 9.7 (8.9–12.0) | 3 | 41 (39–42) | 3 | 34.8 (33.0–35.0) | 3 | 8.1 (4.6–14.5) | 3 | 339 | 1 |
| | 52 | 3 | 100 (25–100) | 36.0 (31.9–50.7) | 3 | 18.8 (14.8–38.7) | 3 | 9.4 (7.4–12.9) | 3 | 40 (38–43) | 3 | 35.2 (34.7–36.0) | 3 | 7.5 (4.2–13.7) | 3 | 339 (240–558) | 2 |
| 2 | | | | | | | | | | | | | | | | | |
| | 0 | 7 | — | 13.6 (8.5–17.7) | 7 | 0.0 (0.0–9.3) | 7 | 2.7 (2.2–4.3) | 6 | 42 (39–52) | 6 | 33.7 (28.7–37.8) | 7 | 11.1 (6.8–14.5) | 7 | 173 | 1 |
| | 1 | 7 | 1200 (1200–1,200) | 14.2 (6.6–18.0) | 7 | 68.0 (0.0–224.0) | 7 | 2.8 (1.5–4.1) | 7 | ND | — | ND | — | ND | — | ND | — |
| | 2 | 7 | 1200 (720–1,200) | 18.3 (11.0–24.2) | 7 | 197.3 (0.0–722.7) | 7 | 3.7 (2.8–4.9) | 7 | ND | — | ND | — | ND | — | ND | — |
| | 3 | 7 | 1200 (300–1,200) | 28.3 (16.0–40.0) | 7 | 176.0 (0.0–706.4) | 7 | 6.1 (3.4–8.4) | 7 | ND | — | ND | — | ND | — | ND | — |
| | 4 | 7 | 800 (300–1,200) | 29.9 (13.0–48.3) | 7 | 60.5 (0.0–597.8) | 7 | 6.1 (2.9–10.9) | 7 | 45 (40–54) | 7 | 31.9 (28.2–34.7) | 7 | 9.0 (3.0–18.7) | 7 | 78 | 1 |
| | 5 | 7 | 400 (300–1,200) | 30.5 (11.8–50.5) | 7 | 11.1 (0.0–538.1) | 7 | 6.5 (2.1–11.9) | 7 | ND | — | ND | — | ND | — | ND | — |
| | 6 | 6 | 300 (200–1,200) | 38.6 (8.0–47.1) | 6 | 10.0 (0.0–48.7) | 6 | 9.1 (1.6–13.2) | 6 | ND | — | ND | — | ND | — | ND | — |
| | 7 | 6 | 300 (50–1,200) | 42.5 (18.3–55.0) | 6 | 5.6 (0.0–47.4) | 6 | 9.7 (3.7–15.8) | 6 | ND | — | ND | — | ND | — | ND | — |
| | 8 | 5 | 200 (50–1,200) | 40.0 (14.4–52.5) | 5 | 0.0 (0.0–13.0) | 5 | 11.5 (3.0–16.3) | 5 | 40 (32–49) | 5 | 32.0 (31.6–36.2) | 5 | 8.3 (2.9–11.0) | 5 | 123 | 1 |
| | 12 | 4 | 100 (50–675) | 35.4 (17.8–54.6) | 4 | 0.0 (0.0–0.0) | 4 | 9.9 (3.8–18.0) | 4 | 36 (30–47) | 4 | 33.6 (32.9–35.4) | 4 | 7.6 (3.8–10.9) | 4 | ND | — |
| | 16 | 3 | 200 (50–400) | 26.8 (13.7–47.0) | 3 | 7.1 (0.0–15.0) | 3 | 7.1 (3.8–15.0) | 3 | 37 (31–38) | 3 | 32.3 (31.3–35.7) | 3 | 9.9 (4.1–13.2) | 3 | ND | — |
| | 20 | 1 | 50 | 46.4 | 1 | 153.6 | 1 | 12.8 | 1 | 36 | 1 | 34.7 | 1 | 4.6 | 1 | ND | — |
| | 24 | 1 | 50 | 48.0 | 1 | 12.1 | 1 | 12.1 | 1 | 40 | 1 | 34.6 | 1 | 3.7 | 1 | ND | — |
| Reference range* | | | — | 32.0–52.0 | | 0.0–40.0 | | 6.9–10.9 | | 40–52 | | 29.0–34.0 | | 5.3–16.6 | | 201–523 | |

Values expressed are median (range).
*Represents reference range established by the laboratory of the College of Veterinary Medicine at Cornell University.
n = Number of cats tested. MCV = Mean corpuscular volume. MCHC = Mean corpuscular hemoglobin concentration. ND = Not determined. — = Not applicable.

group 2 cats may have helped protect many of them from developing iron deficiency during subsequent rfEPO-stimulated erythropoiesis.

