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Retroviral transfer of genes into canine hemopoietic progenitor cells in culture: A model for human gene therapy

(neomycin phosphotransferase/dihydrofolate reductase)

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ABSTRACT **Amphotropic retroviral vectors containing either a mutant dihydrofolate reductase gene (*DHFR*) or the bacterial neomycin phosphotransferase gene (*neo*) were used to infect canine hemopoietic cells. We report successful transfer and expression of the *DHFR* and *neo* genes in canine hemopoietic progenitor cells (colony-forming units, granulocyte/macrophage) as measured by the ability of the viruses to confer resistance to either methotrexate or the aminoglycoside G418, respectively. Transfer was achieved in the absence of helper virus by using retrovirus packaging cell lines. Successful transfer of these genes into canine hemopoietic progenitor cells *in vitro* indicates the feasibility of gene transfer into canine marrow for autologous reconstitution. Studies of transfer of new genetic information into a large, outbred animal such as the dog will provide a preclinical model for future gene therapy in humans.**

The possibility that human genetic disease may be treated by transfer of normal genes to correct abnormalities caused by defective genes (1) is becoming more practical with recent advances in techniques for efficient gene transfer. Recombinant retroviruses have emerged as suitable vectors (2) that can be used to stably transfer genes into a variety of cells of many species, including humans (3–5). These vectors can be designed so that viral proteins are not synthesized following infection and so that further viral replication is prevented (5, 6). Bone marrow is an ideal target for therapeutic gene transfer, because of the ease of obtaining and reintroducing this tissue and the presence of pluripotent self-renewing stem cells in marrow and because of a variety of hereditary disorders that affect cells of hemopoietic origin (7). Experiments using mice have demonstrated that retroviruses can be used to express genes in hemopoietic tissue (8–12) and that pluripotent hemopoietic stem cells can indeed be infected by these viruses (11, 12).

However, there are still many questions to be answered before an attempt to use these techniques to treat humans is made. Infection of mouse hemopoietic stem cells has been demonstrated by using vectors containing elements derived from murine viruses that are capable of infecting only rodents. Amphotropic vectors can infect human cells, but their ability to efficiently infect hemopoietic cells has not been demonstrated, and the efficiency of gene expression may be different in animals other than mice. There are several stages at which retrovirus infection can be blocked in cells from different inbred mouse strains, and it is not known whether similar problems will be encountered in other mammalian species or in outbred animals such as humans. In addition, there are problems of scale involved in infecting the large number of cells required for human transfer as compared with mouse gene transfer.

To address these questions, we have chosen the canine model to test our ability to transfer genes into a large, outbred animal. The canine model is an established preclinical model for human bone marrow transplantation (13), in which the importance of histocompatibility and the success of engraftment following bone marrow transplantation was first established (14) as well as the use of methotrexate (Mtx) for prevention of graft versus host disease (15). Thus, we expect that the dog will be an important preclinical model for human gene therapy studies. Here we show that we can transfer and express selectable genes in canine hemopoietic progenitor cells *in vitro*, a prerequisite for autologous bone marrow reconstitution with infected marrow in dogs.

MATERIALS AND METHODS

Cell Lines. NIH 3T3 (TK^-) mouse fibroblasts (16), PA12 amphotropic retrovirus packaging cells (5), and CF2TH canine fibroblasts (17) have been described. Cells were grown in Dulbecco modified Eagle medium (DME medium) with 4.5 g of glucose per liter and 10% fetal bovine serum. Dialyzed fetal bovine serum was used when selecting for Mtx-resistant cells. Concentrations of G418 used for selection are given as dry weight of powder, of which about 50% is active.

Virus Assay. Medium exposed to confluent monolayers of virus-producing cells for 16 hr was assayed for virus carrying selectable markers by exposing cells seeded the day before at 5×10^5 cells per 60-mm dish to aliquots of virus diluted in 4 ml of medium containing 4 μg of Polybrene (Sigma) per ml. One day later, the cells were trypsinized and divided 1:10 into selective medium, 2 mg of G418 per ml for neomycin phosphotransferase (neo) viruses or 0.1 μM Mtx for dihydrofolate reductase (DHFR) viruses. Drug-resistant colonies were counted about 5 days later after staining the cells. Helper virus was assayed using the S^+L^- assay (5), which measures the ability of virus present in medium to rescue a replication-defective transforming virus. The absence of helper virus production by packaging cells producing selectable vectors was also confirmed by using 1 ml of medium from the cells to infect NIH 3T3 cells, passaging the cells for at least 2 weeks, and assaying these cells for production of the selectable vector and/or helper virus. Helper-free virus stocks were negative by both criteria.

Infection of Canine Hemopoietic Progenitor Cells [Colony-Forming Units, Granulocyte/Macrophage (CFU-GM)]. Nucleated cells were purified from canine marrow by Ficoll density-gradient centrifugation. Mononuclear bone marrow cells (10^6) were cocultivated with virus-producing fibroblasts in DME medium supplemented with 20% fetal bovine serum and 2 μg of Polybrene per ml at 37°C for 24 hr. Fibroblasts were plated the day before at 5×10^5 per 60-mm dish and were irradiated with 1500 rads (1 rad = 0.01 gray) before use to

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Abbreviations: neo, neomycin phosphotransferase; DHFR, dihydrofolate reductase; CFU-GM, colony-forming unit, granulocyte/macrophage; Mtx, methotrexate; LTR, long terminal repeat.

prevent transfer of viable fibroblasts into the CFU-GM assay. For CFU-GM colony assay (18), 7.5×10^4 mononuclear marrow cells were seeded in 2 ml of semisolid medium consisting of DME medium with 20 μg of L-asparagine per ml, 75 μg of DEAE-dextran per ml, 20% heat-inactivated human AB plasma, 5% phytohemaglutinin-stimulated lymphocyte-conditioned medium, and 0.3% agar with or without selective agents. Colonies were counted after 14 days. CFU-GM represented 0.2–0.5% of mononuclear cells.

RESULTS

Retrovirus Vectors. We used the viral vectors depicted in Fig. 1. A virus containing the bacterial *neo* gene (20), a dominant selectable marker that confers resistance to G418 in mammalian cells, was chosen because of success with this vector in murine bone marrow infection (12). Viruses carrying a mutant *DHFR* gene (21), which confers resistance to Mtx, were tested because of the possibility of selection for infected hemopoietic cells *in vivo* using Mtx (22, 23). All of the viruses are replication defective and thus either rely on the presence of helper virus for transmission or can be produced in the absence of helper virus using retrovirus packaging cell lines (5, 6). Virus was generated from plasmids containing the virus constructs by using several techniques, some of which resulted in production of helper virus (Table 1). We were surprised to find helper virus production from PA12 cells secreting the neo virus N2. This occurrence was apparently the result of recombination in homologous sequences present in the vector and packaging system DNA (19). Recently we have been able to produce helper-free N2 virus (Table 1) by using a new packaging cell line called PA317 that has additional alterations in the packaging DNA used to make the cell line, which are designed to make the packaging cells less recombinogenic (A.D.M., unpublished results). The packaging DNA in PA12 cells consists of an amphotropic helper virus from which the packaging signal has been deleted (5). The packaging DNA in PA317 cells consists of an amphotropic helper virus from which the packaging signal, the 3′ LTR, the site for initiation of second-strand DNA synthesis, and the 5′ portion of the 5′ LTR have all been deleted.

The helper virus and packaging cell lines that were employed to generate virus impart an amphotropic pseudotype to these viruses, which allows them to infect cells from many species (24). Table 1 shows that all of the viruses are capable of converting canine fibroblasts to drug resistance, confirming that amphotropic viruses can infect canine cells.

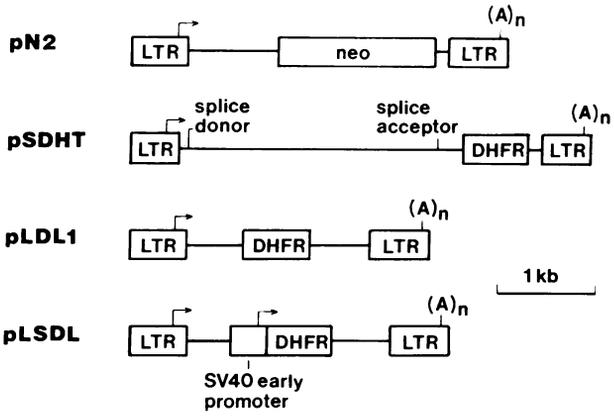

FIG. 1. Virus constructs. Arrows indicate promoters, $(A)_n$ indicates a poly(A) site, and LTR indicates the viral long terminal repeat. Virus constructs pN2 (12), pSDHT (19), pLDL1 (5), and pLSDL (5) have been described. SV40, simian virus 40; kb, kilobase.

