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Long-term transplantation of canine keratinocytes made resistant to G418 through retrovirus-mediated gene transfer

(gene therapy/neomycin phosphotransferase)

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ABSTRACT **We studied cultured canine keratinocytes to determine whether they could serve as targets for retrovirus-mediated gene transfer and whether infected cells could persist after transplantation into dogs, a large random-bred model for gene transfer studies. Canine keratinocytes obtained from skin biopsy samples were cultured *in vitro* with lethally irradiated NIH 3T3 cells used as a feeder layer. The keratinocyte colonies consisted of squamous epithelium with numerous desmosomes, tonofilaments, and keratohyalin granules. In addition, the cells were strongly reactive with monoclonal antibodies to cytokeratin intermediate filament proteins. For the infection studies, we grew the keratinocytes on a feeder layer of lethally irradiated PA317 retrovirus packaging cells, which produced a helper-free amphotropic retroviral vector containing the neomycin phosphotransferase (*neo*) gene. After cocultivation, 34% (range, 10–76%) of the keratinocytes were found to be resistant to the neomycin analogue G418. Infected keratinocytes were then transplanted into the dog of origin; 1% (range, <0.1–3%) of the keratinocytes obtained 27–130 days after transplantation from skin biopsy samples gave rise to G418-resistant colonies. We conclude that canine keratinocytes cultured *in vitro* can be infected efficiently with a *neo* gene-containing retroviral vector, and they show persistent G418 resistance for at least 130 days after transplantation into the skin donor.**

The aim of gene therapy is to introduce genes encoding proteins of medical relevance into somatic cells to obtain permanent gene expression. Retroviral vectors have been used successfully to transfer genes into many different cell types, such as bone marrow (1–8), fibroblasts (9–11), endothelial (12, 13), and epidermal cells (14).

Although bone marrow is an important target for gene transfer, especially for possible gene therapy of genetically caused hematological diseases such as β-thalassemia or sickle cell anemia, *in vivo* gene expression in large animal models has been low and temporary (7, 8). It is therefore important to investigate alternative target cells for gene transfer. Cultured keratinocytes represent an attractive target population because they can be rapidly expanded *in vitro* (15, 16) and can be easily transplanted (17–19).

The purpose of the present study was to establish an *in vitro* culture system for canine keratinocytes to investigate retroviral gene transfer and long-term gene expression after transplantation. We demonstrate that canine keratinocytes cultured *in vitro* can be infected efficiently with a retroviral vector containing the neomycin phosphotransferase (*neo*) gene. The important finding of the present study is the demonstration that G418-resistant cells persist long-term for at least 130 days after transplantation into the dog of origin.

MATERIALS AND METHODS

Experimental Animals. Eight dogs (Beagles) of both sexes and 6–12 months old were used for skin biopsies and skin transplants. Animals were either raised at the Fred Hutchinson Cancer Research Center or were purchased from commercial U.S. Department of Agriculture licensed dealers. Dogs were quarantined on arrival for a minimum of 2 months before being released for use in experiments. All dogs were dewormed and vaccinated for rabies, distemper, leptospirosis, hepatitis, and parvovirus. They were housed in an American Association for Accreditation of Laboratory Animal Care accredited facility in standard indoor runs and were provided commercial dog chow and chlorinated tap water ad libitum. Animal holding areas were maintained at 70°F ± 2°F (≈21°C) with 50% ± 10% relative humidity using at least 15 air changes per hour of 100% conditioned fresh air. The dogs were on a 12-hr light/12-hr dark full-spectrum lighting cycle with no twilight. Research was carried out according to ref. 20. The protocol of this study was approved by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center.

Skin Biopsy and Isolation of Keratinocytes. For skin biopsy, dogs were anesthetized by intravenous injection of sodium pentobarbital at 24 mg/kg. The biopsy site on the flank of the dogs was shaved and aseptically cleansed with a povidone–iodine scrub and an alcohol rinse. A 1.5-cm^2 full-thickness skin biopsy sample was obtained and used as the source of keratinocytes. Dermal connective tissue was trimmed off and the biopsy tissue was washed 8–10 times in Dulbecco's phosphate-buffered saline (PBS) (Flow Laboratories) containing penicillin (50 units/ml) and streptomycin (50 μg/ml). The skin was then minced finely with surgical scissors to pieces <1 mm in diameter in the presence of 0.125% trypsin (Sigma type III; Sigma). Fifteen milliliters of trypsin (0.05%)/EDTA (0.02%) solution was added to the minced skin, and the mixture was transferred into a 50-ml plastic tube. Gentle stirring (120 rpm) at 37°C was used to maximize the effect of trypsin on the minced skin. Every 30 min, dissociated cells were harvested after allowing 1 min for settling by decanting the supernatant. After each harvest, the resulting cell suspension was washed in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) and 10% fetal bovine serum (FBS) (Flow Laboratories), resuspended, and counted. Fresh trypsin/EDTA was added to the tube and the trypsin treatment was repeated two or three times or until no more cells were obtained from the skin. The remaining trypsin-treated skin was filtered through a 0.22-mm mesh screen and washed with medium in an attempt to rescue more cells.

Keratinocyte Culture. First, a feeder layer was set up by plating irradiated (45 Gy) fibroblasts in 25-cm^2 canted-neck

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flasks (Corning) at a density of 3×10^4 cells per cm^2. Twenty-four hours later, cells obtained from skin biopsy samples ($0.1–1.7 \times 10^6$ cells) were inoculated onto the feeder layer and grown in the following medium: A 7:3 mixture of DMEM with L-glutamine (GIBCO), and Ham's F-12 (GIBCO) medium supplemented with 0.18 mM adenine (Boehringer Mannheim), 10% FBS (GIBCO), insulin (5 μg/ml) (Sigma), transferrin (5 μg/ml) (Behring Diagnostics), 2 nM 3,3',5 triiodo-L-thyronine (Sigma), hydrocortisone (0.4 μg/ml) (Behring Diagnostics), 0.1 nM cholera toxin (Calbiochem–Behring). Cultures were incubated at 37°C in a 5% CO_2/95% air atmosphere and were fed three times a week. Recombinant epidermal growth factor (10 ng/ml), generously provided by Chiron, was added to the medium 2 days after the initiation of the cultures. Cultures were maintained until keratinocyte colonies reached confluence (after 2–3 weeks).

Retroviral Vector and Cell Lines. The retroviral vector SPN contains the bacterial *neo* gene, which confers resistance to the aminoglycoside G418 and which was inserted into the $SFFV_A$-L clone of spleen focus-forming virus (21) in place of the gp52 *env* gene. The SPN vector is replication defective. NIH 3T3 (TK^-) (22) and PA317 (23) packaging cells have been described. Cell lines were grown in DMEM (GIBCO) with high glucose (4.5 g/liter) and 10% FBS (GIBCO), except as otherwise noted. PA317 cells that produce the SPN vector (PA317/SPN) were generated as described (24). Assays for vector titer and amphotropic helper virus have been described (23, 24). Virus titers ranged from 10^6 to 10^7 colony-forming units/ml. The virus-producing packaging cell line PA317/SPN was helper virus-free.

Infection of Canine Keratinocytes. A feeder layer was set up using either the *neo* vector-producing packaging cell line PA317/SPN or, as a control, 3T3/N2 cells, which are NIH 3T3 TK^- cells that contain but do not produce the *neo* virus N2 (25). After irradiation with 45 Gy, feeder layer cells were plated in 25-cm^2 canted-neck flasks (Corning) at a density of 3×10^4 cells per cm^2. Twenty-four hours later, cells obtained from skin biopsy samples ($0.1–1.7 \times 10^6$ cells) were inoculated onto the feeder layer and cultured as described above.

After reaching confluence (after 2–3 weeks), keratinocytes were dissociated with 5 ml of trypsin/EDTA solution and subcultured at 6×10^3 cells per cm^2 into 35-mm Petri dishes, containing 3×10^4 irradiated (45 Gy) 3T3/N2 cells per cm^2 as feeder layer. G418 (0.5 mg/ml) (active compound) was added to half the dishes. After 8–10 days of culture, the Petri dishes were stained with 0.5% Coomassie blue and the keratinocyte colonies were counted. The infection rate was determined as a percentage by dividing the number of keratinocyte colonies grown in the presence of G418 by the number of colonies grown in the absence of the drug. As a negative control, keratinocytes cultured only on irradiated 3T3/N2 were assayed in parallel to ensure that there were no "background" drug-resistant colonies.

Preparation of the Keratinocyte–Collagen Layer. Fibroblasts were prepared from dermal tissue obtained from the same skin biopsy that served as a source for keratinocytes as described above. Several small pieces of dermal tissue were placed under sterile coverslips in a Petri dish (100 × 25 mm) and fed with DMEM (GIBCO) and 10% FBS (Flow Laboratories). When fibroblasts growing out of the pieces of dermal tissue were confluent, coverslips and tissue pieces were removed. Fibroblasts were harvested from the Petri dish with 5 ml of trypsin/EDTA solution and transferred to 25-cm^2 canted-neck flasks for further expansion. Three days before keratinocyte transplantation, fibroblasts were dissociated with trypsin/EDTA and counted. Two milliliters of double-strength DMEM (GIBCO) were mixed with 2 ml of FBS (Flow Laboratories) and 0.24 ml of 1 M sodium hydroxide (Baker) in a Petri dish (100 × 25 mm), carefully avoiding bubble formation. Subsequently, 2 ml of Vitrogen 100 (Collagen Corp.) and 1×10^6 fibroblasts were added, all components were mixed, and the resultant mixture was incubated overnight in a 37°C incubator. The following day the keratinocytes, obtained from the same skin biopsy sample as the fibroblasts and cultured on lethally irradiated PA317/SPN cells, were dissociated with EDTA/trypsin, washed twice, resuspended in keratinocyte medium without cholera toxin, and layered on the collagen. The keratinocyte–collagen layer was kept for 2 days in a 37°C incubator until the day of transplantation.

Transplantation of the Keratinocyte–Collagen Layer. On the day of the keratinocyte transplantation, the dog from which the fibroblasts and keratinocytes were obtained was anesthetized by intravenous injection of sodium pentobarbital as described above. The skin of the flank of the dog opposing the previous biopsy site was aseptically cleansed as described. A 1- to 2-cm-wide incision of the skin was made and a subcutaneous pouch (2 × 2 cm) was prepared. The keratinocyte–collagen layer was implanted into the subcutaneous pouch with the keratinocytes on top of the collagen facing toward the epidermis. The skin incision was closed with sutures. The site of the keratinocyte transplant was marked with green tatoo color above the implantation site. At various periods of time after transplantation, a full-thickness skin biopsy sample was taken from the area where the keratinocytes had been implanted. The skin biopsy sample was treated as described above and the keratinocytes obtained were grown on irradiated 3T3/N2 cells with and without G418 as described above to determine the percentage of G418-resistant keratinocyte colonies. Skin biopsy samples from implants of keratinocytes that had been cocultivated with 3T3/N2 cells served as negative controls.

Immunocytochemistry of Skin Biopsy. After fixation for 12–36 hr in buffered formalin, a skin biopsy sample obtained 109 days after transplantation was embedded in paraffin. Four-micrometer sections were then mounted on Fisher Probe-On slides (Fisher) previously coated with 0.01% poly-L-lysine (Sigma). After drying at room temperature, the sections were subsequently processed in a Fisher Code-On automated stainer (Fisher). Paraffin was extracted with Hemo-De (Fisher), sections were rinsed in 100% and 95% ethanol, and endogenous peroxidase was quenched with 3.6% hydrogen peroxide in 100% methanol. After rinsing in 25% acetone and 0.1% brij 35 (Sigma) in Tris-buffered saline, sections were treated with pronase (Calbiochem) [5 mg/ml in 50 mM Tris (pH 7.4)] for 10 min at room temperature. Residual pronase was inhibited with 2% glycine, and sections were incubated at room temperature in a mixture of 5% nonimmunized goat serum in 2% bovine serum albumin (BSA) and 0.1% Brij 35. Murine monoclonal antibodies 34βE12 (26) and AE1:AE3 (Boehringer Mannheim) to intermediate filament cytokeratins were diluted 1:1000 in 1.0% BSA and 0.1% Brij 35 in Tris-buffered saline and were applied for 80 min at 37°C. After the slides were rinsed, a biotinylated horse antibody to mouse IgG (Vector Laboratories) was applied for 30 min. Rinsing was then repeated, and the sections were exposed to peroxidase-conjugated streptavidin (Zymed Laboratories) for 25 min at 37°C. After the rinses, the peroxidase was detected with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Polyscience) in PBS with 0.88% nickel chloride and 0.01% hydrogen peroxide for 6.5 min at room temperature. After rinsing in distilled water, the sections were counterstained with Gill's hematoxylin and dehydrated in sequential alcohol solutions to xylene, and coverslips were applied with Poly-Mount (Poly Scientific, Bay Shore, NY).

