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RETROVIRAL PACKAGING CELLS ENCAPSULATED IN THERACYTE IMMUNOISOLATION DEVICES ENABLE LONG-TERM *IN VIVO* GENE DELIVERY

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1. ABSTRACT

The method of delivering a therapeutic gene into a patient is still one of the major obstacles towards successful human gene therapy. Here we describe a novel gene delivery approach using TheraCyte immunoisolation devices. Retroviral vector producing cells, derived from the avian retrovirus spleen necrosis virus, SNV, were encapsulated in TheraCyte devices and tested for the release of retroviral vectors. *In vitro* experiments show that such devices release infectious retroviral vectors into the tissue culture medium for up to 4 months. When such devices were implanted subcutaneously in SCID mice, infectious virus was released into the blood stream. There, the vectors were transported to and infected tumors, which had been induced by subcutaneous injection of tissue culture cells. Thus, this novel concept of a continuous, long-term gene delivery may constitute an attractive approach for future *in vivo* human gene therapy.

2. INTRODUCTION

To date, the common approaches to human gene therapy are the injection of the patient's own genetically re-engineered cells or the direct *in vivo* injection of virus-derived vectors or naked DNA into the patient (1-3). Both methods pose a number of problems and limitations. Injections of genetic material such as virus-derived vectors or naked DNA lack control of the therapeutics introduced into the patient with limited guarantee that the target will be reached (4,5). Moreover, direct injections of naked DNA and certain viral constructs such as adenovirus-derived vectors into the tissue produce only transient expression, and, therefore, are short-lived (6).

To solve many of the problems associated with the delivery of biological therapeutics such as insulin, the company Baxter Co. has developed an immunoisolation device called TheraCyte (Figure 1). This immunoisolation device is now produced by the company TheraCyte Inc. The system encapsulates cells, which produce a therapeutic agent, and prevents direct contact with the tissue of the patient (7). The inner membrane of the device is impermeable for the cells of the immune system and it has been shown that allografts are completely protected from immune cells of the host for up to one year (8,9). The outer membrane of the device allows vascularization around the system promoting molecular and nutritional flow to and from the encapsulated cells (8,9). TheraCyte immunoisolation devices were implanted subcutaneously and were previously used *in vivo* for delivery of Human Factor IX, antitumor antigen, recombinant human growth hormone, and insulin (7-11).

We initiated a series of *in vitro* and *in vivo* experiments to test Theracyte implantation bags as a tool for gene delivery using retroviral vectors. TheraCyte devices were filled with retroviral vector producer cells, which release vectors derived from spleen necrosis virus, SNV. SNV is an avian reticuloendotheliosis virus, which is

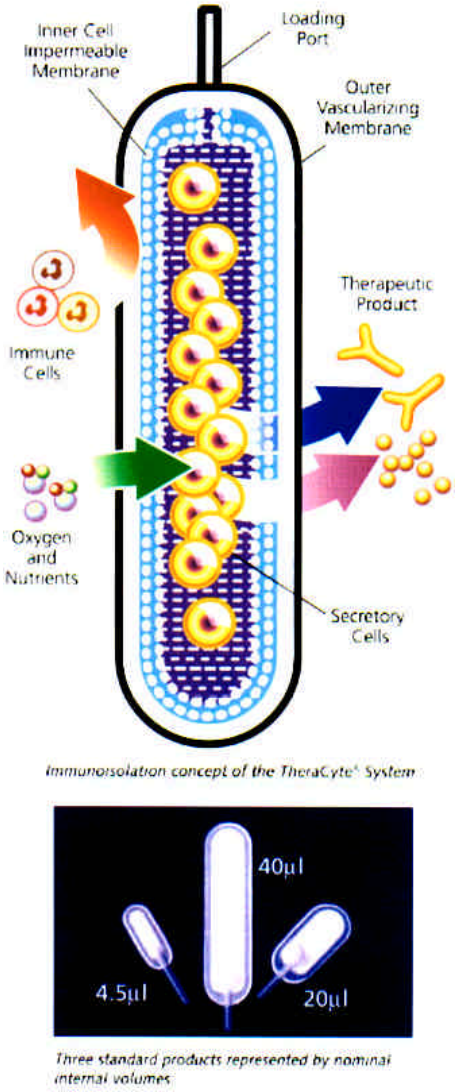

Figure 1. TheraCyte immunoisolation device. A photograph of a TheraCyte device is shown on the left. 2 x 10^6 DSH-cxl-7A5 packaging cells (SNV-derived, dog D17 helper cells) were loaded into the device. The device allows nutrients and oxygen but no immune cells to penetrate. The pore size of the device is large enough to release retroviral vectors.

