Canine Model for Gene Therapy: Inefficient Gene Expression in Dogs Reconstituted With Autologous Marrow Infected With Retroviral Vectors

By Richard B. Stead, William W. Kwok, Rainer Storb, and A. Dusty Miller

Successful retroviral gene transfer into murine hematopoietic stem cells indicates the potential for somatic gene therapy in the treatment of certain human hereditary diseases. We developed a canine model to test the applicability of these techniques to a preclinical model of human marrow transplantation. Previously we reported that canine CFU-GM could be infected with retroviral vectors carrying either the gene for a mutant dihydrofolate reductase (DHFR*) or neomycin phosphotransferase (NEO). This study reports six lethally irradiated dogs transplanted with autologous marrow cocultivated with retroviral vector-producing cells. This procedure conferred drug resistance to 3% to 13% of the CFU-GM. Three dogs infected with either the NEO or DHFR* virus engrafted, but we detected no drug-resistant CFU-GM. Three dogs were given marrow infected with a DHFR* virus and received methotrexate (MTX) as in vivo selection; all three had evidence of engraftment. In the surviving dog, we detected 0.03% to 0.1% MTX-resistant CFU-GM at 3 to 5 weeks posttransplant during in vivo selection. These results indicate that we can reconstitute lethally irradiated dogs with autologous marrow exposed to retroviral vectors and suggest that gene transfer into hematopoietic cells is feasible on a large scale. However, the low-level transient gene expression indicates that considerable obstacles remain before human gene therapy can be considered.

Retrovirus-mediated gene transfer has been proposed as a potential treatment for several hereditary diseases. Most in vivo studies have focused on mice using vectors derived from Moloney murine leukemia virus. These studies demonstrate that mouse pluripotent stem cells can be infected and give rise to a variety of lymphoid and myeloid cell types carrying the vector. We developed a canine model for gene transfer into hematopoietic cells to determine if the methods developed in the murine system can be extended to an established preclinical model of bone marrow transplantation. We showed that canine hematopoietic progenitors could be infected with retroviral vectors carrying either a mutant dihydrofolate reductase gene (DHFR*) or the neomycin phosphotransferase (NEO) gene. We also determined that CFU-GM infected with the DHFR* virus were resistant to pharmacologic levels of methotrexate (MTX).

The present study was designed to determine whether retroviral gene transfer into canine hematopoietic stem cells is feasible using the NEO virus PA12/N2 or the DHFR* virus PA12/SDHT. We also wished to determine the feasibility of marrow reconstitution under these conditions and whether in vivo selection with MTX could be used to enrich for infected hematopoietic stem cells and their progeny. We have developed methods for infection of canine hematopoietic progenitors on a large scale and have reconstituted lethally irradiated dogs with this infected autologous bone marrow. We have encountered problems with the rate of infection of pluripotent hematopoietic stem cells. In addition, there may be suboptimal gene expression in progenitor cells derived from these stem cells. We have also observed serious morbidity from two of the three dosage schedules of MTX methotrexate that we used for in vivo selection.

MATERIALS AND METHODS

Viruses and cell lines. The PA12/N2 cell line produces a virus that confers resistance to G418 and also contains helper virus. The PA12/SDHT cell line produces a helper-free DHFR* virus, derived from the spleen focus-forming virus, which confers resistance to MTX. Assays for viral titer and amphotropic helper virus have been described. Cell lines were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose and 10% fetal bovine serum (FBS), except as otherwise noted. Producer cell lines were seeded at 3 to 5 x 10^6 cells in 850-cm² roller bottles (Corning, Corning, NY) 48 hours prior to the addition of bone marrow. The roller bottles (50% to 70% confluent) received 1,500 rpm just prior to the addition of marrow cells. Roller bottles were supplemented with CO₂ and maintained at 37°C, rotating at 1 to 2 rpm on a Belco roller apparatus. All recombinant viruses, virus-producing cell lines, and infected cells were handled in accordance with the National Institutes of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules at the P2 level of containment.

