

# Expression of erythropoietin in cats treated with a recombinant adeno-associated viral vector

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**Objective**—To characterize the biological effects of IM administration of a recombinant adeno-associated virus serotype 2 (rAAV2) vector containing feline erythropoietin (fEPO) cDNA and determine whether readministration of the vector or removal of muscle tissue at the injection sites alters those effects.

**Animals**—10 healthy 7-week-old specific pathogen-free cats.

**Procedure**—Cats received  $1 \times 10^7$  infective units (iU;  $n = 3$ ),  $1 \times 10^8$  iU (3), or  $1 \times 10^9$  iU (2) of rAAV2-fEPO vector IM (day 0). Two control cats received an rAAV2 vector containing the LacZ gene ( $1 \times 10^9$  iU, IM). In all cats, hematologic variables and serum fEPO concentration were measured at intervals; anti-rAAV2 antibody titer was measured on day 227. In cats that did not respond to treatment, the rAAV2-fEPO vector was readministered. Injection sites were subsequently surgically removed.

**Results**—Compared with control cats, cats treated with  $1 \times 10^9$  iU of rAAV2-fEPO vector had increased Hct and serum fEPO concentrations. One of these cats developed pure RBC aplasia; its Hct normalized following injection site excision. Cats receiving lower doses of vector had no response; on retreatment, 1 of those cats developed sustained erythrocytosis that persisted despite injection site removal and the others did not respond or responded transiently. Antibodies against rAAV2 were detected in all vector-treated cats.

**Conclusions and Clinical Relevance**—Gene therapy may be an effective treatment for cats with hypoproliferative anemia. However, rAAV2-fEPO vector administration may result in pure RBC aplasia or pathologic erythrocytosis, and injection site removal does not consistently abolish the biological response. (*Am J Vet Res* 2005;66:450–456)

Erythropoietin (EPO) is a glycoprotein hormone that regulates RBC production; it is produced predominantly by peritubular cells in the kidney.<sup>1</sup>

Erythropoietin and recombinant human EPO (rhEPO) act to protect erythroid cells from apoptosis, thereby increasing their survival.<sup>2</sup> In addition, EPO regulates the proliferation and differentiation of erythroid progenitor cells to mature erythrocytes.<sup>3</sup> The main application of rhEPO in veterinary medicine is in the treatment of hypoproliferative anemia in cats with EPO deficiency as a result of chronic renal failure. With correction of anemia, these cats have improvement in appetite; weight gain; increased serum potassium concentration, energy, and vocalization; and restoration of personality and behavior.<sup>4</sup> Furthermore, rhEPO has been shown to safely increase Hct values in FIV-infected cats.<sup>5</sup> The initial enthusiasm for treatment of anemic cats with rhEPO has been tempered because of the need for lifelong injections, poor long-term responses, and in some instances, transient transfusion dependence as a result of a humoral immune response induced by the rhEPO that cross-reacts with native feline EPO (fEPO).<sup>4</sup> These adverse events have also been detected in cats treated with recombinant fEPO (rfEPO).<sup>6</sup> Because rfEPO and rhEPO are not optimal for the treatment of cats, delivery of fEPO via gene therapy may be an ideal solution.

Recombinant adeno-associated virus serotype 2 (rAAV2) is considered an excellent vector for gene therapy because it is a highly defective virus incapable of productive infection in the absence of helper adenovirus; wild-type AAV2 is not associated with disease in humans or animals; rAAV2 lacks wild-type coding sequences and thus may elude host defenses; rAAV2 can efficiently transduce terminally differentiated cells; rAAV2 integrates or persists in an episomal location in nondividing cells to decrease the risk of insertional mutagenesis; and the virions are resistant to chemical and physical treatment, which facilitates the purification and concentration of viral stocks.<sup>7–13</sup>

We have constructed an rAAV2 vector containing fEPO complementary DNA (cDNA)<sup>a</sup> under the control

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of a hybrid cytomegalovirus (CMV) enhancer-chicken  $\beta$ -actin (CBA) promoter. The objective of the study of this report was to characterize the biological effects of IM administration of the rAAV2-fEPO vector and determine whether these effects are altered by re-administration of the vector or removal of the muscle tissue at the injection sites.