**Evaluation of bone marrow**—The bone marrow aspirate obtained from 1 cat of group 1 before initiation of rfEPO treatment was judged normocellular with an adequate number of megakaryocytes (4 cells/spicule). The **myeloid-to-erythroid ratio (M:E)** was 1.2:1, which in combination with the anemia and a leukocyte count within the reference range was considered compatible with erythroid hypoplasia associated with CKD. Bone marrow aspirates were also performed on 3 additional cats of group 1 after 14 to 16 weeks of rfEPO treatment because of decreases in Hct and reticulocytopenia; 1 cat required collection of a bone marrow core biopsy for adequate evaluation. All 3 cats had RCA characterized by a lack of or extremely few detectable erythroid precursors (M:E, 32:1 to 100:1). Marrow cellularity was considered normal in 1 cat but was decreased in the other 2 cats. Two of these cats had marrow lymphocytosis (21% to 35% of total cells). Marrow iron stores were detected in 1 of 2 cats tested, compatible with a decrease in iron utilization secondary to RCA. One cat with neutropenia (1,400 neutrophils/μL) had numerous myeloid precursors in the marrow with an orderly and complete maturation sequence. The cause for neutropenia in this cat was not determined.

Bone marrow aspirates were performed on all 7 cats of group 2 before initiation of rfEPO treatment. Marrow could not be aspirated from 2 cats, so core biopsy specimens were obtained from them. Marrow cellularity was considered normal in 3 cats and decreased in 3 cats; cellularity could not be judged in 1 cat because spicules were not obtained during aspiration. Megakaryocyte numbers were considered adequate (2 to 6 cells/spicule). Six cats had RCA (M:E, > 100:1 with no erythroid precursors identified), whereas 1 cat had severe erythroid hypoplasia (M:E, 16:1). Marrow iron was detected in small amounts in the 1 cat evaluated. Four cats had mild bone marrow lymphocytosis (19% to 28% of total cells), and 2 had mild plasmacytosis (6% to 7% of total cells). A second bone marrow aspirate was collected from 1 cat after 16 weeks of rfEPO treatment because the cat had a recurrence of anemia and reticulocytopenia (Figure 6d). The marrow aspirate obtained at that time was consistent with RCA.

**Other clinical variables**—Median systolic blood pressure and median body weight did not change significantly in either group during rfEPO administration. However, systolic blood pressure > 180 mm Hg did



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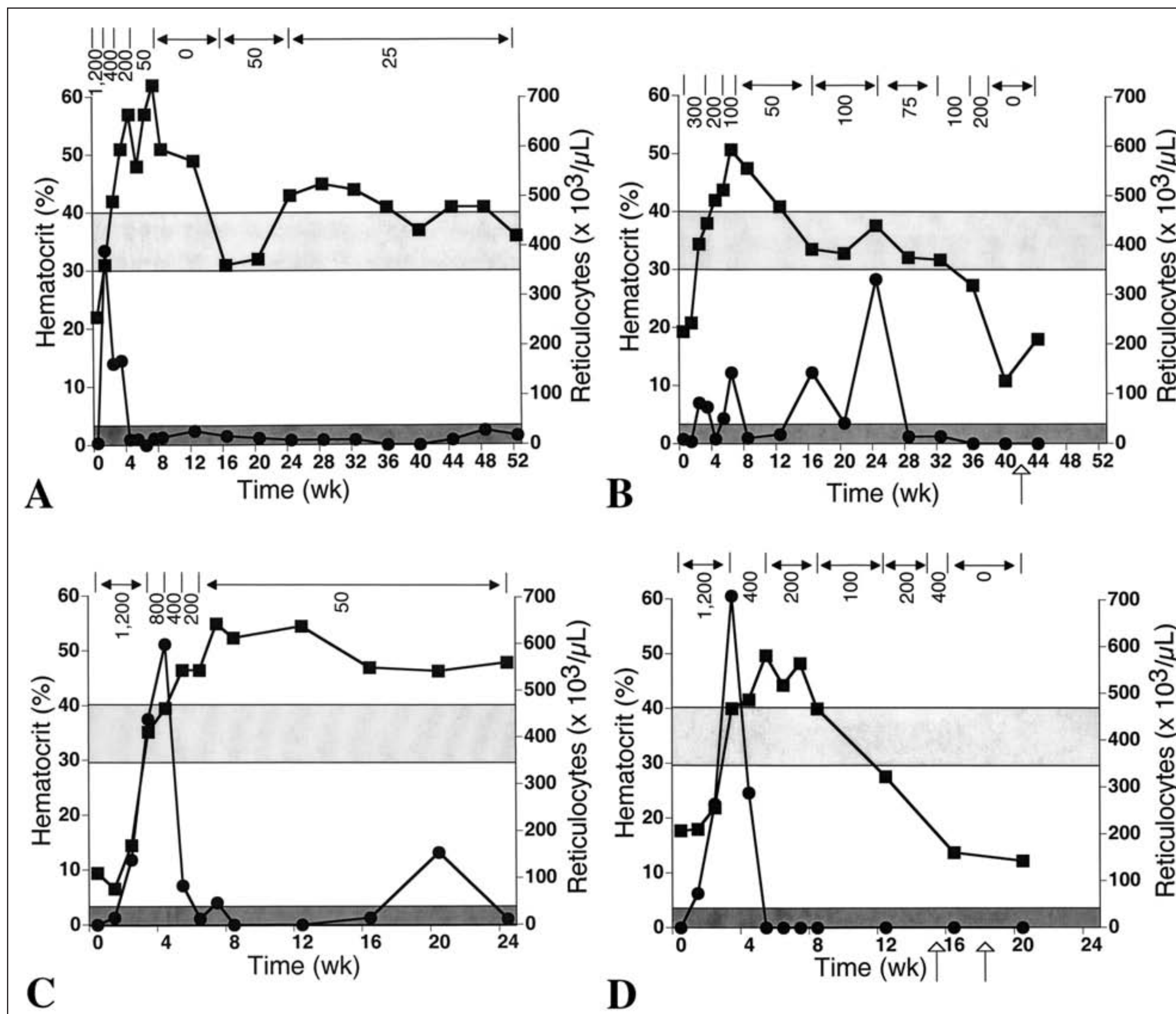


Figure 6—Hematocrit (squares) and absolute reticulocyte count (circles) in 2 cats with chronic kidney disease (A and B) and 2 cats with chronic kidney disease and rhEPO-induced red cell aplasia (C and D) during treatment with rfEPO. Values at the top of the graph represent the rfEPO doses (U/kg/wk) for each cat. In addition, 2 cats were administered blood transfusions (open arrows) after developing rfEPO-refractory anemia (B and D). *See* Figure 5 for remainder of key.

Table 2—Median (range) values for serum biochemical analyses in 26 cats (19 cats with anemia attributable to chronic kidney disease and 7 cats with chronic kidney disease and rhEPO-induced red cell aplasia) during treatment with rfEPO.

| Variable | Week 0 | n | Week 8 | n | Week 16 | n | Week 24 | n | Week 32 | n | Week 40 | n | Week 48 | n | Reference range* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sodium (mEq/L) | 154 (146–161) | 24 | 155 (147–159) | 16 | 153 (147–162) | 12 | 154 (152–159) | 7 | 154 (151–157) | 5 | 151 (147–157) | 5 | 151 (151–155) | 3 | 146–156 |
| Potassium (mEq/L) | 4.5 (3.6–6.2) | 24 | 5.0 (3.9–6.3) | 16 | 4.9 (3.6–5.8) | 12 | 5.4 (4.5–6.4) | 7 | 5.7 (5.0–6.3) | 5 | 5.4 (4.8–5.9) | 5 | 5.0 (4.9–5.4) | 3 | 3.8–5.6 |
| Chloride (mEq/L) | 118 (106–128) | 24 | 115 (106–126) | 16 | 119 (108–127) | 12 | 118 (113–122) | 7 | 118 (109–121) | 5 | 117 (102–121) | 5 | 121 (118–122) | 3 | 112–123 |
| BUN (mg/dL) | 63 (31–123) | 26 | 56 (31–130) | 16 | 53 (25–89) | 12 | 64 (32–107) | 7 | 70 (36–98) | 5 | 58 (38–231) | 5 | 61 (40–110) | 3 | 17–35 |
| Creatinine (mg/dL) | 4.2 (2.0–8.7) | 26 | 3.1 (2.2–10.9) | 16 | 3.1 (1.6–5.1) | 12 | 3.1 (1.9–5.2) | 7 | 3.6 (2.0–5.3) | 5 | 4.1 (2.3–10.3) | 5 | 3.7 (2.3–5.1) | 3 | 0.7–2.1 |
| Calcium (mg/dL) | 10.4 (9.1–12.5) | 25 | 10.7 (8.7–13.7) | 16 | 10.8 (8.4–13.0) | 12 | 10.8 (7.5–11.8) | 7 | 11.3 (9.6–11.7) | 5 | 10.5 (9.5–11.2) | 5 | 10.1 (9.6–10.9) | 3 | 8.2–11.5 |
| Phosphorus (mg/dL) | 5.3 (3.0–15.3) | 25 | 5.3 (3.7–13.6) | 16 | 5.0 (3.7–11.2) | 12 | 5.9 (4.8–10.3) | 7 | 6.1 (5.1–6.6) | 5 | 5.4 (4.2–16.1) | 5 | 6.6 (4.7–8.8) | 3 | 3.0–6.6 |
| Total protein (g/dL) | 7.4 (6.3–9.0) | 26 | 7.5 (6.2–8.5) | 16 | 7.4 (6.4–8.3) | 12 | 7.5 (7.1–8.3) | 7 | 7.9 (6.9–8.7) | 5 | 7.2 (6.4–7.6) | 5 | 7.0 (6.8–8.1) | 3 | 6.7–8.5 |
| Albumin (g/dL) | 3.0 (2.2–4.3) | 26 | 3.1 (2.0–3.7) | 16 | 3.2 (1.8–3.4) | 12 | 3.1 (2.0–3.4) | 7 | 3.1 (2.8–3.4) | 5 | 2.9 (2.6–3.2) | 5 | 3.0 (2.5–3.2) | 3 | 2.9–4.3 |
| Glucose (mg/dL) | 108 (95–195) | 26 | 98 (76–188) | 16 | 109 (79–146) | 12 | 108 (74–120) | 7 | 113 (54–118) | 5 | 104 (76–126) | 5 | 100 (92–112) | 3 | 63–140 |
| ALT (U/L) | 60 (26–174) | 26 | 66 (41–125) | 16 | 65 (12–246) | 12 | 48 (37–104) | 7 | 54 (34–57) | 5 | 56 (46–258) | 5 | 51 (41–83) | 3 | 29–186 |
| AST (U/L) | 29 (10–83) | 21 | 37 (15–57) | 13 | 31 (16–94) | 10 | 27 (12–80) | 5 | 27 (23–36) | 4 | 36 (19–134) | 4 | 34 (23–45) | 2 | 13–46 |
| ALP (U/L) | 23 (8–44) | 26 | 30 (3–47) | 16 | 28 (6–40) | 12 | 27 (9–31) | 7 | 21 (12–50) | 5 | 28 (17–33) | 5 | 27 (19–39) | 3 | 15–96 |
| GGT (U/L) | 4 (0–13) | 18 | 1 (0–11) | 10 | 3 (0–32) | 9 | 4 (0–9) | 6 | 3 (1–25) | 5 | 3 (1–9) | 5 | 4 (2–8) | 3 | 0–2 |
| Total bilirubin (mg/dL) | 0.1 (0.1–0.2) | 25 | 0.1 (0.1–0.3) | 16 | 0.2 (0.1–0.8) | 12 | 0.1 (0.1–0.3) | 7 | 0.1 (0–1.1) | 5 | 0.2 (0.1–0.4) | 5 | 0.2 (0.1–0.4) | 3 | 0–0.2 |
| Cholesterol (mg/dL) | 181 (110–337) | 25 | 193 (103–300) | 16 | 198 (126–323) | 12 | 214 (138–299) | 7 | 204 (150–340) | 5 | 252 (155–333) | 5 | 264 (217–307) | 3 | 73–265 |
| Bicarbonate (mEq/L) | 17 (11–30) | 11 | 19 (13–23) | 8 | 18 (15–22) | 4 | 13 (13–20) | 3 | 17 (10–19) | 3 | 18 (10–18) | 3 | 16 | 1 | 12–21 |

ALT = Alanine transaminase. AST = Aspartate transaminase. ALP = Alkaline phosphatase. GGT = γ-Glutamyl transferase.
*See* Table 1 for remainder of key.