not infectious in human or rodent cells (5,12,13). However, SNV-derived retroviral vectors efficiently infect human cells, when they are endowed with a targeting envelope, e.g., single chain antibodies (14-20), or when pseudotyped with the envelope of other viruses including vesicular stomatitis virus (VSV) or rabies viruses (Parveen *et al.*, submitted). We show that SNV vectors are released from TheraCyte devices *in vitro* for at least 4 months. Studies using SCID mice reveal that retroviral vectors are also released from such devices *in vivo.* These vectors were then transported via the blood system to their target organ (an induced subcutaneous tumor).

3. MATERIALS AND METHODS

3.1. Cells

D17 is a canine osteosarcoma cell line. DSH-cxl-7A5 is a SNV derived retroviral vector packaging cell line that was derived from D17 cells (17,21). DSH-cxl-7A5 and D17 cells were maintained in DulbeccoÕs modified Eagles medium (DMEM) containing 6% calf serum. Cells were passaged after treatment with 50 mM EDTA in PBS for 3 min. The cells were counted and plated 2 x 10^5 cells per 60 mm dish.

3.2. Radiation of helper cells

DSH-cxl-7A5 cells were cultivated in serum-free DMEM overnight. The next morning, the cells were radiated at 4000 rads for 34.6 min.

3.3. TheraCyte[a] preparation and loading

DSH-cxl-7A5 packaging cells were treated with 50 mM EDTA in PBS for 3 min. The cells were counted, centrifuged at 1000 x g for 5 min., and resuspended in

growth medium to make a final concentration of 2 x 10^6 cells per 30 µl. Twenty µl TheraCyte[a] system devices (obtained from TheraCyte, Inc., Irvine, CA, Figure 1) were sanitized with a series of washes through the fill port of the device: 100% EtOH; 70% EtOH soak for 2 hours; twice with 0.9% NaCl allowing 20 minutes in between each wash; and rinsed with phosphate buffered saline (PBS). The loading bags were then sealed with the portable cautery leaving PBS inside. All washing solutions were filtered through 0.2 µm filters. A sterile profiled collar was attached to a devise port via 0.40" ID section. To load cells into the device through 0.024" ID section of profiled collar, 5 µl of medium were used as a leader solution, following with 30 µl of cells and another 5 µl of medium. Injection was done with Hamilton syringe and needle. Adding sterile medical silicone type A adhesive into the collar sealed the device port.

3.4. *In vitro* infection via TheraCyte[a]

The immunoisolation device was cut out of the loading bag and placed on D17 cells. The device was transferred to fresh target cells weekly. After one week of incubation D17 cells were transferred to 100 mm plates and stained with X-gal for beta-galactosidase as described (22).

3.5. FACS analysis

Fluorescence-activated cell sorter (FACS) analysis was performed on DSH-cxl-7A5 cells and infected D17 cells as described (14). 10^6 Cells were incubated with 100 µl of R#8550 antibody (23), an anti-SNV-envelope mAB (diluted 1 : 20 in PBS) for 30 min. at 4ºC. Cells were then washed twice with 4 ml of PBS with 0.5 % sodium azide. Cells were resuspended in 100 µl of FITC conjugated (Fab specific) goat anti-mouse IgG (diluted 1 : 200 in PBS) for 30 min. at 4ºC. Cells were washed once and resuspended in 400 µl of PBS with 0.5% sodium azide. Samples were analyzed on a Coulter Epics Elite flow cytometer using Elite software.

3.6. Immunostaining

When the tumors reached a size of 1 cm in diameter, the animals were sacrificed by CO_2 inhalation. The tumor, liver, and kidneys were removed. Paraffin embedded tissues were cut into 5 µm sections, dewaxed, fixed in 2% paraformaldehyde for 10 - 15 min, and rinsed three time in 1 x PBS. The tissues were stained with a mouse anti beta-galactosidase antibody (IgM, purchased from Sigma Chemicals) followed by incubation with an anti-mouse secondary antibody (IgM specific, Jackson Research Labs) according to procedures recommended by the supplier of the antibodies.

3.7. Test for RCR

Retroviral vector producer cells were tested for RCR by infectivity assays. First, vector virus was harvested from the producer cells (step 1 cells) and 1 ml of supernatant medium was used to infect freh D17 target cells (step 2 cells) as described above. The infected D17 cells were kept in culture for up to three weeks. Next, tissue culture supernatant medium was harvested from the D17 cells and added to fresh D17 cells (step 3 cells). 48 hours later, all D17 cells were stained with x-gal for the detection of lacZ positive cells. If the producer cells were contaminated with RCR, the lacZ vector is further spread and transferred at each step. No lacZ positive D17 step 3 cells indicate the absence of a helper virus.

4. RESULTS

All experiments with TheraCyte immunoisolation devices were performed using a SNV-derived retroviral vector producer cell line, termed DSH-cxl-7A5 (17,24). This helper cell line has been very stable in tissue culture and produces retroviral vector particles, which display the SNV wild-type envelope and, in addition, a single chain antibody (scA, also termed scFv) fused to the transmembrane (TM) unit of the SNV-Env. The scA recognizes an unidentified antigen expressed on human T-cells and macrophages (17). The vector particles are capable of infecting dog D17 cells as well as human hematopoietic cells with titers above 10^6 colony forming units (cfu) per ml tissue culture supernatant medium *in vitro* (17,21). The vector particles transduced the bacterial beta-galactosidase (lacZ) gene (22). The use of the lacZ gene as a marker enabled to visually identify and quantify infected cells by X-gal staining or by immunostaining using an antibody specific for the bacterial beta-galactosidase.

The vector producer cells were tested for replication-competent retroviruses (RCR) to guarantee that target cells were infected by vector virus and that the marker gene was not further spread by RCR. No RCR was detected (data not shown). Of note, DSH packaging cells do not have overlapping regions of homology between the vector genome and the viral protein coding sequences (24) and have remained RCR-free for six years in tissue culture in our laboratory (25).

4.1. *In vitro* experimental system

To test, whether cells encapsulated into TheraCyte devices release retroviral vectors into the surrounding medium, a series of tissue culture experiments was performed. Radiated or non-radiated DSH-cxl-7A5 cells (2 x 10^6) were loaded into TheraCyte implantation devices. The filled devices were placed onto 60 mm tissue culture plates containing 1 x 10^5 dog D17 target cells (Figure 2A). The bags were kept on the plates and incubated at 37ºC in a CO2 incubator for one week, Figure 2A). After the target cells reached confluence, the cells were transferred to 100 mm plates and stained with X-gal to determine the number of infected cells. The device was transferred to fresh target cells up to 22 times (Figure 2A).

During the first week of incubation, non-radiated DSH-cxl-7A5 cells started to release retroviral vectors into the tissue culture medium (Figure 2B). The number of particles released from the bags increased over the next four weeks, peaked between weeks 5 and 10, and started to decrease thereafter. However, infectious particles were released until the end of the experiment which was terminated at week 22. (Figure 2B). Radiated DSH-cxl-7A5 cells released less infectious particles and displayed the highest infectivity at the beginning of the experiment,

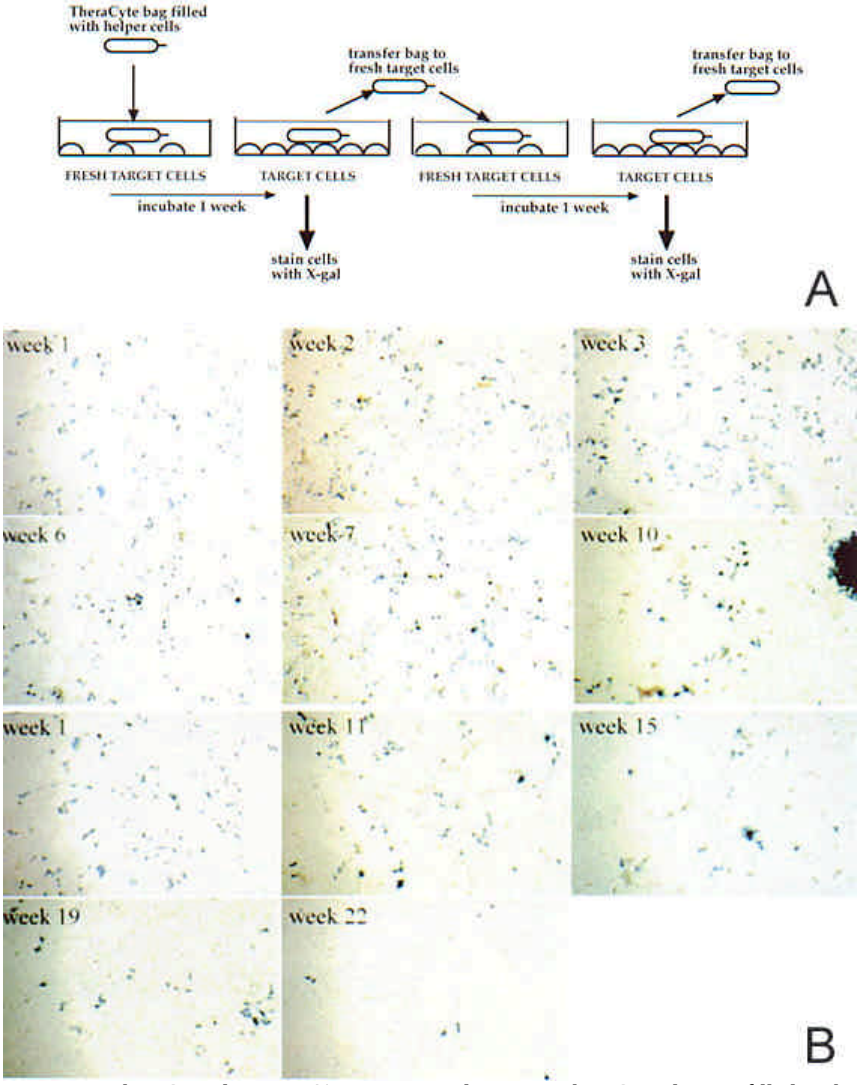

Figure 2. *In vitro* assay using TheraCyte devices. A) Experimental system: TheraCyte devices filled with DSH-cxl-7A5 helper cells were placed onto D17 cells in 100 mm tissue culture dishes. When the cells formed a confluent layer (approximately one week), the device was removed and placed onto a another plate containing fresh D17 target cells. The cells were stained with X-gal. B) Infected D17 cells stained with X-gal at different time periods. The experiment was terminated at week 22.

specifically during weeks 1 through 4. Viral production of radiated cells then started to decrease significantly, at approximately week 6 (data not shown).

To ensure that no leakage of cells from TheraCyte devices occurred during the study and only viral particles were passing through the membrane of the device, FACS analysis of target D17 cells was performed sixteen weeks after the beginning of the experiment and the initial loading of TheraCyte devices with helper cells. Infected D17 cells were marked with a primary antibody (termed R#8550) which is directed against the envelope protein of the spleen necroses virus (SNV) (23,26). Since only helper cells express the SNV envelope protein, and target D17 cells do not, the presence of contaminating helper cells would have been detected in the FACS analysis. DSH-cxl-7A5 cells served as positive control, D17 cells were used as a negative control. Three samples of D17 cells incubated with TheraCyte for one week showed properties of the negative control and no Envelope-positive cells were detected (data not shown). Thus, coinciding with earlier observations (27,28), no cells encapsulated in the TheraCyte device passed through the membrane into the environment.

4.2. *In vivo* studies

To test whether TheraCyte immunoisolation devices would also be useful as a tool to deliver genes *in vivo*, a SCID mouse model system was established. SCID mice lack a fully functional immune system (29-32) and develop solid tumors under their skin, when immortal tumor cells are injected subcutaneously. We induced subcutaneous tumors using dog D17 cells, which are easily infected by SNV-derived vectors (5).

Table 1. *In vivo* gene delivery into tumor cells from retroviral helper cells encapsulated in Theracyte immunoisolation devices

Site of tumor	Site Of Implanted TheraCyte device	Time Of Device implantation after tumor cell injection(weeks)	Time Period Of The device in the mouse (weeks)	No. of tumors containing lac Z expressing cells / No. of total mice
left	none	-	-	0 / 2
left	left	6 - 7 [1]	2	5 / 5
left	left	3.5	7	4 / 4
left	right	3.5	7	4 / 4
left + right	right	3.5	7	3 / 3 [2]

2×10^5 D17 cells were injected subcutaneously at the left, or left and right anterior dorsal surface of SCID mice. 3.5 to 7 weeks after the injection of the tumor cells, TheraCyte devices filled with retroviral packaging cells were implanted subcutaneously at either the same or other site of the animal at the posterior lateral surface. When the tumors reached a diameter of about 1 cm, the mice were sacrificed and the tumors analyzed for the presence of the lacZ gene by antibody or X-gal staining. [1] devices were implanted, when tumors became visible; [2] tumors on both sides of the mouse contained cells expressing the lacZ gene.

In our experiments, we injected 2×10^6 D17 cells subcutaneously into SCID mice. Before injection, an aliquot of the D17 cells was tested for RCR contamination and for expression of a lacZ gene. We found that they were not infected with any reticuloendotheliosis virus capable of spreasding the lacZ vector nor did they express the lacZ gene (data not shown). D17 cell tumors became visible after about 7 to 8 weeks and continued to grow rapidly. The tumors reached a size of about 1 cm in diameter after another two weeks. Thus, all experiments had to be terminated about 8 to 10 weeks after the injection of the tumor cells in order not to distress the animals. The use of D17 cells as target had the following advantages: SNV-derived vectors infect D17 cells with very high efficiency *in vitro* (5). Moreover, we have shown previously that D17 cells are also infected *in vivo,* when they were injected into the peritoneum of SCID mice followed by the injection of vector virus solutions (18).

In the first set of experiments, TheraCyte devices filled with DSH-cxl-7A5 helper cells were implanted under the skin of five SCID mice as soon as tumors became visible (about 7 weeks after the injection of D17 cells). In another set of experiments, TheraCyte devices were implanted into 11 out of 13 SCID mice 26 days after the injection of the D17 cells: in four mice, the devices were implanted at the same body side, at which the tumor started to grow. In another four mice the devices were implanted on the opposite side. Tumors were induced on both sides of three mice, (Table 1). The mice with tumors on both sides of the body had the device implanted on the right side. Two mice did not receive a device (negative control).

The mice were sacrificed when the tumors reached a diameter of about 1 cm. The tumors, as well as liver and kidneys were removed surgically and analyzed for the expression of the bacterial beta-galactosidase gene.

Tumors in control mice did not reveal significant areas of cells expressing the bacterial lacZ gene (Figure 3, a, a', and a"). In all other tumors, large (Figure 3b, c and d) or small (Figure 3e) areas were detected, in which cells expressed the bacterial beta-galactosidase gene indicating successful gene delivery. Figure 3e shows some infected cells, which apparently underwent one or two cell divisions (arrows). The location of the immunoisolation device relative to the tumor did not have a significant effect on gene delivery (Figure 3 c, c' and c"). These data suggest that the retroviral vectors were released from the device *in vivo* and transported to the tumor, where they infected D17 tumor cells.

To test for non-specific infections, the liver and kidneys of all mice where also tested for the presence of the lacZ gene. LacZ staining of aliquots of the liver did not reveal significant areas of cells expressing lacZ. DNA was isolated from aliquots of liver and kidneys and subjected to PCR with primers specific for the lacZ gene. No PCR products were detected (data not shown). These data indicate that no significant non-specific infection occurred in the liver and kidneys, which are the main blood-filtering organs.

Of note, the TheraCyte devices were also removed from the sacrificed mice and subjected to X-gal staining. We found that in all devices examined, all encapsulated helper cells stained deep blue (data not shown). These data indicate that the helper cells were still viable and expressed high levels of the beta-galactosidase (from the retroviral vector genome) after several weeks in test animals.

5. DISCUSSION

TheraCyte immunoisolation devices have been originally developed for the implantation of genetically engineered cells into a patient. Experiments have been performed with TheraCyte devices releasing various therapeutic proteins, such as insulin, factor VIII, or alpha-interferon. It has been shown that cells allografts encapsulated in such devices were protected from the immune system of the host and survived *in vivo* for up to one year (7-11).

Here we report for the first time the use of Theracyte immunoisolation devices for continuous gene delivery via retroviral vectors. Retroviral vector producing cells encapsulated in such devices released retroviral vectors *in vitro* and *in vivo.* Non-radiated cells released infectious vectors *in vitro* for up to four months, while radiated cells produced vectors for only less than two months. Thus, in all *in vivo* experiments, non-radiated retroviral vector producer cells were encapsulated in TheraCyte devices and implanted subcutaneously in SCID mice.