## Materials and Methods

**Adeno-associated virus vectors**—The gene sequence of the fEPO cDNA (GenBank accession No. U00685)<sup>14</sup> was confirmed by use of an automated DNA sequencing protocol.<sup>15,b</sup> A fragment containing the Kozak consensus sequence upstream of the fEPO cDNA was cloned into the pCR2.1 plasmid.<sup>c</sup> The Kozak-fEPO cassette was digested by Xho I, blunt-ended by T4 polymerase, and isolated by Hind III digestion. The cassette was inserted into the UF11-CBA plasmid by Not I digestion to generate the rAAV2-fEPO vector (Figure 1). The UF11-CBA plasmid contained the hybrid CMV enhancer-CBA promoter upstream of the fEPO cDNA and the herpesvirus thymidine kinase promoter-driven gene for neomycin resistance downstream of the fEPO cDNA.<sup>16</sup> The hybrid CMV enhancer-CBA promoter contained nucleotides -706 to -188 of the CMV enhancer (nucleotides 436 to 954 of the sequence with GenBank accession No. X03922)<sup>17</sup> and nucleotides 1 to 1,345 of the CBA promoter (GenBank accession No. E03011).<sup>18,19</sup> This modified promoter construct of CMV enhancer and CBA promoter results in higher levels of gene expression than other promoters.<sup>20</sup> The vectors were packaged into infective virus by use of 293 cells, as previously described.<sup>21</sup> The 293 cells were cotransfected with the pDG plasmid containing the AAV *rep* and *cap* genes and the adenoviral genes necessary for AAV vector production.<sup>22</sup> Cells were disrupted by freeze-thaw lysis, and virions were purified via iodixanol-gradient ultracentrifugation followed by 6% crosslinked agarose, heparin-treated, bead-formed, agarose-based gel filtration matrix column chromatography, as described.<sup>13,21</sup> The vector stock contained  $5 \times 10^9$  infectious units (iU) of rAAV2-fEPO/mL as determined by an infectious center assay.<sup>21</sup>

**Cats**—Ten 7-week-old sexually intact specific pathogen-free cats (5 males and 5 females) that weighed 0.55 to 0.80 kg were included in the gene therapy trial. These cats were considered to be healthy on the basis of findings of physical and laboratory evaluations and were free of FeLV and FIV infections as determined by ELISA.<sup>d</sup> An additional 5 cats were used as negative controls for the determination of anti-AAV2 antibodies; these were privately owned, adult healthy cats of both sexes from which blood samples were collected with the owners' informed consent. All animal studies were conducted with the approval of the University of Florida Institutional Animal Care and Use Committee in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The rAAV2-fEPO vector was registered with the University of Florida Institutional Biosafety Committee and Biological Safety office.

**Vector administration**—The 10 cats were assigned to the treatment groups arbitrarily. All cats were premedicated with butorphanol (0.4 mg/kg, SC) for preemptive analgesia. Anesthesia was induced via IM injection of a pre-mixed combination of tiletamine HCl-zolazepam HCl (5 mg/kg), ketamine (4 mg/kg), and xylazine (0.5 mg/kg).<sup>23</sup> The skin in the left hip region was aseptically prepared for a small incision to expose the left superficial gluteal mus-

cle. By use of a tuberculin syringe with a 25-gauge needle, the rAAV2-fEPO vector was injected into the superficial gluteal muscle at doses of  $1 \times 10^7$  iU for the low-dose group (cats 1 through 3),  $1 \times 10^8$  iU for the intermediate-dose group (cats 4 through 6), and  $1 \times 10^9$  iU for the high-dose group (cats 7 and 8). To evaluate the in vivo transduction of feline skeletal muscle by rAAV2,  $1 \times 10^9$  iU of an rAAV2 vector containing the LacZ gene (rAAV2-LacZ) was injected into the contralateral superficial gluteal muscle in control cats.<sup>24</sup> The stock solutions of rAAV2-fEPO and rAAV2-LacZ vectors were diluted with sterile PBS solution (pH, 7.2) so that all cats received a 200- $\mu$ L injection volume. Following vector administration, anesthesia was partially reversed with yohimbine (0.15 mg/kg, IV).

**Blood sample collection and hematologic evaluations**—Blood was collected via jugular venipuncture into tubes containing EDTA and serum-separator tubes prior to vector administration (day 0) and every week thereafter for 47 weeks (329 days). The samples collected in EDTA were used for Hct measurements, CBCs with differentials, and aggregate reticulocyte counts. Hematocrits were measured weekly throughout the study. A CBC (automated<sup>e</sup>) with differential (performed manually) was determined before treatment and on days 14, 35, 49, 63, 84, 98, 112, and 140 after treatment. Aggregate reticulocytes were also counted at these time points except for day 140. Serum was separated within 30 minutes of blood collection and stored at -80°C pending analysis for fEPO concentration and anti-rAAV2 antibodies. Bone marrow aspirates were collected from the proximal humerus of 2 cats (1 and 7) following the unexpected development of persistent erythro-