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Table 3—Median (range) values for serum iron variables in cats with anemia attributable to chronic kidney disease (group 1) and cats with chronic kidney disease and rhEPO-induced red cell aplasia (group 2) during treatment with rfEPO.

| Group | Week | Iron (mg/dL) | n | TIBC (mg/dL) | n | Transferrin saturation (%) | n |
|---|---|---|---|---|---|---|---|
| 1 | | | | | | | |
| | 0 | 68 (33–211) | 17 | 259 (196–309) | 14 | 27 (19–100) | 14 |
| | 4 | 39 (14–127) | 13 | 240 (187–319) | 9 | 15 (7–48) | 9 |
| | 8 | 49 (21–326) | 12 | 256 (208–326) | 10 | 19 (8–100) | 10 |
| | 12 | 154 (15–303) | 10 | 228 (165–310) | 9 | 95 (9–100) | 9 |
| | 16 | 54 (10–268) | 9 | 236 (147–288) | 8 | 18 (7–100) | 8 |
| | 20 | 68 (21–89) | 5 | 251 (205–312) | 4 | 28 (28–29) | 4 |
| | 24 | 47 (36–81) | 6 | 271 (218–327) | 6 | 18 (13–34) | 6 |
| | 28 | 84 (69–129) | 4 | 238 (193–313) | 3 | 36 (28–54) | 3 |
| | 32 | 59 (25–112) | 5 | 264 (217–320) | 4 | 21 (10–52) | 4 |
| | 36 | 108 (57–159) | 3 | 291 (261–315) | 3 | 41 (20–50) | 3 |
| | 40 | 82 (59–284) | 5 | 254 (199–307) | 5 | 32 (25–94) | 5 |
| | 44 | 92 (64–255) | 4 | 255 (200–337) | 4 | 32 (31–100) | 4 |
| | 48 | 79 (63–118) | 3 | 240 (182–298) | 3 | 35 (33–40) | 3 |
| | 52 | 98 (88–108) | 2 | 254 | 1 | 35 | 1 |
| 2 | | | | | | | |
| | 0 | 301 (230–344) | 6 | 287 (230–324) | 3 | 100 (97–100) | 3 |
| | 4 | 155 (50–414) | 5 | 278 (261–295) | 2 | 62 (23–100) | 2 |
| | 8 | 174 (65–237) | 4 | 281 (237–325) | 2 | 72 (43–100) | 2 |
| | 12 | 209 (206–259) | 3 | 209 (206–259) | 3 | 100 (100–100) | 3 |
| | 16 | 280 (270–290) | 2 | 290 | 1 | 100 | 1 |
| | 20 | ND | — | ND | — | ND | — |
| | 24 | 100 | 1 | 336 | 1 | 30 | 1 |
| Reference range* | | 57–156 | | 208–378 | | 20–61 | |

TIBC = Total iron-binding capacity.
*See* Table 1 for remainder of key.

develop in 6 cats (5 cats of group 1 and 1 cat of group 2). For the study reported here, hypertension was defined as systolic blood pressure > 180 mm Hg as measured by use of indirect methods in conscious untrained cats. In 4 of the 6 cats, the increase in blood pressure was attributed to stress and subsequent blood pressure determinations were consistently ≤ 180 mm Hg. In the remaining 2 cats (both of which were in group 1), 1 developed terminal hypertension, whereas the other was treated successfully with amlodipine.[v]

During rfEPO treatment of the 19 cats in group 1, appetite subjectively improved in 15 and amount of activity increased in 14. These improvements generally were recorded by owners and veterinarians during the first few weeks after initiation of rfEPO treatment. For the 7 cats of group 2, improvements in appetite and amount of activity were recorded in 4 of the 5 cats that developed reticulocytosis and subsequent increases in Hct during rfEPO administration and after blood transfusion in the 2 cats that failed to respond to rfEPO treatment.

