Absence of Toxicity of Chronic Weekly Intravenous Gene Therapy with Pegylated Immunoliposomes

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Purpose. Plasmid DNA-based gene therapy involves episomal gene expression and must be given on a chronic basis. