Expression of a Foreign Gene in Cats Reconstituted With Retroviral Vector Infected Autologous Bone Marrow


A Moloney murine leukemia virus based retroviral vector was used to transfer the bacterial neomycin resistance gene (neo) into feline hematopoietic cells. We reconstituted four cats that had been lethally irradiated with autologous bone marrow that had been infected with the N2 or SAX retroviral vector. Bone marrow cells from all four cats expressed the neo gene 30 days posttransplant and three of four cats still had the neo gene and a low level of drug resistant colony-forming unit granulocyte-macrophage after more than 200 days. Two of the four cats unexpectedly developed diabetes mellitus 90 days posttransplantation. The expression of a foreign gene in cats, albeit at a low level, demonstrates that retroviral vectors can be used for gene transfer in noninbred large animal species and may be useful for gene therapy of humans. The development of diabetes mellitus in two of the subjects emphasizes the value of animal models for the study of possible deleterious effects of retroviral vector-mediated gene transfer.

GENE THERAPY is the insertion of a normal gene into an organism to correct a genetic defect. Prospects for human gene therapy have increased with the development of retroviral vectors for efficient gene transfer into cells. Hematopoietic stem cells are one possible target cell for gene transfer into intact organisms because they are capable of self-renewal and undergo expansion with multiple rounds of replication during hematopoietic differentiation. We and others have shown that exogenous genes are expressed during in vitro culture of hematopoietic progenitor cells from mice, dogs, cats, sheep, monkeys, and humans. In mice, exogenous genes have been shown to be stably integrated in chromosomal DNA from hematopoietic cells after retroviral infection in vitro and bone marrow transplantation. The infected hematopoietic cells in the recipients were capable of reconstituting lethally irradiated secondary recipients, indicating that the pluripotent stem cells had been infected. Expression of the bacterial neomycin resistance gene (neo) promoted by a viral long terminal repeat (LTR) in some expres- sion of the human β-globin gene in mouse erythroleukemia cells and human erythroid cells in vitro, and lineage-specific expression of the human β-globin gene in murine transplant recipients reconstituted with retroviral-transduced stem cells has shown that retroviral vectors can be used to transfer and express exogenous genes in hematopoietic progenitor cells.

Long-term expression of retroviral-transduced genes in hematopoietic stem cells from adult animals in species other than mice and the fate of exogenous genes in individual animals of any species has not been reported. The dog and cat are potentially excellent large animal models to test gene therapy because they, like humans, are not inbred species and many genetic disorders similar to human diseases have been well characterized in these species. As a first step to evaluating gene therapy in individual animals of any species has not been reported. The dog and cat are potentially excellent large animal models to test gene therapy because they, like humans, are not inbred species and many genetic disorders similar to human diseases have been well characterized in these species.

MATERIALS AND METHODS

Experimental animals. Adult cats of both sexes were used in these studies. All animals had been previously vaccinated and dewormed and were disease free for at least 2 months before experimental study. These studies were approved by the institutional Animal Care and Concerns Committee. Animals were housed in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Retroviral infection and bone marrow transplantation (BMT). BM was obtained by percutaneous aspiration of long bones using ketamine/xylazine sedation. BM mononuclear cells were prepared by ficoll-hypaque gradient sedimentation and infected with the N2 or SAX retroviral vector, which contain the bacterial neo gene, using a "supernatant" infection protocol described previously. Briefly, BM cells were incubated for 4 to 6 hours with conditioned medium from high titer clones, V-6 or S3A, of the amphotrophic packaging cell line PA127 that had been previously transduced with the N2 or SAX vectors, respectively. Conditioned medium from V-6 or S3A cells routinely has a titer of 3 to 5 × 10^5 drug-resistant colony-forming units/mL (CFU/mL) and contains approximately 0.1% helper virus contamination. After infection, cells were washed three times with complete medium (Iscove's modified Dulbecco's medium [IMDM]) and plated in the colony-forming unit granulocyte-macrophage (CFU-GM) assay (see below) or infused into the donor animals that had received 7.5 Gy (0.125 Gy/min) total body irradiation from a Cobalt 60 source (Picker, Cleveland, OH). Animals were kept in a clean isolated room, treated with antibiotics and fluids as indicated until BM reconstitution was evident based on complete blood counts (CBCs).

CFU-GM assay. The CFU-GM were determined as previously described. Briefly, 7.5 × 10^4 nucleated marrow cells were plated in 35-mm^2 dishes and CFU-GM were enumerated after 6 to 8 days of culture. The antibiotic G418, when added, was present at a concentration payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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concentration of 2 mg/mL. The total amount present was 4 mg/mL because only about 50% of stock G418 is active.