Canine CFU-GM assay. The growth of canine CFU-GM colonies in semisolid agar medium has been described. To determine the percentage of drug-resistant CFU-GM colonies, 7.5 x 10^4 to 1 x 10^5 mononuclear cells were plated with and without selective agent; either MTX at a concentration of 0.25 or 0.50 μg/L or G418 at a concentration of 1 to 2 mg/ml (concentration of powder, of which ~50% was active). The rate of infection of CFU-GM is defined as the number of colonies that grow in the presence of selection divided by the total number of colonies, expressed as a percentage. Control marrow (uninfected or infected with an irrelevant virus) was routinely assayed in parallel to insure that there were no "background" drug-resistant colonies. The initial rate of infection (day 0) was assayed by taking an aliquot from the overnight cocultivation and comparing it with uninfected marrow from the same animal. Following full hematopoietic reconstitution, bone marrow samples...
were obtained serially every 2 to 6 weeks, and three to 12 replicate plates with and without the selective agent were plated along with two to six control plates from an animal not infected with that virus.

Bone marrow infection. Approximately 100 mL of bone marrow was harvested by vacuum aspiration from the humeri and femora of healthy dogs under general anesthesia as previously described.\textsuperscript{15,16} The marrow was passed through a wire screen, diluted 1:3 in Waymouth's medium containing preservative-free heparin (10 U/mL), penicillin, and streptomycin, and layered over Ficolli-Hyphaque in 50-mL conical bottom tubes. Initial studies were performed with Ficolli-Hyphaque of specific gravity 1.077 g/dL, but subsequent experiments indicated that the recovery of mononuclear cells improved with no change in the proportion of contaminating neutrophils using sp gr of 1.14 g/dL (data not shown). After 30 minutes at 900 g, the cells at the interface were removed, washed in medium, and resuspended in DMEM containing 10% to 20% FBS and 2 µg/mL polybrene prior to inoculation into roller bottles containing producer cells. Mononuclear and total cell counts as well as viability by trypan blue exclusion were determined. Twelve roller bottles (as prepared above) were irradiated with 1,500 rad between opposing cobalt sources, and the medium was changed to 50 to 100 mL of the medium containing polybrene one to three hours before 1 to 1.5 × 10^6 mononuclear cells were added to each roller bottle. Five of the six canine marrow samples were infected with the helper-free DHFR* virus PA12/SDHT; animal no. two was given marrow infected with PA12/N2, which contains amphotropic helper virus in addition to the N2 NEO virus. The initial two animals' marrow was cocultivated for only 12 hours; however, after we determined that there were no significant problems with engraftment, the remaining cocultivations were of 20- to 24-hour duration. Following cocultivation, the marrow cells were removed and the inside of the roller bottles were carefully rinsed with medium to remove as many adherent hematopoietic cells as possible without dislodging the producer cells. The cells were washed twice, pooled, and resuspended in serum-free medium prior to reinfusion. An aliquot was removed to assess cell recovery, viability, producer cell contamination, and initial rate of infection of CFU-GM.