Table 1. Virus titers

Virus-producing cells	Selectable virus titer, colony-forming units/ml: Assayed on NIH 3T3 mouse fibroblasts	Assayed on CF2TH canine fibroblasts	Helper virus titer
PA12-N2 c4 (neo virus)	5×10^6	2×10^6	$>10^3$*
PA317-N2 c11 (neo virus)	10^7	ND	<1
PA12-SDHT c6 (DHFR virus)	10^6	10^6	<1
PA12-LDL1 c7 (DHFR virus)	3×10^6	6×10^5	<1
AM-LSDL (1 μM Mtx) (DHFR virus)	10^7	2×10^6	10^6

Virus titer in medium harvested from cell lines made by using vectors depicted in Fig. 1 is shown. The cell lines PA12-N2 c4 and PA12-SDHT c6 were made (23) by transfecting Psi-2 cells (6) with the DNA constructs pN2 and pSDHT, respectively, and using virus harvested after 2 days to infect PA12 amphotropic retrovirus packaging cells (5). Clones of drug-resistant PA12 cells were isolated and screened for virus production (19). Similarly, the cell line PA317-N2 c11 was made by transfection of Psi-2 cells followed by infection of PA317 cells and selection and screening of clones. PA12-LDL1 c7 has been described (5). AM-LSDL (1 μM Mtx) cells were made by cotransfecting NIH 3T3 cells with pAM replication-competent amphotropic retrovirus DNA (5) and pLSDL DHFR virus DNA (5) and selecting the cells in increasing concentrations of Mtx up to 1 μM. This technique results in high-titer DHFR virus, as demonstrated previously (5). ND, not determined.
*The titer of helper virus increased during passage of this cell line.

Infection of Hemopoietic Cells. Hemopoietic progenitor cells in canine marrow that produce colonies in soft agar containing granulocytes and macrophages (CFU-GM) can be assayed by using conditions similar to those used for mouse or human marrow (18). We tested the ability of the viruses shown in Fig. 1 to confer drug resistance to canine CFU-GM. Two hundred fifty nanomolar Mtx or 1 mg of G418 per ml was sufficient to completely inhibit the growth of canine CFU-GM, concentrations comparable to those needed to kill cultured cell lines. In preliminary experiments, we determined that infection of hemopoietic cells by cocultivation with virus-producing cells resulted in 3- to 10-fold more efficient gene transfer than that obtained following exposure of hemopoietic cells to virus-containing medium. In the following experiments, infection was performed by using the cocultivation technique. PA12 or PA317 cells, which produce virions that do not contain viral RNA, were substituted for virus-producing cells in control infections. The neo virus N2 (Table 2) and the DHFR virus SDHT (Table 3) could convert 6–25% of CFU-GM to drug resistance. Thus, these two types

Table 2. G418 resistance of canine CFU-GM following neo virus infection

PA12		PA12-N2		PA317		PA317-N2	
0/642	(0%)	211/1052	(20%)				
0/620	(0%)	217/852	(25%)				
0/471	(0%)	257/1325	(19%)				
0/1374	(0%)	147/933	(16%)				
				0/1560	(0%)	119/1960	(6%)
0/3107	(0%)	832/4162	(20%)	0/1560	(0%)	119/1960	(6%)

The cells listed were used in cocultivation. Data are expressed as the number of G418-resistant CFU-GM colonies divided by the number of nonselected colonies. The concentration of G418 used was 1 mg/ml. Each row represents data from a separate experiment. Totals are given at the bottom of each column.

Table 3. Mtx resistance of canine CFU-GM following DHFR virus infection

PA12		PA12-SDHT		PA-LDL1		AM-LSDL	
0/682	(0%)	86/551	(16%)				
0/853	(0%)	59/670	(9%)			2/208	(1.0%)
0/474	(0%)	62/480	(13%)	0/457	(0%)	2/404	(0.5%)
0/958	(0%)	55/486	(11%)				
0/439	(0%)	17/238	(7%)				
0/3406	(0%)	279/2425	(12%)	0/457	(0%)	4/612	(0.7%)

The cells listed were used in cocultivation. Data are expressed as the number of Mtx-resistant CFU-GM colonies divided by the number of nonselected colonies. The concentration of Mtx used was 500 nM. Each row represents data from a separate experiment. Totals are given at the bottom of each column.

of viruses could infect canine hemopoietic cells effectively. In particular, the use of helper-free neo or DHFR virus resulted in drug-resistant CFU-GM production.