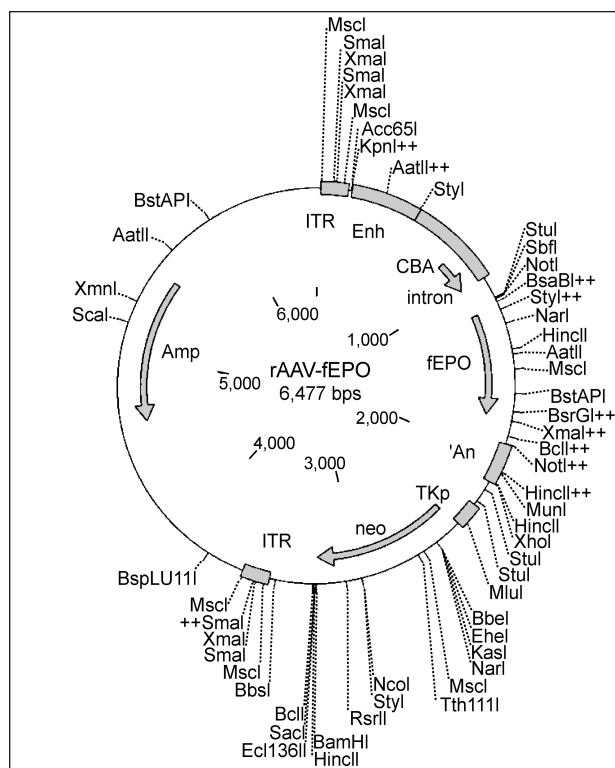


Figure 1—Map of the 6,477-bp UF11-chicken  $\beta$ -actin promoter (CBA) plasmid containing recombinant adeno-associated virus (rAAV) and the feline erythropoietin (fEPO) gene. Transcription of the fEPO gene is controlled by a hybrid promoter consisting of the cytomegalovirus (CMV) enhancer (Enh) and the CBA. The herpesvirus thymidine kinase promoter (TKp) controls transcription of the neomycin resistance gene (neo). The inverted terminal repeats (ITR) are the only wild-type AAV sequences in the plasmid vector.

cytosis and hypoplastic anemia, respectively; for these procedures, cats were anesthetized as described for vector administration. Bone marrow samples were prepared as smears and stained with Wright-Giemsa for cytologic examination and calculation of the myeloid-to-erythroid ratio.

**Readministration of the rAAV2-fEPO vector**—On day 83 after the initial injection, the cats in the low- and intermediate-dose groups that did not respond to treatment received a second administration of rAAV2-fEPO vector. Following surgical preparation (as performed for the initial treatment), cats that initially received a low dose were injected in the left superficial gluteal muscle with  $1 \times 10^9$  iU of rAAV2-fEPO vector and cats that previously received an intermediate dose were treated with  $1 \times 10^8$  iU of rAAV2-fEPO vector.

**Removal of injection sites**—On day 256, cats were anesthetized as described for vector administration; the superficial gluteal muscle previously treated with vector was excised from all cats in the control, low-dose, and intermediate-dose groups. Because pure RBC aplasia developed in cat 7 in the high-dose group, the left superficial gluteal muscle and a bone marrow specimen from the proximal humerus were collected from this cat on day 125. Because severe erythrocytosis developed in cat 8 in the high-dose group, the left superficial gluteal muscle was excised from this cat on day 227.

The excised superficial gluteal muscles were fixed in neutral-buffered 10% formalin, and sections were stained with H&E for routine histologic examination. In addition, muscle samples from the control cats were snap-frozen in liquid nitrogen for analysis of  $\beta$ -galactose content because of the expression of the LacZ gene insert in the rAAV2 vector, as described.<sup>12</sup>

**Assessment of fEPO concentrations**—Feline EPO concentrations in sera collected on days 0, 35, 52, and 87 were determined by use of an ELISA<sup>1</sup> performed according to the manufacturer's instructions.<sup>25</sup> Erythropoietin concentrations were also measured on days 125 and 135 in cat 7 after development of severe nonregenerative anemia.

**Detection of neutralizing anti-AAV2 antibodies**—Sera collected on day 227 from all cats that received rAAV2-fEPO vector and 5 cats that had not received gene therapy were tested for antibodies against rAAV2 by use of a virus neutralization assay. For this assay, human 293 cells were seeded in 24-well plates ( $1 \times 10^5$  cells/well) in 500  $\mu$ L of Dulbecco's modified Eagle medium and incubated with  $1 \times 10^4$  iU of adenovirus. Serum samples were diluted 1:10, 1:100, and 1:1,000 in Ringer's solution containing  $1 \times 10^7$  iU of an rAAV2 vector expressing green fluorescent protein. Negative controls were similar dilutions of sera from healthy cats that had not been treated with any rAAV2 vectors. Positive controls were similar dilutions of a mouse monoclonal antibody<sup>8</sup> against AAV2. The serum-rAAV2 mixtures were added to duplicate wells containing adenovirus-infected 293 cells and incubated at 37°C for 36 hours. The number of fluorescent (ie, green fluorescent protein-expressing) and nonfluorescent cells in each well was determined via fluorescence microscopy. The presence of rAAV2 antibodies was determined by the inhibition of fluorescence in 293 cell cultures containing serum from treated cats, compared with fluorescence detected in serum from untreated control cats. Cultures with at least 50% inhibition of fluorescence were considered to contain AAV2-neutralizing antibodies.

**Statistical analyses**—The data were analyzed as a randomized complete block design with each cat as a block.

Treatment group variances were tested for heterogeneity by use of the *F*-max test, but observed variance differences were not sufficiently great to warrant transformation. Computations were performed by use of computer software (mixed procedure).<sup>h</sup> A *P* value < 0.05 was considered significant.

## Results

**Hematologic responses to treatment with rAAV2-fEPO vector**—There were no significant differences in platelet (*P* = 0.39), neutrophil (*P* = 0.31), eosinophil (*P* = 0.41), lymphocyte (*P* = 0.18), or monocyte (*P* = 0.19) counts among the treatment groups. Similarly, there were no significant differences in RBC indices, including mean corpuscular volume (*P* = 0.17), mean corpuscular hemoglobin (*P* = 0.38), and aggregate reticulocyte counts (*P* = 0.12), among groups at any time point (Figure 2). The initial decrease observed in mean corpuscular volume and mean corpuscular hemoglobin in all groups, combined with the gradual but mild increase in Hct in all but the high-dose group, were consistent with normal age-related changes of these parameters in young kittens.<sup>26</sup> Compared with cats treated with  $1 \times 10^8$  iU of rAAV2-fEPO vector, the red cell distribution width in cats treated with  $1 \times 10^9$  iU of rAAV2-fEPO vector was slightly increased (*P* = 0.04) at several time points. Furthermore, red cell distribution width was higher at day 0 than at day 14 (*P* = 0.01) and day 35 (*P* = 0.02) in all groups. From 0 to 83 days after vector administration, there were no significant (*P* = 0.4) differences in the Hct values between control cats and cats treated with  $1 \times 10^7$  iU or  $1 \times 10^8$  iU of rAAV2-fEPO vector.

In contrast to the cats treated with lower doses of vector that did not respond to treatment, the 2 cats treated with  $1 \times 10^9$  iU of rAAV2-fEPO vector developed erythrocytosis. By day 35 after treatment, the cats in this group had significantly (*P* = 0.02) higher Hct values than all other treatment groups, and the Hct values continued to increase through day 87 (Figure 3). One cat (cat 8) in this high-dose group had a sustained erythrocytosis through day 227, with Hct values consistently > 60% (Figure 4); however, following removal of the vector-treated muscle on day 227, the Hct steadily decreased to a value within the Hct range for the control cats and cats that did not respond to treatment. The Hct value in the other cat (cat 7) in the high-dose group decreased precipitously from a peak value of 58% on day 87 to 18% on day 125 (Figure 5). Aggregate reticulocyte counts for cat 7 were low during this time (0.3% to 0.7% on days 63 through 98) despite a markedly high serum fEPO concentration (76 and 145 mU/mL on days 87 and 125, respectively). Bone marrow analysis on day 125 revealed that the erythrocyte maturation was orderly and complete in this cat, but there was a marked decrease in erythrocyte progenitors, resulting in an abnormal myeloid-to-erythroid ratio of 5:1 (normal reference range,<sup>27</sup> 1:1 to 1:3). There was no evidence of neoplasia in cat 7, and a diagnosis of pure RBC aplasia was made. Within 10 days of excision of the vector-treated muscle on day 125, the Hct returned to a value within the Hct range of the control cats and cats that did not respond to treatment and remained stable thereafter.

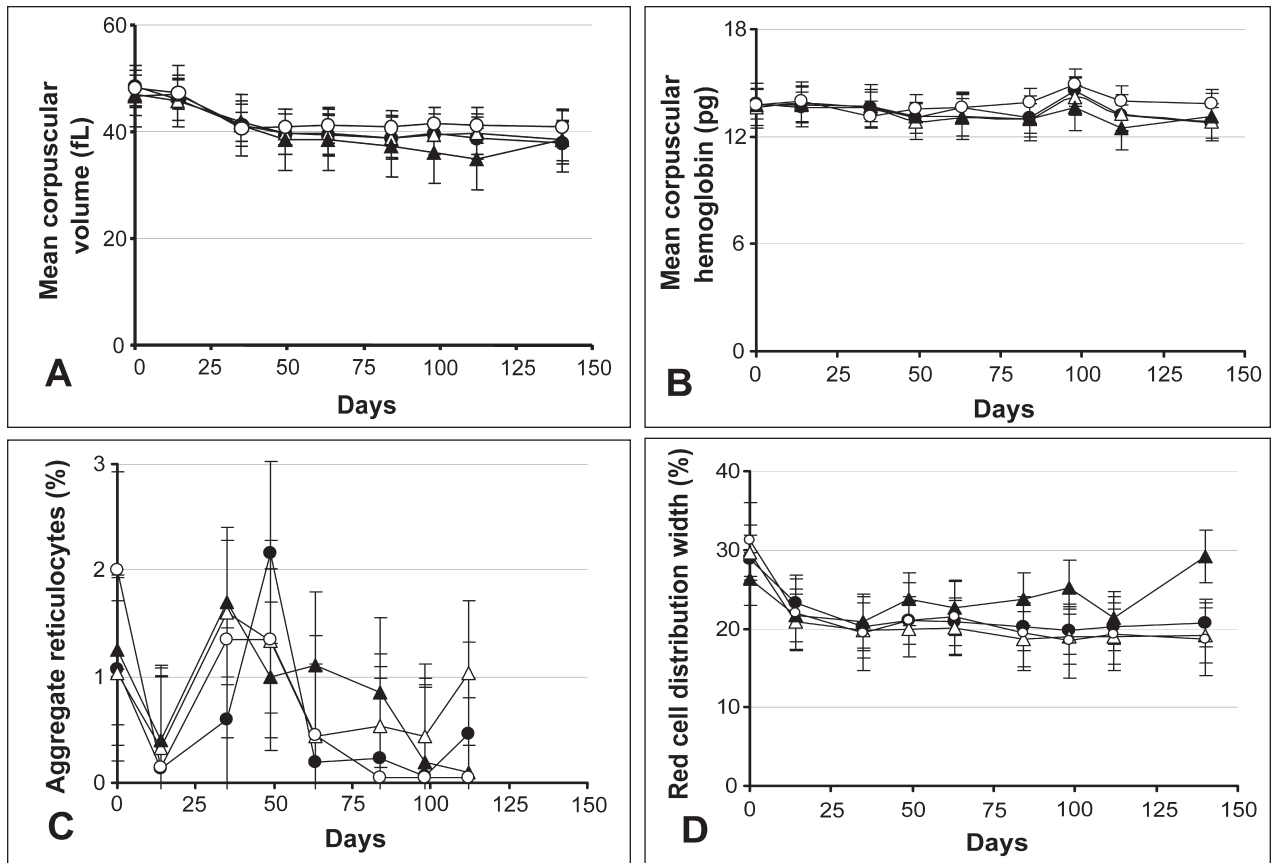


Figure 2—Mean  $\pm$  SD corpuscular volume (A), corpuscular hemoglobin (B), percentage of aggregate reticulocyte (C), and red cell distribution width (D) in 2 control cats treated (day 0) with an rAAV2 vector containing the LacZ gene ( $1 \times 10^9$  infectious units [iU], IM; open circles), 3 cats treated with  $1 \times 10^7$  iU of rAAV2-fEPO (closed circles), 3 cats treated with  $1 \times 10^8$  iU of rAAV2-fEPO (open triangles), and 2 cats treated with  $1 \times 10^9$  iU of rAAV2-fEPO (closed triangles). There were no significant ( $P > 0.05$ ) differences in mean corpuscular volume, mean corpuscular hemoglobin, and mean aggregate reticulocyte counts among treatment groups or over time. Values of red cell distribution width were significantly ( $P \geq 0.02$ ) higher in all groups at the pretreatment time point, compared with days 14 and 35, and in the high-dose group, compared with the intermediate-dose group at days 98 and 140.

**Serum fEPO concentrations**—During the first 83 days of the trial, there were no significant differences in serum fEPO concentrations between control cats and cats in the low- ( $P = 0.4$ ) or intermediate-dose groups ( $P = 0.1$ ). The serum fEPO concentrations in these cats were within the established reference range (1 to 15 mU/mL).<sup>25</sup> In contrast, cat 7 in the high-dose group had high serum fEPO concentrations on days 35 (24 mU/mL), 52 (44 mU/mL), 87 (76 mU/mL), and 125 (145 mU/mL). On day 135 (10 days after the injection-site muscle was removed), the serum fEPO concentration in cat 7 decreased to 16 mU/mL. The serum fEPO concentration in the other cat (cat 8) in this treatment group was within reference limits at all time points, except at day 87 when the value was 16 mU/mL.