Bone marrow transplantation, animal care, and in vivo selection. Dogs were selected, housed, and cared for according to standard procedures in the Fred Hutchinson Cancer Research Center kennels. Research was conducted according to the principles enunciated in the Guide for Laboratory Animal Facilities and Care prepared by the National Academy of Sciences-National Research Council. All animals were pretreated with oral nonabsorbable antibiotics administered for 5 days prior to transplantation and continued until hematopoietic reconstitution was complete. Animals received 500 rad midline tissue dose of total body irradiation (TBI) from opposing 60Co sources at a rate of 10 rad/min ~two hours prior to marrow infusion. Previous studies from this laboratory have shown that this is a lethal dose of radiation in dogs unless autologous marrow is infused\textsuperscript{12}; dogs receiving 460 rad all died within nine to 13 days with marrow aplasia unless autologous marrow was infused. Dogs were supported with subcutaneous fluids, antibiotics, and whole blood transfusions (irradiated with 1,500 rad in vitro) as indicated. Daily complete blood counts were obtained until reconstitution was complete and periodically thereafter; animals were weighed once daily. Animals 4, 5, and 6 were given MTX (Lederle) 0.5 mg/kg, intravenously (IV), on days one, three, and five after grafting and twice weekly thereafter. In an experiment designed to determine the efficacy and toxicity of in vivo selection independent of total body irradiation, animals 1 and 2 were given 0.5 mg/kg once a week for 5 weeks starting ~4 months after transplantation; the dose was then increased to twice a week for 2 to 4 weeks. Animal 2, infected with NEO virus, served as the negative control for in vivo selection of the DHFR*-infected dogs. Serum MTX levels were determined at two and 24 hours after MTX infusion by a homogeneous enzyme immunoassay (Emit Methotrexate Assay kit, Syva/Syntex) courtesy of Dr Gary Yee.

Southern analysis. High-mol-wt DNA was obtained from marrow cells, digested with the appropriate restriction endonuclease, transferred to nitrocellulose, probed with a DHFR*, NEO, or SFFV gag probes of a specific activity of 0.5 to 1 × 10^6 dpm, and exposed for one to seven days by standard methods.\textsuperscript{8} DNA standards containing one proviral copy for each dog genome equivalent of DNA were prepared from a dog cell line CF2Th\textsuperscript{17} containing a single proviral copy of the DHFR* vector per cell; 0.01 and 0.1 copy per cell standards were prepared by diluting this DNA with uninfected CF2Th DNA.

RESULTS

Transplantation and engraftment. Table 1 summarizes the results of injection of marrow from six dogs; all except the marrow from dog no. 2 were infected with helper-free DHFR* virus. Losses of 30% to 60% of the starting mononuclear cells occurred during the initial Ficoll-Hyphaque density centrifugation of canine bone marrow (data not shown). In addition to this initial loss, Table 1 shows that we were able to recover only an average of 53% of the Ficoll-Hyphaque separated mononuclear cells after large-scale cocultivation for 12 to 24 hours. This is somewhat less than the 60% to 80% that we typically recover following small-scale cocultivation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dog No.</th>
<th>Avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus*</td>
<td>DHFR*</td>
<td>NEO</td>
</tr>
<tr>
<td>Yield (%)†</td>
<td>37</td>
<td>50</td>
</tr>
<tr>
<td>Viability (%)‡</td>
<td>99</td>
<td>95</td>
</tr>
<tr>
<td>Producer cells (%)§</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cell does (× 10^9 MN cells/kg)</td>
<td>4.9</td>
<td>12</td>
</tr>
<tr>
<td>Infection rate (%)¶</td>
<td>4.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*Selectable marker carried by the virus used; DHFR* (PA12/SDHT) or NEO (PA12/N2).
†Percentage of mononuclear cells introduced into cocultivation that were recovered.
‡Viability after cocultivation determined by trypan blue exclusion.
§Morphological determination of the percentage of contaminating virus producing fibroblasts after cocultivation.
||Number of viable mononuclear cells (× 10^9) infused per kilogram body weight.
¶Percentage of drug-resistant CFU-GM after cocultivation.
experiments (data not shown) and likely reflects some cell death as well as greater inefficiency of recovery on this large scale. Given these losses, we were able to recover 3 to 12 x 10^6 mononuclear cells/kg body weight. The cells recovered were 90% to 95% viable, contained an average of 7% contaminating, irradiated producer cells and ~200 to 250 CFU-GM/10^6 mononuclear cells (data not shown), comparable to unmanipulated bone marrow. The initial rate of infection of CFU-GM ranged from 2.8% to 12.9% (5.4± 3.8%) at the lower end of the range we previously reported in small-scale experiments.\textsuperscript{12}