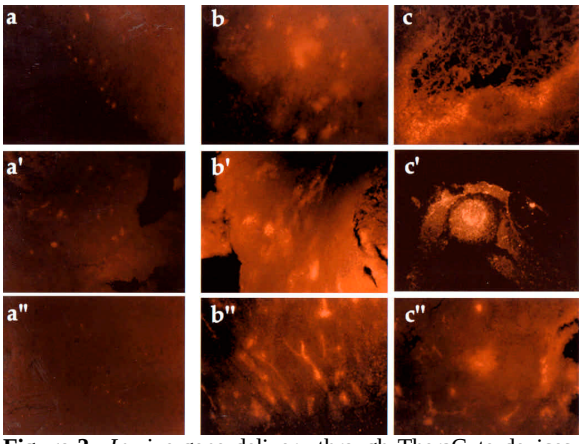

Figure 3. *In vivo* gene delivery through TheraCyte devices. TheraCyte filled with DSH-cxl-7A5 helper cells were subcutaneously implanted in SCID mice. The mice had been injected subcutaneously with 2 x 10^6 D17 cells, which form visible tumors about 6 to 8 weeks after the injection of the cell. When the tumors reached a size of approximately 1 cm in diameter, the mice were sacrificed, the tumors removed, sectioned, and stained with an anti beta-galactosidase antibody or with lacZ. a, a', a": tumors from untreated mice (no device implanted, negative control); b, b', b": tumors from three different mice, which carried the TheraCyte device at the same site as the tumor; c, c', c": tumors from three different mice which contained the TheraCyte device on the opposite site of the tumor; see also Table 1. d and e: Higher magnification of tumors showing individual cells expressing the beta-galactosidase; d: immunofluorescence using an anti-beta-galactosidase antibody, e: X-gal staining.

The *in vivo* model system described here mimics a cell-type-specific gene delivery system, because SNV-derived vectors efficiently infect dog D17 cells, but do not infect mouse cells. Thus, we expected such vectors to circulate in the blood stream until they find their actual target cells (in our experiments the dog osteosarcoma). Of note, previously, we have reported that SNV-derived vectors are not inactivated by SCID mouse serum (18). Our data indicate that vectors were indeed transported to the tumor target cells, as tumors showed many areas of cells expressing the bacterial beta-galactosidase gene.

Gene delivery into the tumor cells was independent from the location of the immunoisolation device in regard to the site of the tumor. However, at this point no conclusions can be drawn regarding the efficiency of the gene transfer. E.g., in areas in which numerous cells express the marker gene (e.g., Figure 3d), it is unclear at what point the cells were infected and how many cell divisions occurred from the time of the infection until the tumor was removed. Further, it is common knowledge that cells shed and uptake the bacterial beta-galactosidase protein. Thus, many cells in a lac-Z protein-positive area may have uptaken the protein from surrounding cells (bystander effect) and appear positive in the detection assay, although they actually do not express the protein from an internal gene. For example, an area shown in Figure 3e, which shows a few infected cells, may would look like an area shown in Figure 3d several weeks later.

The TheraCyte devices released retroviral vectors, which displayed the SNV wild-type envelope and a single chain antibody (scA) fused to the SNV transmembrane unit (TM) of Env on the viral surface. The scA was directed against a cell surface protein expressed in human T-cells (17). Although it was not necessary to use this particular vector producing cell line - the use of a standard packaging line expressing the SNV-wild-type envelope would have been sufficient - the use of this helper cell line had two advantages: first, DSH-cxl-7A5 cells have been stable and continuously produced relatively high titer retroviral vectors (10^6 cfu/ml); second, in future experiments, cells of specific organs may be targeted with vectors displaying a scA as a targeting envelope for cell-type specific gene delivery. Thus, our experiments already show, that such vectors displaying wild-type and targeting envelopes are released from the immunoisolation device.

Gene delivery through implantation devices may have many advantages in future human gene therapy: the device appears to constantly release specific amounts of gene transfer vectors. Thus, the effect of the gene transfer can be consistently monitored and the device can be removed as soon as adverse reactions in the patient are noticed. As low levels of vectors are released, immune responses may be insignificant. It is common knowledge that very low levels of antigen do not stimulate an immune response. Thus, low levels of vectors (which do not transduce viral protein coding sequences) may not induce an immune response at all. However, questions of immunogenicity still need to be addressed.

At this point, no conclusion can be made, for how long such implantation devices would need to be in an experimental animal (and in the future in a patient) to see an effect of a therapeutic gene. It has to be stressed that our experiments were performed with a second generation packaging line (10^6 cfu/ml). Currently, our group is developing new cell-type-specific packaging lines derived from mouse cells, which produce higher amounts of vector virus. Future experiments in immuno-competent mice with more efficient packaging lines, that transduce a therapeutic gene, will answer this question. However, here we show the first proof of principle that retroviral packaging cells encapsulated in TheraCyte immunoisolation devices released infectious retroviral vectors *in vivo.* Such particles found their target organ and inserted the marker gene into the genome of the target cell. Thus, this system may have great potential for future *in vivo* human gene therapy.

6. ACKNOWLEDGEMENT

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