**Response to readministration of rAAV2-fEPO vector**—Because of their lack of response to initial treatment, administration of rAAV2-fEPO was repeated on day 83 in all cats in the low- and intermediate-dose treatment groups. The 3 cats in the low-dose group were retreated with  $1 \times 10^9$  iU of rAAV2-fEPO vector, and the 3 cats in the intermediate-dose group were retreated with another dose of  $1 \times 10^8$  iU of rAAV2-fEPO vector. Repeated treatment of cats in the inter-

mediate-dose group did not cause Hct values to increase, whereas cats in the low-dose group had variable Hct responses to vector readministration (Figure 5). The Hct value in 1 cat (cat 2) did not increase above the upper limit of the reference range. Another cat (cat 3) had a transient increase in Hct above the upper limit of the reference range from day 200 to 224, which reached a maximum of 52% on day 217 before spontaneously returning to a value within reference limits. The third cat (cat 1) had a persistently high Hct (57% to 77%) beginning on day 119. The Hct value did not decrease after removal of the injected muscle on day 256 and remained high through day 569 when monitoring was discontinued (data not shown). Bone marrow analysis performed on this cat on day 493 (when the Hct value was 57%) revealed orderly and complete maturation of the erythroid cell line with a marked increase in erythroid progenitors, resulting in an abnormal myeloid-to-erythroid ratio of 0.35:1. There was no evidence of neoplasia in cat 1, and a diagnosis of erythroid hyperplasia was made.

**Assessment of serum anti-AAV2 antibodies**—At day 227 of the gene therapy trial, rAAV2 virus-neutralizing antibodies, as defined by  $> 50\%$  suppression of

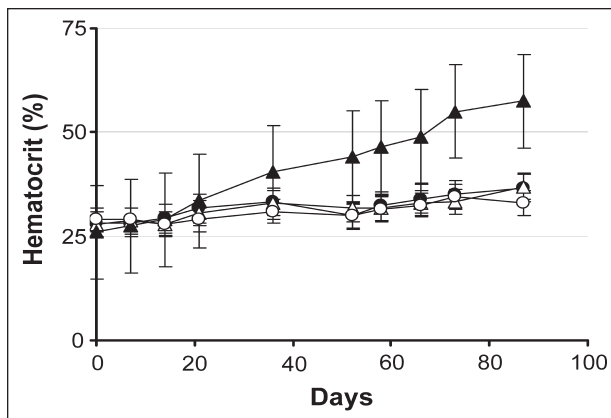


Figure 3—Mean  $\pm$  SD Hct values in 2 control cats treated (day 0) with an rAAV2 vector containing the LacZ gene ( $1 \times 10^9$  iU, IM), 3 cats treated with  $1 \times 10^7$  iU of rAAV2-fEPO, 3 cats treated with  $1 \times 10^8$  iU of rAAV2-fEPO, and 2 cats treated with  $1 \times 10^9$  iU of rAAV2-fEPO. In cats treated with  $1 \times 10^9$  iU of rAAV2-fEPO, Hct values were significantly ( $P \leq 0.02$ ) higher than those in all other groups from days 35 to 87. There were no significant ( $P > 0.05$ ) differences in Hct between control cats and cats treated with  $1 \times 10^7$  iU or  $1 \times 10^8$  iU of rAAV2-fEPO at any time point. See Figure 1 for key.

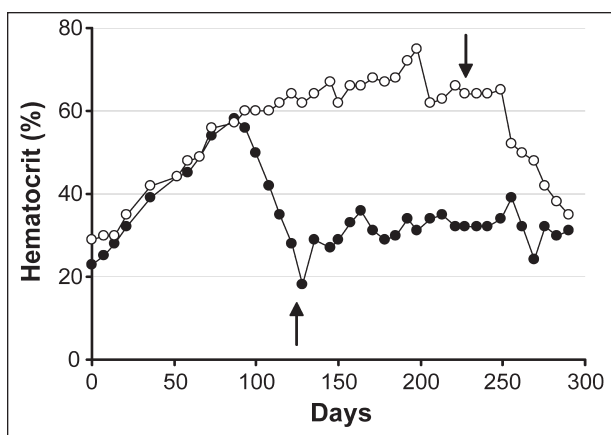


Figure 4—Changes in Hct in 2 cats (cat 7, closed circles; cat 8, open circles) treated IM with  $1 \times 10^9$  iU of rAAV2-fEPO (day 0). Cat 8 had a sustained erythrocytosis; cat 7 had an initial erythrocytosis response, but Hct dramatically decreased after day 87. For both cats, the Hct values returned to reference range values following excision of the vector injection site (denoted by arrows) on days 125 (cat 7) and 227 (cat 8).

rAAV2 infectivity of 293 cells, were detected in all dilutions of sera of all cats treated with rAAV2-fEPO vector.