The three animals that received either the NEO or DHFR\textsuperscript{*} viruses demonstrated prompt engraftment of the three major hematopoietic cell types (Table 2) and have shown no adverse effects of the procedure after a 3- to 7-month follow-up period. Of the three animals that received MTX for in vivo selection immediately after transplantation, only animal 5 survived. The other two animals died on days 20 and 21, respectively, of sepsis and gastrointestinal (GI) toxicity, but both had evidence of early engraftment. Animal 4 had a total neutrophil (polymorphonuclear leukocytes (PMNs) count of 1,900 and nucleated RBCs in peripheral blood within 2 weeks, but the PMN count dropped to 330, and the dog became septic 3 days after the fifth dose of MTX. At autopsy, there was histologic evidence of trilineage engraftment with 30% cellularity, a myeloid to erythroid ratio of 3:1, and the presence of megakaryocytes. The primary pathology was inflammation and secondary infection of the small intestine. Similarly, animal 6 attained a PMN count of >1,300 in 2 weeks, which dropped to 80 at the time of death. At autopsy, the marrow was hypocellular, but myeloid and erythroid elements were present, indicating early engraftment; there was extensive GI inflammation, necrosis, hemorrhage, and infection. Thus, all six animals showed evidence of engraftment following this procedure; two of the three animals receiving postgrafting MTX succumbed to life-threatening GI toxicity.

Gene transfer. We were unable to demonstrate the presence of proviral DNA in the hematopoietic cells of the four surviving animals by Southern blot analysis. We analyzed multiple DNA samples from each animal isolated from bone marrow at ~one-month intervals following transplantation, including animal 5 at weeks 3 and 5. Using DNA isolated from an infected nonproducer cell line as a single copy control and dilutions as noted in the Materials and Methods section, we can estimate the sensitivity of detection at the 2% to 3% level (data not shown). This indicates that following full reconstitution, <2% to 3% of the nucleated bone marrow cells contain an intact copy of the vector proviral DNA.

We also attempted to demonstrate the expression of drug-resistance markers in CFU-GM on multiple occasions, from three weeks to six months after transplantation. We found no evidence of drug resistance in several thousand CFU-GM in three of the four animals that could be analyzed. Table 3 shows that in animal 5, which had the highest rate of infection of CFU-GM initially and was given MTX, we detected 0.1% and 0.03% MTX-resistant colonies at weeks 3 and 5, respectively; by week 7, we could no longer detect any MTX-resistant colonies. We are confident that the few colonies that grew in the presence of 0.25 to 0.5 \( \mu \text{mol/L} \) MTX were protected by the vector because previous experiments showed that canine CFU-GM are completely inhibited at 0.25 to 0.5 \( \mu \text{mol/L} \) MTX under these culture conditions.\textsuperscript{12} Control marrow for this experiment included marrow from animals 1 and 2, which were receiving MTX at the time (described below and in the Materials and Methods section), and showed no MTX-resistant colonies. This suggests that the MTX-resistant colonies we observed in animal 5 were not merely the result of selection of spontaneous mutations or amplification of the endogenous DHFR\textsuperscript{*} gene. Furthermore, this result cannot be ascribed to a higher basal level of MTX resistance because there were no MTX-resistant colonies in >12,000 plated at weeks 7 and 10 from the same dog.

In vivo selection. We assumed at the outset that we would need a strategy to enrich selectively for stem cells and their progeny that were infected by and expressing the vector; we chose to select in vivo with MTX immediately following transplantation as outlined in the Materials and Methods section. Table 4 shows that the primary toxicity was on the GI system with this regimen. The animals receiving MTX lost 17% to 36% body weight in the 3 weeks following transplantation as compared with \( \leq 10\% \) in the animals not receiving the drug. Analysis of serum MTX levels showed

| Table 2. Recovery Following Transplantation of Infected Autologous Canine Marrow |
|-----------------|-------|-------|-------|-------|-------|-------|
| Parameter       | 1     | 2     | 3     | 4     | 5     | 6     | Avg  |
| Selection*      | None  | None  | None  | MTX   | MTX   | MTX   | —    |
| PMN >500/\( \mu \text{L} \) (day)\textsuperscript{†} | 16    | 12    | 16    | 13    | 14    | 12    | 14   |
| Pits >20K/\( \mu \text{L} \) (day)\textsuperscript{†} | 28    | 20    | 40    | —§    | 19    | —    | 27   |
| NRBCs (day)\textsuperscript{†} | 17    | 13    | 21    | 13    | 26    | —    | 18   |
| Engraftment (histologic)\textsuperscript{**} | ND    | Yes   | Yes   | Yes   | Yes   | Yes   | —    |
| Survival (weeks) | >28   | >28   | >19   | 3$\|$ | >20   | 3$\|$ | —    |

\textsuperscript{*}MTX administered starting day 1 after transplant or no MTX.

\textsuperscript{†}Day at which neutrophil count reached and was sustained at \( \geq 500/\mu \text{L} \).

\textsuperscript{‡}Day at which platelet count reached and was sustained at \( \geq 20,000/\mu \text{L} \).

\textsuperscript{§}Died on day 20.

\textsuperscript{∥}Died on day 21.

\textsuperscript{∥∥}Day at which nucleated RBCs were first observed in peripheral blood.

\textsuperscript{**}Histologic evidence of engraftment. ND, not determined.
that peak levels averaged 1 μmol/L at two hours following a dose and that at 24 hours, serum levels were undetectable (<10^-5) (data not shown). This indicates that the toxicity was not due to excessive accumulation of MTX, but is a predictable manifestation of MTX in this model system, in agreement with previous data.22

We tried two other regimens for administration of MTX, starting several months after recovery from TBI used to condition the animals for transplantation. Animal 1, infected with the DHFR* vector, and animal 2, as the control, were treated with 0.5 mg/kg MTX once a week for 5 weeks and demonstrated no evidence of significant GI or hematopoietic toxicity (Table 5); neither did this regimen appear to enrich the MTX-resistant CFU-GM in either animal. The dose was therefore increased to twice a week and resulted in significant GI toxicity, but only minimal myelosuppression, and no MTX-resistant CFU-GM. Animal 1 died 2 weeks after MTX was discontinued, and the drug was withheld in animal 2 because of a skin infection, weight loss, and anorexia. Animal 5 continued to receive MTX at 0.5 mg/kg twice weekly for 6 weeks after transplantation; continued MTX resulted in minimal myelosuppression, but significant weight loss (Table 5), and anorexia and diarrhea.

**DISCUSSION**

Somatic cell gene therapy using retroviral vectors holds promise for the treatment of certain hereditary diseases.1,3

We developed a protocol for large-scale processing, infection, and reconstitution of the canine hematopoietic system to define the methods and problems relevant to human gene therapy. Previous work from this laboratory indicates that at this radiation dose, none of the animals would have survived without the infusion of autologous marrow (19, unpublished observations). Despite significant cell losses in both the Ficoll-Hypaque separation and cocultivation steps, sufficient marrow was recovered that evidence of engraftment could be seen within two weeks in all six animals. We specifically developed large-scale cocultivation methods because our previous studies showed very poor efficiency of infection of canine CFU-GM by incubating marrow cells with virus-containing medium.

**Table 3. MTX-Resistant CFU-GM in Dog 5 Following Reconstitution**

<table>
<thead>
<tr>
<th>Week</th>
<th>Dog 5 CFU-GM (MTX/Total)</th>
<th>MTX (μmol/L)</th>
<th>Control CFU-GM (MTX/Total)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5/3,480</td>
<td>0.25</td>
<td>0/744</td>
<td>0.14</td>
</tr>
<tr>
<td>5</td>
<td>2/6,900</td>
<td>0.50</td>
<td>0/3,960*</td>
<td>0.03</td>
</tr>
<tr>
<td>7</td>
<td>0/11,960</td>
<td>0.50</td>
<td>0/3,960*</td>
<td>&lt;0.014</td>
</tr>
<tr>
<td>10</td>
<td>0/276*</td>
<td>0.25</td>
<td>ND</td>
<td>&lt;0.36</td>
</tr>
</tbody>
</table>

Marrow was obtained and assayed for CFU-GM with and without MTX, as described in the Materials and Methods section, at a final concentration of 0.25 or 0.5 μmol/L MTX. The number of MTX-resistant (MTX*) canine CFU-GM divided by the total number of CFU-GM was determined for the experimental (dog 5) and a control animal.