Successful transfer of the neo virus into canine hemopoietic progenitor cells was demonstrated by another experimental approach. The PA12-N2 cell line produced amphotropic helper virus in addition to neo virus. Helper virus-mediated rescue of neo virus from infected CFU-GM was detected as follows. Individual 14-day-old G418-resistant CFU-GM colonies resulting from infection with virus from PA12-N2 cells were removed from agar, dispersed, and added to NIH 3T3 cells seeded at a density of 10^4 cells per 16-mm dish. Selective medium containing 2 mg of G418 per ml was applied after 24 hr. G418-resistant NIH 3T3 colonies were scored after 5 days. Of 20 G418-resistant CFU-GM colonies assayed, 15 induced G418-resistant NIH 3T3 colonies, indicating the presence of helper virus and neo virus in these CFU-GM colonies. Uninfected CFU-GM did not induce colonies. Thus, at least 75% of the neo virus-infected, G418-resistant CFU-GM colonies actually contain the neo virus.

In contrast to the results obtained with the neo virus N2 and the DHFR virus SDHT, the DHFR virus LSDL conferred limited resistance to CFU-GM, and the DHFR virus LDL1 did not confer resistance to CFU-GM (Table 3). These results were quite different than the results obtained by using canine fibroblast cell lines, in which efficient infection was achieved by using all of the viruses. Since the virus coat and other proteins involved in virus transmission are the same for these viruses, we hypothesize the expression level of the transferred genes was more critical in hemopoietic cells than in fibroblasts and CFU-GM were infected by the viruses but were not drug resistant due to inadequate expression of the drug resistance gene. This interpretation is supported by

Table 4. Percentage of drug-resistant CFU-GM in infected bone marrow as a function of time of cocultivation

Time, hr	% drug-resistant colonies			Average % of maximal infectivity
	Exp. 1	Exp. 2	Exp. 3	
2	4.6	—	5.5	46
6	6.3	12.5	10.9	77
24	9.3	15.7	12.9	100

Bone marrow cells were cocultivated with virus-producing fibroblasts at a ratio of 1:2. Bone marrow cells were removed from the fibroblasts at the times indicated and incubated in DME medium with 20% fetal bovine serum. Twenty-four hours after the start of cocultivation, the cells were assayed for CFU-GM formation in the presence and absence of drug selection. Results are expressed as the percentage of drug-resistant CFU-GM compared with nonselected CFU-GM. Experiments 1 and 2, cocultivations were carried out with PA12-N2 cells and selection was in 1 mg of G418 per ml. Experiment 3, cocultivation was carried out with PA12-SDHT cells and selection was in 500 nM Mtx.

Table 5. Percentage of drug-resistant CFU-GM as a function of ratio of virus-producing cells to bone marrow cells

Ratio*	% drug-resistant colonies			
	Exp. 5	Exp. 6	Exp. 7	Exp. 8
1:100	0.2	0.6	—	—
1:10	2.1	6.8	5.9	—
1:4	—	—	12.2	22.9
1:2	4.2	9.5	12.9	25.4
1:1	4.5	10.5	—	—
2:1	—	—	10.8	—

Mononuclear bone marrow cells (10^6) were cocultivated with virus-producing fibroblasts at the indicated ratios. Cells were assayed for the presence of CFU-GM in the presence and absence of drug selection. Experiments 5, 6, and 8, cocultivations were carried out with PA12-N2 cells and selection was 1 mg of G418 per ml. Experiment 7, cocultivation was carried out with PA12-SDHT cells and selection was 500 nM Mtx.
*Ratio of virus-producing fibroblasts to bone marrow cells.

rescue experiments involving infection of bone marrow with the DHFR virus LSDL, which contains helper virus, followed by assay of CFU-GM colonies for production of DHFR virus. The assay was similar to that described above for neo virus-infected, G418-resistant CFU-GM colonies, except that the infected CFU-GM were not selected for drug resistance, and selection of resultant NIH 3T3 colonies was performed in 0.1 μM Mtx. Indeed, following infection of canine marrow by cocultivation with the LSDL DHFR virus-producing cells, 8% of the unselected CFU-GM colonies secreted DHFR virus. This is about 10-fold higher than the number of Mtx-resistant CFU-GM that we detected following infection with LSDL virus (Table 3). We conclude that the DHFR virus LSDL was capable of infecting hemopoietic cells and was transcribed at a level sufficient for production of virus particles but that production of the mutant DHFR gene product was not sufficient for the protection of all infected CFU-GM from Mtx toxicity.