**Findings of histologic examinations**—To evaluate the transduction of feline skeletal muscle *in vivo* by rAAV2, the right superficial gluteal muscle of the control cats was injected with rAAV2-LacZ vector. Approximately 25% to 33% of the myofibers contained  $\beta$ -galactose in the cytoplasm as a result of the expression of the LacZ gene insert, indicating efficient transduction comparable to that detected in a previous study.<sup>12</sup> There were no abnormalities noted on histologic examination of the specimens of superficial gluteal muscles obtained from any cats except for focal, mild lymphocytic infiltrates in 1 control cat (cat 9) and 1 cat (cat 4) that received an intermediate dose of rAAV2-fEPO vector.

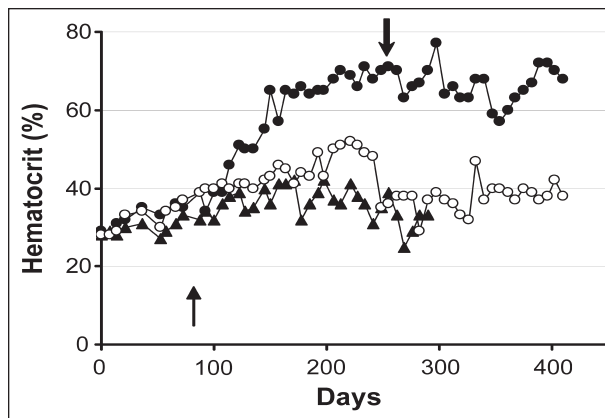


Figure 5—Changes in Hct in 3 cats (cat 1, closed circles; cat 2, closed triangles; cat 3, open circles) that were initially administered (day 0)  $1 \times 10^7$  iU of rAAV2-fEPO, IM, without response and retreated (day 83; thin arrow) with a second dose of  $1 \times 10^9$  iU of rAAV2-fEPO, IM. The Hct values in cat 2 did not increase beyond the upper limit of the reference range. The Hct values in cat 3 increased transiently above the upper limit of the reference range from days 200 to 224 and then spontaneously returned to reference range values. Cat 1 had a sustained increase in Hct after day 119 that persisted after removal of the vector-treated muscle on day 256 (thick arrow).

## Discussion

The present study was designed to provide proof-of-concept that an rAAV2 vector containing the fEPO gene downstream from a hybrid CBA promoter can produce a robust and durable biological effect after IM administration in cats, that this response could be regulated by adjusting the dose of vector administered, and that the biological effect could be abolished by removal of the vector injection site.

Beall et al<sup>28</sup> previously reported an increase in Hct in cats injected IM with an rAAV2 vector containing fEPO cDNA under the control of a CMV promoter. However, that study was terminated at 7 weeks after vector administration, and no attempt was made to abolish gene expression in cats that developed erythrocytosis.

The response to treatment with rAAV2-fEPO vector in our study indicated a threshold or all-or-none phenomenon rather than a clinically desired dose-dependent increase in Hct. In cats that received the high dose of vector, values of Hct began to increase 2 weeks after vector administration. This interval represents the time required for second-strand synthesis.<sup>29,30</sup> Readministration of the vector resulted in a variable biological response; each of the 3 cats that were retreated with  $1 \times 10^9$  iU had no response, a transient response, or a robust and prolonged biological response. This variable effect of retreatment may have been modulated by a humoral immune response directed at rAAV2; however, serum neutralizing antibodies were not measured prior to the readministration of the vector, so this remains undetermined. Humoral immune responses to the AAV virion capsid have been consistently detected in animals after rAAV2 vector administration. The presence of neutralizing antibodies in serum has been shown to prevent or reduce the success of vector readministration.<sup>31,32</sup> As such, repeat dosing is unreliable to titrate a desired Hct when readmin-

istration is performed with the same rAAV serotype.<sup>33</sup> However, the lack of cross-reactivity among neutralizing antibodies of different rAAV serotypes suggests that vector readministration by use of different serotypes may be feasible.<sup>34</sup> Cats initially treated and retreated with  $1 \times 10^8$  iU of rAAV2-fEPO vector had no increase in Hct, presumably because the threshold dose of vector to accomplish gene expression was not achieved either because the total dose ( $2 \times 10^8$  iU) was inadequate or a humoral response generated against rAAV2 diminished the number of infectious units.