*The control for this experiment was animal 2, which was being treated with 0.5 mg/kg MTX once a week at the time.

Our results contrast with recent primate studies which show that exposure of marrow to virus-containing medium resulted in more reliable gene transfer and engraftment than an infection protocol involving cocultivation. These workers found that cocultivation of marrow cells resulted in incomplete reconstitution in most animals, although the low cell dose in two of these animals (1 x 10^3, ref 23) may have been a contributory factor. Successful engraftment was attained by infusion of 11 to 74 x 10^6 cells exposed to virus-containing medium per kilogram; we attained engraftment in both animals with a cell dose of ~3 x 10^7 cells/kg. Our cocultivation technique may result in better recovery of hematopoietic cells required for engraftment.

The rate of infection of CFU-GM of 3% to 13% observed here is comparable to studies of murine CFU-GM and CFU-S and human CFU-GM, BFU-E, and CFU-mix. Studies in mice have utilized pretreatment of donor mice with 5-flurouracil (5-FU), growth factors such as interleukin 3 (IL3), and preselection of NEO-infected marrow in G418. All of these are reported to increase the rate of infection of hematopoietic cells. These strategies were not incorporated in our experiments because of our concern for their feasibility in the canine system. The use of 5-FU has not been reported in an autologous transplantation model, it has been used only in the murine system, in which syngeneic marrow is harvested from one or more donors and then transplanted into a different recipient. In this set of experiments, designed to test the use of MTX for vivo selection, we expected that use of pretransplant 5-FU would probably result in unacceptable GI toxicity, especially when coupled with postgrafting.

**Table 4. Effect of MTX During First Three Weeks Following Graft**

<table>
<thead>
<tr>
<th>Dog No</th>
<th>MTX days 1–2</th>
<th>MTX days 3–6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.14</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Dogs 1, 2, and 3 received no MTX during the first 3 weeks postgraft.
†Dogs 4, 5, and 6 were treated with 0.5 mg/kg MTX from days 1, 3, 5, and 11 and twice weekly thereafter.
‡Dogs 4 and 5 had trilineage engraftment; dog 6 had evidence of engraftment of at least two lineages.
§Percentage of CFU-GM resistant to 0.25 μmol/L MTX.

**Table 5. Effect of MTX (0.5 mg/kg) After Reconstitution (>30 Days)**

<table>
<thead>
<tr>
<th>Dog No</th>
<th>MTX 1x/week*</th>
<th>MTX 2x/week†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*Dogs 1 and 2 were administered MTX at 0.5 mg/kg once a week for 4 weeks starting >4 months postgrafting.
†Dogs 1 and 2 were increased to two doses of MTX 0.5 mg/kg/week for 5 and 3 weeks, respectively. Dog 5 was continued on twice-weekly MTX for 6 weeks postgrafting.
‡Less than 20% fall in PMN's or platelets or abnormal CBC.
MTX. Indeed, the MTX alone clearly had profound GI toxicity; the timing and dosage of 5-FU will have to be carefully studied before it can be used in this setting. At the time of these experiments, we did not have growth factors such as IL3 that stimulate canine stem cells to divide; such growth factors are now being evaluated. Another technique for improving the apparent rate of gene transfer and expression involves preselection of the infected marrow for 24 to 48 hours prior to marrow infusion and results in cell losses of up to 90% in mouse experiments,\textsuperscript{6,7} which can be compensated for by using marrow from more donors. Given that our final recoveries following cocultivation average only two- to three-fold more than the 1.5 to 3 $\times$ 10\textsuperscript{6} mononuclear cells/kg required for reliable autologous engraftment,\textsuperscript{27-29} this method may not be suitable in a large animal model, in which marrow availability is limiting.