Seizures unassociated with hypertension or polycythemia developed in 1 cat after 5 weeks of rfEPO administration and continued intermittently (once per week) until the cat died during week 7. Polycythemia (Hct > 60%) developed in 1 rfEPO-treated cat (Figure 6a). However, the Hct of that cat never exceeded 62%, and clinical consequences of polycythemia were not evident; with temporary cessation of rfEPO administration, the Hct returned to the reference range. Excoriations at injection sites were recorded for 2 cats of group 1, but it was unclear whether these cats were reacting specifically to the rfEPO because both were also receiving fluids subcutaneously. Two cats of group 2 excessively licked their skin or pulled their hair during rfEPO treatment; 1 of these cats exhibited similar behavior during prior treatment with rhEPO. Despite continued rfEPO administration in all 4 cats with dermatologic lesions, the excoriations and abnormal behavior resolved. Another cat became recumbent 90 minutes after an rfEPO injection and died later that day despite treatment with antihistamines, glucocorticoids, and oxygen for presumed anaphylaxis. Additional problems that were first recognized during the period of the study included a hepatic mass and dental abscess (1 cat), abdominal mass and lameness (1), *Mycoplasma haemofelis* infection (1), and diarrhea (1). Episodic vomiting and poor appetite were evident in most cats during the study but were attributed to uremia.

No significant difference in Kaplan-Meier survival curves between the 2 groups of cats was detected. Median values were 111 and 124 days for groups 1 and 2, respectively.

## Discussion

Analysis of results of the study reported here indicates that rfEPO effectively stimulates erythropoiesis in cats with nonregenerative anemia secondary to CKD or rhEPO-induced RCA. Seventeen of 19 cats in group 1 and 5 of 7 cats in group 2 had an erythroid response to rfEPO characterized by an increase of ≥ 50% above the pretreatment Hct without confounding factors (eg, recent blood transfusions or profound dehydration). Most cats responded within 2 to 3 weeks after initiation of rfEPO treatment. Although they responded to rfEPO, 3 cats (2 cats of group 1 and 1 cat of group 2) had Hct values that did not reach the target range of 30% to 40% and required larger doses of rfEPO (600 to



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1,200 U/kg/wk) to maintain RBC numbers or stimulate reticulocytosis. Interestingly, abdominal masses were subsequently diagnosed in 2 of these cats. It has been reported[30-34] that inflammatory cytokines associated with neoplasia or chronic disease inhibit EPO bioactivity, directly inhibit erythroid cell progenitors, and impair iron metabolism. Furthermore, this inhibition may be overcome by increased concentrations of EPO.[35,36] A similar phenomenon may have been responsible for the blunted response to rfEPO manifested by those 2 cats.

Adverse effects that have been reported[2,7,8,16,37] after administration of homologous EPO in humans and dogs (eg, iron deficiency, hypertension, polycythemia, seizures, and local and systemic reactions) were also evident in the cats of the study reported here. Reductions in MCV, serum iron concentration, and percentage transferrin saturation developed in many cats of group 1 during the initial months after initiation of rfEPO treatment despite supplementation of iron in most cats (Tables 1 and 3). These clinicopathologic patterns, which are consistent with iron deficiency, have been attributed to insufficient iron release from macrophages to meet the increased demand accompanying recombinant EPO-driven erythropoiesis.[38] Supplementation of iron is recommended during treatment with recombinant EPO to prevent iron depletion, but patients often develop iron deficiency despite use of oral supplements.[15,16,38-40] Unfortunately, several cats were intolerant of orally administered iron supplements, and insufficient amounts of iron in these cats may have contributed to iron deficiency. Although iron deficiency can blunt stimulation of erythropoiesis,[8,11,38-40] significant correlations between serum iron concentrations and absolute reticulocyte counts or between MCV and absolute reticulocyte counts in rfEPO-treated cats were not detected.

It is uncertain whether hypertension (ie, systolic blood pressure persistently > 180 mm Hg) documented in 2 rfEPO-treated cats was a manifestation of CKD or a consequence of rfEPO treatment. With increased RBC production after administration of recombinant EPO, oxygen delivery to tissues is improved, which causes a decrease in vasodilation and an increase in peripheral vascular resistance.[41] In addition, exogenous EPO may also cause Hct-independent, vasoconstriction-dependent hypertension mediated by endothelin or prostanoids.[42-44] Regardless of cause, hypertension was successfully treated by use of amlodipine in 1 cat.