Both cats in the high-dose group had high serum fEPO concentration at  $\geq 1$  time point, but values at several time points were within the reference range for cats. Neither of these cats that responded to treatment had a significant increase in aggregate reticulocyte count. Despite this, both cats had a marked increase in Hct, compared with their pretreatment values. These findings have been noted by other researchers<sup>35,36</sup> who concluded that EPO expression is more effectively monitored via assays of the biological consequences of that expression than via intermittent detection of serum EPO concentrations. In normal regulation of hematopoiesis, primary erythrocytosis results in negative feedback on EPO release; thus, animals with primary erythrocytosis are expected to have serum EPO concentrations below the lower reference limit.<sup>25</sup> In contrast, cats 7 and 8 in our study had normal to high serum EPO concentrations in the presence of erythrocytosis as a result of unregulated EPO gene expression.

The finding that control and treated cats did not differ with regard to platelet, neutrophil, eosinophil, and lymphocyte counts suggests that rfEPO acts specifically on cells of the erythroid lineage.<sup>37</sup> Aggregate reticulocyte counts and values of mean corpuscular volume and mean corpuscular hemoglobin were also not significantly different between control cats and cats with erythrocytosis. This finding may reflect the short time that erythrocytes in cats remain at this point of maturation before becoming punctate reticulocytes and erythrocytes.<sup>38</sup>

One of the cats (cat 7) in the high-dose group had a decrease in Hct after initial erythrocytosis. This cat had the major hematologic and bone marrow features of pure RBC aplasia that have been identified in some human patients receiving rhEPO.<sup>39,40</sup> This suggests that, compared with native fEPO, rfEPO expressed by feline skeletal muscle may possess differences (post-translational or otherwise) that cause the transgene product to be immunogenic. The resulting anti-rfEPO antibodies may cross-react with native fEPO in cats. The authors of this report are unaware of any studies describing an immune response against EPO after gene transfer in any species. In cat 7, anemia resolved promptly after excision of the vector-treated muscle; however, excision of the injection site proved unreliable for abrogating the erythrocytosis effect of fEPO in the present study. In 1 cat (cat 8) with erythrocytosis, excision of the injection site was successful in returning the Hct value to within the reference range; in contrast, another cat (cat 1) with erythrocytosis had no decrease in Hct after excision of the superficial gluteal muscle. This suggests that the vector may have reached

the circulation, resulting in transduction of distant tissues; adjacent muscle may have been transduced after leakage from the injection site; or the superficial gluteal muscle was not entirely excised.

Histologic evaluation of the excised injection sites revealed no evidence of local inflammation. This observation is in agreement with findings of previous studies,<sup>41,42</sup> which indicated that the delivery of rAAV into a variety of tissues was associated with an absence of inflammation. In another study,<sup>12</sup> analysis of  $\beta$ -galactose in skeletal muscle 256 days after vector administration revealed efficient transduction and persistent LacZ gene expression by rAAV2.

Recombinant AAV vector-mediated gene transfer in skeletal muscle of mice, dogs, nonhuman primates, and humans with hemophilia is well tolerated and associated with long-term expression.<sup>43-47</sup> In neonatal mice, a CBA promoter identical to that used in the present study elicited prolonged expression (1.2 years) of human factor IX after IM injection and injection via the portal vein.<sup>20</sup> In that study, serum concentrations of human factor IX were increased after administration of the CBA promoter, compared with responses to administration of constructs containing CMV or EF1 $\alpha$  promoters. Potent promoters, such as CBA, induce higher levels of gene expression, allowing lower doses of vector to be administered, thereby reducing the risk of insertional mutagenesis or germ line transmission.<sup>20</sup>

Our data have indicated the potential for rAAV gene therapy in the treatment of EPO-responsive hypoproliferative anemia in cats. However, readministration of the same vector in an attempt to titrate a clinically appropriate Hct did not result in predictable effects in the cats of our study. Furthermore, surgical excision of the injection site did not reliably terminate the biological effect of the gene therapy, suggesting that tissues beyond the injection site were transduced. The pure RBC aplasia that developed in association with the gene therapy in 1 of the cats in the present study was unexpected and has suggested a potential adverse effect of vector administration that warrants further investigation. Finally, it is apparent that clinically useful gene expression of fEPO should include a mechanism to regulate gene expression to avoid life-threatening anemia or erythrocytosis.

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- a. Provided by Dr. Frank Bunn, Brigham and Woman's Hospital, Harvard Medical School, Boston, Mass.
  - b. University of Florida, Interdisciplinary Center for Biotechnology Research, Gainesville, Fla.
  - c. Invitrogen, San Diego, Calif.
  - d. SNAP FIV antibody/FelV antigen combo test, IDEXX Laboratories, Westbrook, Me.
  - e. Cell-Dyn 3500, Abbott Laboratories, Abbott Park, Ill.
  - f. EPO ELISA, Medac Diagnostics, Hamburg, Germany.
  - g. Mouse Anti-AAV, Clone A20, Research Diagnostics Inc, Flanders, NJ.
  - h. SAS, SAS Institute Inc, Cary, NC.
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