Instead, we chose to apply selective pressure with MTX in vivo following infection and transplantation to enrich for stem cells expressing the MTX-resistant DHFR\textsuperscript{*} gene. Only one of three animals, with the highest rate of infection and least GI toxicity, survived this regimen of MTX following transplantation. A similar result was recently reported by Williams et al.,\textsuperscript{30} who demonstrated protection of transplanted mice from otherwise lethal doses of MTX by infection of the transplanted marrow with a virus carrying the same DHFR\textsuperscript{*} gene used here. Although these results provide indirect evidence for expression of the DHFR\textsuperscript{*} gene, no direct evidence of expression or demonstration of the cell type(s) responsible for this protection is available.

Our studies suggest very-low-level transient expression in one animal following reconstitution. The small number of MTX-resistant colonies detected at weeks three and five in this animal may represent infected progenitors that have differentiated to CFU-GM or the progeny of an infected pluripotent stem cell active only transiently. We do not believe that these few resistant colonies are artifactual given our previous demonstration of complete sensitivity of uninfected CFU-GM to these concentrations of MTX (ref 12 and unpublished observations) as well as the absence of any resistant colonies in the concurrent controls or from the same animal at later times. The limits of sensitivity of our DNA analysis did not allow us to detect the proviral sequences.

Recent primate experiments conclusively demonstrated transient low-level expression of human adenosine deaminase in several animals.\textsuperscript{23,24} DNA was detected in only one of the 17 primates analyzed, again suggesting that sensitive methods of detecting gene expression may be required to demonstrate gene transfer in these first large animal experiments. In the mouse, there is often a very low level of gene expression\textsuperscript{4,6,8,11} even with efficient infection of CFU-S. This may indicate that the true pluripotent hematopoietic stem cell is relatively difficult to infect and/or that the retroviral vectors used to date are generally not expressed at sufficient levels in vivo. Furthermore, possibly only one or a very few stem cells are used at any given time.\textsuperscript{10} Thus, it may be critical to select infected pluripotent stem cells (or infect with virtually 100% efficiency) to ensure the continued presence of a transferred gene in differentiated hematopoietic cells.

We successfully engrafted dogs with autologous marrow cocultivated with retroviral vector producing cells and provide further evidence of gene transfer into the hematopoietic cells of an animal other than the mouse, but we could not demonstrate significant in vivo expression. We believe that in vivo selection may still have an important role in that we could only detect MTX-resistant colonies in animal 5 during the period of in vivo selection. This model will allow us to test whether modifications in vector design, methods of infection, and in vitro or in vivo selection are feasible and effective in a large animal model. This study and others\textsuperscript{23,24} point to the significant obstacles that remain and the importance of analyzing them in a large-scale model, in addition to the mouse, before human gene therapy clinical trials are contemplated.

ACKNOWLEDGMENTS

We express our appreciation to Dr David Trauber for providing the pSDHT vector, to Dr Eli Gilboa for the N2 vector, and to Dr Gary Yee for performing the MTX assays. We also gratefully acknowledge assistance with animal care from Drs Fred Appelman, Friedrich Schuening, and H. Joachim Deeg and the histologic examinations performed by Drs Robert Hackman and David Myerson. Excellent technical assistance was provided by Carol Buttimore, Ray Colby, Greg Davis, Ted Graham, and Robin Rowedder. The FHCR clinical hematology laboratory performed daily blood counts.

REFERENCES


18. Ladiges WC, Storb R, Graham T, Thomas ED: Experimental techniques used to study the immune system of dogs and other large animals. Methods of animal experimentation (in press)


Canine model for gene therapy: inefficient gene expression in dogs reconstituted with autologous marrow infected with retroviral vectors

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