Polycythemia (Hct, 62%) was detected in only 1 cat and resolved with temporary cessation of rfEPO administration (Figure 6a). Seizures and a presumed anaphylactic reaction also were each detected in 1 rfEPO-treated cat and ultimately precipitated the deaths of both of those cats. Unfortunately, necropsy evaluations were unavailable for either of those cats to enable an evaluation of potential contributing variables. Four cats reportedly had local reactions manifested as excoriations at the injection sites or removal or pulling of hair in those areas, but all resolved despite continued administration of rfEPO.

A far more serious problem from a clinical perspective was that 8 of 26 treated cats became refractory to rfEPO administration (Figure 6b and 6d). These cats were from both experimental groups, thereby including some cats with no history of ever having received rhEPO. The cats redeveloped anemia associated with profound reticulocytopenia that was ultimately more severe than the anemia evident at the time of entry into the study. Analysis of bone marrow aspirates obtained from 4 of these 8 cats confirmed RCA. This unexpected finding suggests that despite being feline-specific, the rfEPO preparation used in this study has the potential to induce neutralizing antibodies that interfere with the erythroproliferative activity of recombinant and endogenous EPO. The fact that 5 of 7 cats in group 2 clearly responded initially to rfEPO may indicate that rhEPO and rfEPO have structurally distinct, immunogenic epitopes.

In addition to the fEPO cDNA clone used for construction of the expression vector for the study reported here (GenBank accession No. U00685), 1 other fEPO cDNA sequence (GenBank accession No. L10606) has been reported[13] by another group of investigators. Direct comparison of these 2 sequences identifies a single nucleotide mismatch in the protein-coding region within exon 2. The 18th codon of the mature fEPO protein (ie, after cleavage of the signal peptide) is reported as GGG for the expression vector used in our study, which predicts the small aliphatic amino acid glycine. This same codon is reported as GAG in the cDNA sequence reported by that other group, which predicts the larger and acidic amino acid glutamate. Analysis of these data suggests the possibility that fEPO has allelic variation within the domestic cat population, which theoretically could explain the reason that rfEPO made from an expression vector containing the GGG codon may be immunogenic in a subset of cats. To explore this possibility, the exon 2 region of fEPO was amplified with a PCR technique by use of genomic DNA from 12 cats. Nine of those cats were participants in the rfEPO clinical study reported here. Of these, 1 was a cat in group 2 that failed to respond to rfEPO, 6 responded to rfEPO treatment but developed RCA, and 2 responded to rfEPO without developing RCA (including 1 cat treated for the entire 1-year study period). The nucleotide sequence in all 12 cats (24 fEPO alleles) was GAG at the 18th codon (data not shown). Unfortunately, genomic DNA was unavailable from the original cat from which the fEPO cDNA reported as GenBank accession No. U00685 was created, leaving undetermined whether the GGG sequence is an infrequent allele in domestic cats or possibly an artifact introduced during the original cloning. Human EPO also has a nucleotide sequence with GAG at the 18th codon and differs from fEPO at 26 other amino acid residues (83.7% amino acid identity for the mature protein).

A recent study[45,w] on rfEPO administered to 8 clinically normal cats via IM injection of a **recombinant adeno-associated virus (rAAV)** vector containing fEPO cDNA raises additional questions. Three cats became polycythemic, which is reflective of rfEPO expression from the transgene. However, 1 of those 3 cats developed RCA approximately 12.5 weeks after administration. Interestingly, the fEPO cDNA sequence



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used in the rAAV vector and both fEPO alleles in the cat that developed RCA had the trinucleotide sequence GAG at the 18th codon (data not shown). Analysis of this finding suggests a mechanism for inducing RCA other than an amino acid disparity at codon 18 in exon 2. Importantly, another report[46] describes the development of a CHO cell-based rfEPO expression system that uses a cDNA clone with GAG at codon 18. The obvious question is whether a subset of cats treated with this rfEPO preparation will also develop RCA.