RESULTS

Characteristics of Canine Keratinocyte Cultures. Full-thickness skin biopsy samples obtained from normal dogs

provided 0.1–1.7 × 10^6 single cells per cm^2 (mean, 0.8 × 10^6) (data not shown). When inoculated into 25-cm^2 flasks containing either irradiated PA317/SPN or 3T3/N2 cells as feeder layer, the keratinocytes formed colonies and reached confluence after 13–29 days (mean, 20 days). Histology of a section from a confluent keratinocyte culture revealed an organized and regularly stratified squamous epithelium. Electron microscopy showed characteristic features of keratinocytes (16) such as abundant tonofilaments, numerous desmosomes, and keratohyalin granules (data not shown). Immunocytochemistry studies using monoclonal antibodies to cytokeratin intermediate filaments demonstrated strong reaction of the cultured keratinocytes (data not shown). The keratinocytes could be easily subcultured.

Retroviral Transfer of the *neo* Gene into Canine Keratinocytes. Canine keratinocytes were cocultivated with lethally irradiated *neo* vector-producing PA317/SPN cells or with irradiated 3T3/N2 cells until they reached confluence and were then cultured on irradiated 3T3/N2 cells with and without G418. Keratinocytes cocultivated with PA317/N2 cells gave rise, on the average, to 34% G418-resistant colonies (range, 10–76%) (Table 1). Cocultivation with irradiated 3T3/N2 cells did not yield any drug-resistant colonies.

Transplantation of *neo* Virus-Infected Keratinocytes into the Skin Donor. Canine keratinocytes infected by cocultivation with lethally irradiated *neo* vector-producing PA317/SPN cells were transplanted subcutaneously into the skin donor. The cells were not selected in G418 prior to transplantation. At the time of the transplant, 31% of keratinocytes (range, 0.3–80%) showed expression of the *neo* gene (Table 2). Keratinocytes obtained from full thickness skin biopsy samples taken 27–130 days after transplantation from the area of the keratinocyte implants and grown on irradiated 3T3/N2 cells with and without G418 gave rise to 1% (range, <0.1–3%) G418-resistant keratinocyte colonies (Table 2). Skin biopsy samples from control implants of keratinocytes that had been cocultivated with 3T3/N2 cells did not yield any drug-resistant keratinocyte colonies (0/1331; <0.08%).

Immunocytochemistry with monoclonal antibodies to cytokeratin intermediate filaments demonstrated strong reactivity of normal canine epithelial cells in the epidermis, pilosebaceous units, and other adnexal structures (Fig. 1). In addition, well-preserved aggregations of immunoreactive cultured keratinocytes were present deep in the subcutaneous tissue of a skin biopsy sample obtained 109 days after transplantation (Fig. 1).

DISCUSSION

Adopting the method of Rheinwald and Green for human keratinocytes (15), we were able to establish canine keratinocyte cultures by cocultivating dog epidermal cells with lethally irradiated *neo* virus-producing fibroblasts (PA317/SPN). The virus-producing cells served not only as a source of retroviral vectors but also provided fibroblast support necessary for optimal growth of keratinocytes (16).

The key finding of the present study involves transplantation of *neo* virus-infected keratinocytes into the skin donor, where we found on the average 1% *neo* gene expressing keratinocytes that were persisting for at least 130 days after transplantation, the last day of testing so far. This percentage is an underestimate of the survival rate of infected keratinocytes because full thickness skin biopsy samples of the area of the subcutaneous keratinocyte implants were used to determine the percentage of drug-resistant keratinocytes. As a result, an unknown number of uninfected keratinocytes from the skin covering the subcutaneous keratinocyte implant were included in the keratinocyte population assayed for gene expression. The persistent gene expression in transplanted keratinocytes observed here contrasts with our results involving infection of canine bone marrow, where drug-resistant colonies were detected in marrow for at best 1 month after transplantation of infected bone marrow cells (8). Such persistent gene expression will be a requirement for effective gene therapy.

Morgan *et al.* investigated retrovirus-mediated gene transfer into cultured human keratinocytes (14). When cocultivating human keratinocytes with two different types of *neo*

Table 1. G418 resistance of canine keratinocytes after retroviral infection

Cocultivation with 3T3/N2	Cocultivation with PA317/SPN
0/601 (<0.2%)	46/475 (10%)
0/149 (<0.7%)	116/684 (17%)
0/874 (<0.1%)	385/508 (76%)
0/276 (<0.4%)	93/279 (33%)
	Mean 34%

Canine keratinocytes were inoculated into 25-cm^2 canted-neck flasks containing lethally irradiated PA317/SPN cells or lethally irradiated 3T3/N2 cells as a control. After reaching confluence (2–3 weeks), keratinocytes were dissociated with trypsin/EDTA and subcultured at 6 × 10^3 cells per cm^2 in 35-mm Petri dishes containing 3 × 10^4 lethally irradiated 3T3/N2 cells per cm^2 as feeder layer and either 0.5 mg/ml (active compound) or no G418. Results are expressed as the number of G418-resistant/nonselected colonies followed by the percentage in parentheses. Each row represents data from a separate experiment. The colony numbers of each experiment are the mean values from three single cultures except for the experiments in which no drug-resistant colonies were observed, in which case the total number of nonselected colonies is indicated.

Table 2. G418 resistance of canine keratinocytes after cocultivation with PA317/SPN and subsequent *in vivo* transplantation

	No. of G418-resistant colonies/ total colonies per plate	
Day of biopsy	Before transplant	After transplant
27	28/67 (42%)	3/109 (3%)
28	79/99 (80%)	6/241 (2%)
57	16/132 (12%)	5/186 (3%)
71	0.3/111 (0.3%)	0.4/401 (0.1%)
86	20/91 (22%)	0/1112 (<0.1%)
88	51/211 (24%)	0/527 (<0.2%)
91	9/38 (24%)	0.2/337 (0.06%)
101	124/207 (60%)	0.4/233 (0.2%)
105	ND	2/849 (0.2%)
130	6/32 (19%)	13/1124 (1.2%)
	Mean 31%	1%

Canine keratinocytes were cocultivated in 25-cm^2 canted-neck flasks with lethally irradiated PA317/SPN cells. After the keratinocytes reached confluence (2–3 weeks), they were dissociated with EDTA/trypsin, layered over a collagen layer derived from autologous fibroblasts, and transplanted subcutaneously into the skin donor. At various time periods after transplantation (day of biopsy), a full-thickness skin biopsy sample was taken from the area of the keratinocyte transplant. Keratinocytes obtained from the skin biopsy sample, representing a mixture of infected keratinocytes from the subcutaneous implant and uninfected keratinocytes from the skin covering the implant, were grown on lethally irradiated 3T3/N2 cells as a feeder layer with or without 0.5 mg of G418 per ml (active compound). Results are expressed as the number of G418-resistant/nonselected colonies followed by the percentage in parentheses. Each row represents data from a separate experiment. The colony numbers of each experiment are the mean values from 8–15 single cultures except for the experiments in which no drug-resistant colonies were observed, in which case the total number of nonselected colonies is indicated. Skin biopsy samples from control implants of keratinocytes that had been cocultivated with 3T3/N2 cells did not yield any drug-resistant keratinocyte colonies (0/1331; <0.08%). ND, not determined.

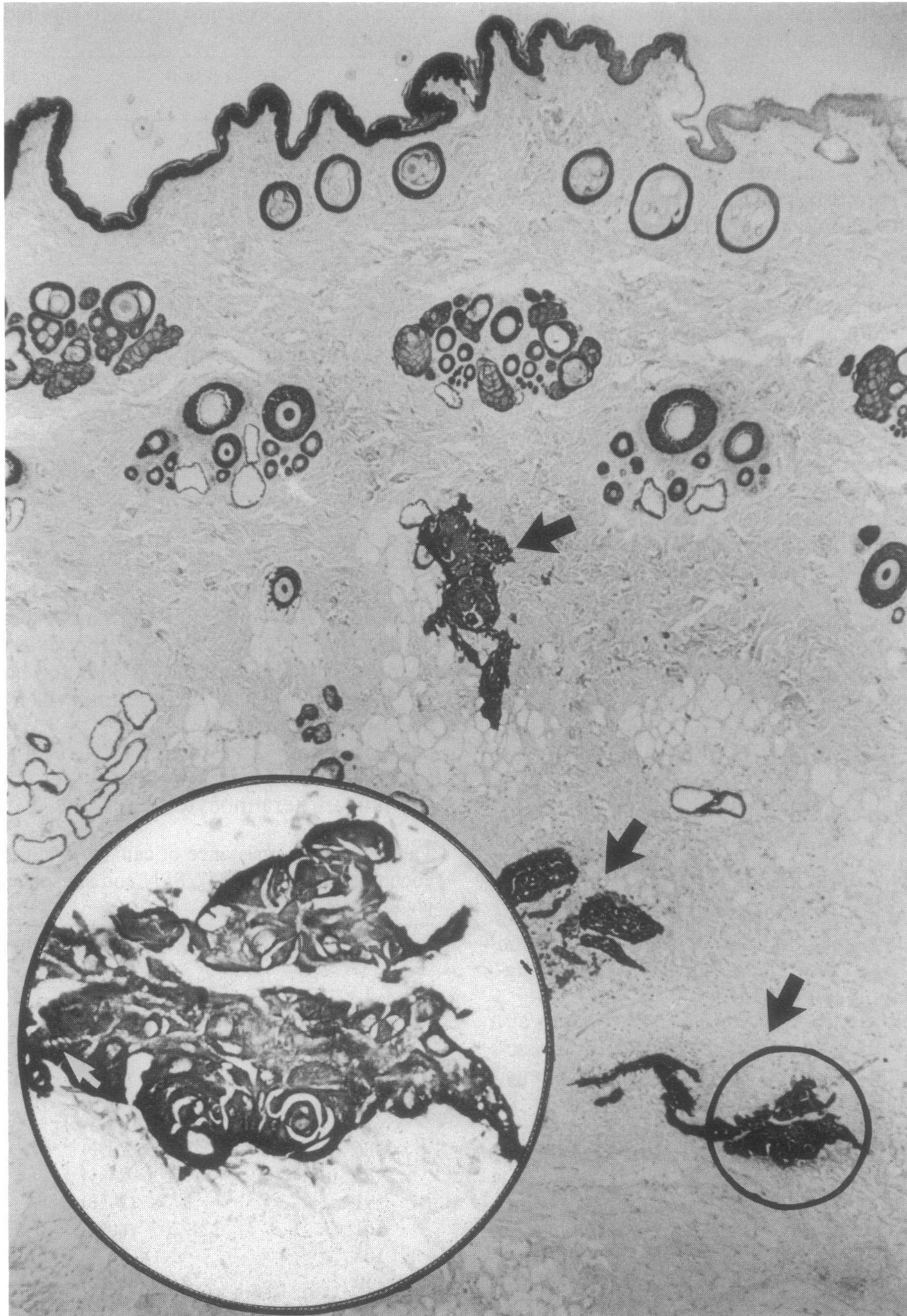

FIG. 1. Photomicrograph of a biopsy specimen of canine skin and subcutaneous tissue obtained 109 days after transplantation of keratinocytes. Biotinylated antibody 34βE12 to cytokeratin intermediate filaments has been detected with streptavidin conjugated with peroxidase to demonstrate three large aggregations of keratinocytes (dark arrows) below the normal immunoreactive epidermis and other epithelial components. (×10.) (*Inset*) Keratinocytes are well-preserved and intercellular bridges are still present (white arrow). (×50.)

virus-producing fibroblasts for 3–4 days, they found only 0.1–0.5% G418-resistant keratinocyte colonies. In comparison, this study showed an average infection rate of 34% in cultured canine keratinocytes. We suspect that the difference in infection efficiency is due not to differences between human and canine keratinocytes, but to differences in the vectors used, in particular, the extended packaging signal present in the vectors used here (9, 25, 27).

In addition, Morgan *et al.* (14) transplanted human keratinocytes infected with a retroviral vector containing the human growth hormone gene into the subcutaneous tissue of athymic mice. One to 3 weeks after transplantation, the grafts were removed and the growth hormone content of the grafted epidermis was determined. Growth hormone was detected in the epidermis only up to 7 days after transplantation. No growth hormone could be detected in serum obtained from mice 7 days to 3 weeks after transplantation by a specific and sensitive radioimmunoassay. In comparison, this study showed persistent G418 resistance of keratinocytes infected with a retroviral vector containing the *neo* gene for at least 130 days after transplantation into the skin donor.

Further research will need to focus on investigating long-term expression of transferred genes that encode proteins of medical relevance. For example, genetically modified keratinocytes could be used to metabolize toxic substrates in blood that accumulate in certain diseases, such as in adenosine deaminase deficiency, where adenosine and deoxyadenosine accumulate and are toxic to B and T cells (9, 28).

Engraftment of genetically modified keratinocytes could also be used to provide needed proteins such as coagulation factors, since it appears that even relatively large proteins synthesized by keratinocytes can move from the site of synthesis in the skin into the circulation (29). The canine keratinocyte transplantation model that we have developed here provides a large animal model for further exploration of these possibilities.

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