DNA analysis. DNA was prepared from BM cells by sodium dodecyl sulfate (SDS)-protease K digestion, phenol/chloroform extraction, and ethanol precipitation by standard procedures. BM DNA (1 µg) was specifically amplified for neo<sup>+</sup> sequences by the polymerase chain reaction (PCR) method<sup>39</sup> and Southern blotting. The PCR reaction conditions (30 cycles of 94°C for 2 minutes and 45 seconds, 53°C for 2 minutes, and 72°C for 3 minutes and 30 seconds) were performed as described<sup>34</sup> using a Gene-Amp kit (Perkin-Elmer Cetus, Norwalk, CT) and an automated thermal cycler (DNA Thermal Cycler; Perkin-Elmer Cetus, or Twinblock system temperature cycler; Ericomp, San Diego, CA). The oligonucleotide primers (CAAGATGGATTGCACGCAGG, CCCGCT-CAGAAGAACTCGTC) permitted amplification of a 790-bp region of the env gene using reaction conditions 94°C for 2 minutes, 56°C for 2 minutes, and 72°C for 2 minutes for 30 cycles.

The oligonucleotide (ATGTGGGCTTCCAGTAAGC) used to probe the Southern blot hybridizes to a region of the env gene between the primers.

Neomycin phosphotransferase (NPT) assay. NPT activity was determined according to the method of Reiss et al<sup>40</sup> as previously described. CFU-GM colonies resistant to G418 were recovered from methyl cellulose cultures, washed extensively, and stored as a "dry" pellet at −70°C until analysis.

RESULTS

Retroviral infection and BMT: Four cats that had been lethally irradiated (7.5 Gy) were reconstituted with autologous BM that had been infected with the N2 retroviral vector (n = 3) or the derivative vector, SAX (n = 1), to investigate the usefulness of the cat as a model for evaluating gene therapy protocols and to follow the long-term stability and expression of exogenous genes in individual animals. We chose to use the N2 vectors in these studies because the neo<sup>+</sup> gene in this vector was expressed in murine transplant recipients as well as feline and canine hematopoietic stem cells in vitro.<sup>38,41,50</sup>

BM reconstitution was evident in all four cats by 20 days posttransplantation based on evaluation of the polymor-

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The CFU-GM were determined as described. The data are presented as total CFU-GM of five individual culture dishes (7.5 x 10<sup>6</sup> cells/dish). Control marrow (uninfected marrow from the same animal [day 0 only] or marrow from an uninfected cat) was assayed in parallel to check for "background" G418-resistant CFU-GM. The MOI is the ratio of viral particles, as estimated by the titer of drug-resistant 3T3 CFUs, to BM mononuclear cells.

Abbreviation: ND, not done.
Fig 1. Detection of neo<sup>+</sup> sequences in BM DNA from transplanted cats by PCR. The lanes are: 1, Martina-pretransplant (pre-TXP); 2, Martina-26 days posttransplant (post-TXP); 3, Andy-pre-TXP; 4, Andy-154 days post-TXP; 5, Andy-223 days post-TXP; 6, Yenkee-pre-TXP; 7, Yenkee-26 days post-TXP; 8, Yenkee-96 days post-TXP; 9, positive control (SAX).

Fig 2. Sequential evaluation of BM DNA for the neo<sup>+</sup> gene. (A) The lanes are: 1, negative control; 2, Martina-pre-TXP; 3, Martina-214 days post-TXP; 4, Martina-609 days post-TXP; 5, Martina-978 days post-TXP; 6, positive control. Lanes 7 and 8 are empty. (B) The lanes are: 1, positive control; 2, Yenkee-pre-TXP; 3, Yenkee-26 days post-TXP; 4, Yenkee-67 days post-TXP; 5, Yenkee-96 days post-TXP; 6, Yenkee-214 days post-TXP; 7, Yenkee-609 days post-TXP; 8, Yenkee-978 days post-TXP.
phonuclear leukocyte (PMN) and platelet counts. The PMN and platelet counts had returned to at least 5,000 PMN/µL and 50,000 platelets/µL in the four cats by 18.5 ± 5.4 (mean ± SD) and 16.0 ± 4.7 days, respectively. This finding indicated that retroviral infection, per se, did not preclude BM reconstitution in an autologous transplantation protocol. Although gastrointestinal toxicity and hematologic complications were observed to various degrees in most animals, the severity was not greater than that observed previously from lethal irradiation and autologous BMT alone.