Other parameters affecting efficiency of gene transfer were studied by using the DHFR virus SDHT and the neo virus N2. Polybrene improves virus infectivity in cultured cells but at concentrations up to 20 μg/ml had no effect on the efficiency of CFU-GM infection by cocultivation (data not shown). Cocultivation of bone marrow cells with fibroblasts for 6 hr was sufficient to attain about 75% of infectivity achieved after 24 hr of cocultivation (Table 4). This observation is important for application of these techniques to bone marrow transplantation, because of the uncertain viability of stem cells during prolonged *in vitro* manipulations. We also determined the optimal ratio of virus-producing fibroblasts to bone marrow cells for infection of CFU-GM (Table 5). Bone marrow cells (10^6) were incubated with a variable number of

Table 6. Percentage of Mtx-resistant CFU-GM in infected and mock-infected bone marrow as a function of Mtx concentration

Mtx, μM	% drug-resistant colonies	
	PA12 cocultivation	PA12-SDHT cocultivation
0.1	1.6	
0.25	0	7.8
0.5	0	7.6
1	0	6.4
5		4.6
10		3.3
25		2.4
50		2.0

Data are from a representative experiment with duplicate dishes for each condition.

virus-producing fibroblasts, and production of drug-resistant CFU-GM was scored. A 4:1 or lower ratio of marrow cells to fibroblasts provided maximal drug-resistant colony production.

Mtx Resistance of Infected CFU-GM. Further studies were carried out to determine the toxicity of Mtx to CFU-GM infected with the DHFR virus SDHT (Table 6). Under our assay conditions, uninfected CFU-GM did not produce colonies when exposed to 250 nM Mtx, and the LD_{50} was about 40 nM. CFU-GM infected with the DHFR virus are significantly more resistant to Mtx, with 8% of the CFU-GM resistant to 250 nM Mtx and 2% resistant to levels as high as 50 μM. These results show that the DHFR virus is able to confer resistance to hemopoietic cells *in vitro* to pharmacologic levels of Mtx attainable in animals and clinical subjects.

DISCUSSION

We have demonstrated that genes can be transferred into and expressed in canine hemopoietic progenitor cells by using helper-free amphotropic retroviral vectors. The efficiency of G418-resistant CFU-GM production following cocultivation with neo virus-producing cells is comparable using canine (6–25%) or mouse (10–20%) (12) bone marrow; thus, infection of canine marrow poses no unusual problems in comparison to infection of mouse marrow. These results also demonstrate the utility of the amphotropic vector system. Recent studies have shown that it is possible to infect pluripotent mouse hemopoietic stem cells (11, 12). Although we cannot directly predict the efficiency of canine hemopoietic stem cell infection resulting in the long-term presence of the introduced genes in transplanted dogs, we are encouraged by the apparent similarity between the ability to infect canine and murine CFU-GM. We have evidence that we can induce drug resistance in human CFU-GM (3–10%) using these vectors (25), suggesting that the vectors will also be useful in humans.

Autologous marrow reconstitution in dogs after lethal total body irradiation can be obtained by infusion of 5×10^7 mononuclear cells per kg. Thus, successful reconstitution of a 10-kg dog requires exposure of at least 10^9 marrow cells to virus, assuming 50% recovery. Utilizing roller bottles (850 cm^2), each seeded with $\approx 5 \times 10^7$ virus producer cells and $\approx 2 \times 10^8$ mononuclear bone marrow cells, we are able to obtain rates of infection comparable to those obtained here in small-scale experiments, demonstrating the feasibility of *in vivo* experiments.

The neo virus produced by PA317-N2 cells and the DHFR virus produced by PA12-SDHT cells are helper virus-free by stringent criteria capable of detecting one helper virus per ml of medium exposed to these cells. Virus from both of these cell lines was capable of transferring drug resistance to CFU-GM. The ability to transfer these drug markers in the absence of helper virus will allow assessment of the ability of these vectors to infect pluripotent canine hemopoietic stem cells following infection and transplantation of bone marrow cells into animals. In addition, helper-free virus vectors will probably be a requirement for use in human gene therapy.

Successful transfer of a mutant *DHFR* gene into hemopoietic cells that confers resistance to pharmacologic levels of Mtx *in vitro* suggests an approach for *in vivo* selection of infected cells by administration of Mtx after autologous reconstitution. Coupling of other nonselectable genes to the *DHFR* gene may allow us to select for cells containing the nonselectable gene. Another potential application of the DHFR vector is to improve the therapeutic index of Mtx in the treatment of solid tumors by conferring Mtx resistance to bone marrow, and this can be tested in the canine lymphoma model (26). Results presented here demonstrate the feasibility of retrovirus-mediated gene transfer into hemopoietic cells of animals other than mice and will be important for eventual application of these techniques to the treatment of human disease.

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