Other possible causes for immunogenicity of recombinant proteins include differences in glycosylation from the native protein, product components that may act as adjuvants, and route of administration.[47,48] In the past 5 years, RCA has been recognized with increasing frequency in human patients receiving rhEPO produced with CHO cell expression systems, even though recombinant and native human EPOs have identical amino acid compositions.[47,49-53] Neutralizing anti-EPO antibodies have been confirmed in affected humans, but the trigger for the formation of those antibodies remains unknown.[49,50] Although patterns of glycosylation may differ between native and recombinant proteins,[54,55] neutralizing EPO antibodies in rhEPO-treated humans appear to be directed against the protein component of EPO rather than the carbohydrate side chains added after translation by the CHO cells.[49,50] Furthermore, in the cat that developed RCA following administration of rAAV-fEPO, the rfEPO was produced in situ within the cat's own muscle cells rather than in a heterologous cell culture system.[45,w] Nevertheless, conformational changes in recombinant proteins associated with altered glycosylation could expose novel epitopes for immunologic recognition.[56] Most of the human cases of rhEPO-induced RCA have developed during treatment with 1 preparation of the recombinant protein,[q] which suggests that the manufacturing process may be an important variable.[47,50,53] Precedent also exists for other recombinant proteins (eg, interferon or thrombopoietin) to elicit immunogenic responses.[47,48,57,58] Additionally, most human patients that develop RCA following rhEPO administration have received the product subcutaneously rather than intravenously,[49,50] possibly suggesting that SC administration may increase the likelihood of antibody production.[48,50,59]

The study reported here was designed to test the hypothesis that rfEPO will be effective for the therapeutic management of EPO-dependent nonregenerative anemia in cats while simultaneously providing improved safety by avoiding the immunogenicity problems associated with use of rhEPO. Species-specific recombinant EPO only rarely causes RCA in people (ie, rhEPO; < 1/10,000 [0.01%])[60] and has never been observed to cause RCA in the limited data set available with dogs (ie, rcEPO; 0/26 [0%]).[15,16] In contrast, the incidence of suspected RCA associated with the rfEPO preparation reported here (5/19 [26%] of group 1 cats) appears to be in the same general range as that observed for cats that receive a sustained course of rhEPO.[10-12,45] Therefore, even though the efficacy of rfEPO in cats was high and included the ability to restore active erythropoiesis in 5 of 7 cats with rhEPO-induced RCA, apparent immunogenicity remained a major problem. An evaluation of the serum samples collected over time from the cats in this study to determine whether there were any neutralizing anti-EPO antibodies, and epitope mapping should neutralizing antibodies be identified, would provide important additional information.

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[a]Provided by Dr. Robin Bell, Cornell University, Ithaca, NY.
[b]pCI-neo, Promega, Madison, Wis.
[c]ATCC 37146, American Type Culture Collection, Rockville, Md.
[d]ATCC CRL-9096, American Type Culture Collection, Rockville, Md.
[e]Geneticin selective antibiotic, GibcoBRL, Grand Island, NY.
[f]CD-CHO, GibcoBRL, Grand Island, NY.
[g]Q Sepharose Fast Flow, Amersham Biosciences, Piscataway, NJ.
[h]Albumin from cat, Sigma Chemical Co, St Louis, Mo.
[i]96-well cell culture plate, Corning Science Products, Corning, NY.
[j][Methyl-$^3$H] thymidine, Perkin Elmer, Shelton, Conn.
[k]Skatron, Flow Laboratories, McLean, Va.
[l]LS 6800, Beckman, Fullerton, Calif.
[m]FACS Calibur, Becton-Dickinson, San Jose, Calif.
[n]Retic-COUNT, Becton-Dickinson, San Jose, Calif.
[o]Epogen, Amgen Inc, Thousand Oaks, Calif.
[p]Procrit, Ortho Biotech Inc, Johnson & Johnson, Raritan, NJ.
[q]Eprex, Janssen-Ortho Inc, Johnson & Johnson, Toronto, ON, Canada.
[r]Dinamap vital signs monitor, Critikon, Tampa, Fla.
[s]Ultrasonic Doppler flow detector, Parks Medical Electronics, Aloha, Ore.
[t]Hitachi 917, Roche Diagnostics, Indianapolis, Ind.
[u]Advia 120, Bayer Diagnostics, Norwood, Mass.
[v]Norvasc, Pfizer, New York, NY.
[w]Levy JK, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, Fla: Personal communication, 2003.

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