The results of the pretransplant BM infections, cell doses for transplantation, and multiplicity of infection (MOI) for each cat are summarized in Table 1. The pretransplant infection rate (day 0) was 5.9%, 3.3%, and 0% in the three animals that were evaluated. These results are similar to our previous findings on small-scale BM infections and indicated that the “supernatant” infection protocol was adequate for large-scale BM infections required in a large animal retroviral infection/autologous BMT protocol.

DNA analysis. DNA was prepared from BM mononuclear cells both before and after retroviral infection to test for the presence of the neo gene in the transplanted animals. The neo gene was not detected in BM cells from the transplanted animals by standard Southern hybridization techniques, suggesting that the neo gene was present in fewer than 5% of the BM cells. However, when DNA from BM samples from three of the same animals was specifically amplified for neo sequences by PCR and Southern blotting (Fig 1), the neo gene was detected in two animals 26 days posttransplant (lanes 2 and 7), and one animal 154 days after retroviral infection/BMT (lane 4). The neo gene was not detected in BM samples before retroviral infection (Fig 1, lanes 1, 3, and 6). Sequential evaluation of BM marrow mononuclear cell DNA from two cats showed that the neo gene was still detectable after 200 days. The neo gene was detected in Martina 214 and 609 days following transplantation but not 978 days following transplantation (Fig 2A). The neo gene was also detected in Yenkee 26, 67, 214, 609, and 978 days following transplantation (Fig 2B).

CFU-GM analysis. BM obtained from the transplanted animals from approximately 30 days to greater than 300 days posttransplant was tested for expression of the neo gene in the in vitro CFU assay. Expression of the neo gene was evident in all four cats 30 days posttransplantation based on the CFU assay (Table 1). The fraction of BM progenitor cells expressing the neo gene in the autologous BMT recipients was initially similar to that observed previously in vivo infection experiments with a comparable MOI. Sequential evaluation of BM for G418-resistant CFU showed that the fraction of G418-resistant CFU declined over a period of several months, but the three cats that could be examined still had a low level of drug-resistant CFU-GM after 200 days. NPT activity was detected in one of two cats 26 days posttransplant, confirming that G418 resistance can be associated with enzyme activity (Fig 3). Enzyme activity was not detected in BM samples from Martina and Yenkee 96, 214, and 248 days or from Andy 94, 223, 348, and 368 days following transplant, even though several samples were positive for the neo gene by PCR and Southern blotting and had drug-resistant CFU-GM. This finding suggests that the CFU-GM assay and PCR and Southern blotting may be more sensitive than direct enzyme assay for detecting the neo gene in transplanted animals.

PCR detection of helper virus. The replicative-defective retroviral vectors N2 and SAX were encapsulated using the amphotrophic packaging cell line PA12. The PA12 cell line has a low level of helper virus contamination with clones expressing the N2 genome. To determine if animals infected with N2 or SAX retroviral vectors had acquired a helper viremia from the initial BM infection at the time of autologous BMT, we evaluated serum from each animal for functional helper virus with the S’L- assay. Serum samples from each animal obtained at various times posttransplantation were always negative for helper virus with the S’L- assay. Next, we checked for the helper virus genome in BM DNA by PCR amplification of the amphotropic retrovirus 4070A envelope (env) gene. The helper virus

**Fig 3.** NPT activity in G418-resistant feline CFU-GM. The arrow indicates the position of the expected 29-Kd NPT protein. Lane 1, Martina-day 26 post-TXP, 2.4 x 10^6 cells; lane 2, Yenkee-day 26 post-TXP, 1.3 x 10^6 cells; lane 3, empty; lane 4, V-6 cells, 1 x 10^6; lane 5, empty; lane 6, uninfected control marrow, 1 x 10^6 cells.
GENE TRANSFER INTO FELINE HEMATOPOIETIC CELLS

Genome was detected in BM DNA in two of three cats that were evaluated (Fig 4). The two animals positive for the env gene had been infected with the N2 vector, while the negative cat had been infected with the SAX vector. The presence of the helper virus genome in the transplanted cats means a localized BM helper virus infection may have contributed to the persistent expression of the neo\(^R\) gene in two of the transplanted animals. However, the cat infected with the SAX vector had stable expression of the neo\(^R\) gene for at least 248 days and was negative for the helper virus genome. However, it is possible the cat is still helper virus-positive because the cat was exposed to helper virus at the time of BM infection but the virus cannot be detected.

Diabetes mellitus in transplanted cats. Application of retroviral vector-mediated gene transfer to treatment of human genetic diseases is not only dependent on demonstrated efficacy in an animal model system but also the relative assurance that this technology will not induce significant disease in treated patients. Two of the four transplanted cats unexpectedly developed diabetes mellitus approximately 90 days posttransplantation. Islet amyloid, the characteristic histologic abnormality seen in pancreases from spontaneously diabetic cats,\(^\text{42}\) was not observed on histologic examination of the pancreases from the two transplanted cats. Postmortem examination of both cats was normal except for a \(\beta\)-cell deficiency and islet atrophy (Fig 5).

**DISCUSSION**

The results of the current study demonstrate that an exogenous gene can be expressed in feline hematopoietic cells after reconstitution of lethally irradiated donors with retroviral-infected autologous BM. The expression of a foreign gene in cats, albeit in a small number of cells, demonstrates that retroviral vectors can be used for gene transfer in noninbred large animal species and may be useful for gene therapy of humans. The basis for the decrease in the fraction of cells expressing the neo\(^R\) gene several months posttransplant was not determined in the current study, but several possibilities exist: (1) the pluripotent stem cells were not adequately infected in the current transplantation protocol and committed cell clones could not be detected after several months; (2) infected stem cell

![Fig 4. Detection of amphotrophic retrovirus 4090A env sequences in BM DNA from transplanted cats by PCR. The lanes are: 1, negative control (no DNA); 2, Martina-pre-TXP; 3, Martina-26 days post-TXP; 4, normal cat; 5, Martina-210 days post-TXP; 6, empty; 7, Andy-pre-TXP; 8, Andy-154 days post-TXP; 9, Andy-223 days post-TXP; 10, Andy-338 days post-TXP; 11, empty; 12, Yenkee-pre-TXP; 13, Yenkee-26 days post-TXP; 14, Yenkee-96 days post-TXP; 15, Yenkee-209 days post-TXP. The weak positive band in the normal cat (lane 4) is most likely an artifact due to carry-over from the previous sample, which was extremely positive. The three transplanted cats were not positive on DNA prepared from BM before retroviral infection.](image)
clones from which the drug-resistant CFU-GM progenitor cells are derived are active for variable periods of time; or (3) cells expressing NPT are selectively removed from BM because of the bacterial NPT.

Our findings in cats contrast with similar studies in dogs where a very low level of drug-resistant colonies but not the proviral sequences could be detected after retroviral vector transfer of a dihydrofolate reductase or neo* gene into dog BM cells.19 (Lothrop et al, unpublished observations). The basis for the difference between the studies in dogs and cats is not completely understood but may be explained by species differences or by variable infection efficiencies. A supernant infection protocol with a high MOI (>2) was used in those animals found to express the neo* gene.

The unique histopathologic abnormalities in the two diabetic cats combined with the fact that pancreatic islets are extremely radioresistant suggests a novel mechanism of diabetes mellitus in the two transplanted cats. Although there was no histopathologic or immunohistochemical evidence of systemic viremii in either cat, it is possible that a transient viremi could have caused direct islet destruction or indirectly induced an immune response that led to islet
destruction and eventual diabetes mellitus. Viruses are thought to be the common environmental factor inducing diabetes in susceptible people. Similarly, one of the diabetic cats in our study clearly had detectable amphotrophic virus envelope sequences in BM before development of diabetes mellitus (Fig 3). Although the exact cause of diabetes in the two transplanted cats is not known, the potential for helper virus-induced disease exists. The unexplained diabetes mellitus in two cats that had been previously infected with N2 retroviral-rich supernatant from the PA12 retrovirus packaging cell line, which is contaminated with 4070A amphotrophic helper retrovirus, demonstrates the potential importance of using helper virus-free retroviral vectors for human gene therapy.

The findings of the current study suggest that retroviral LTR transcriptional regulatory sequences are active in feline hematopoietic progenitor cells. Previous studies have shown that LTR sequences were not always transcriptionally active in murine transplant recipients. The variability in expression of retroviral-transduced genes indicates that retroviral vectors may have to be empirically evaluated for transcriptional activity until the DNA sequences and regu-
latory factors controlling expression of retroviral-transduced genes in hematopoietic progenitor cells are defined better. Although we have shown that an exogenous gene can be expressed in feline hematopoietic progenitor cells in vivo, improvement of current retroviral vector infection protocols is necessary to achieve stable transfer and expression of exogenous genes in the majority of BM cells. Animal models of human genetic diseases should be extremely useful for designing and evaluating safe gene therapy protocols for treatment of human genetic diseases.

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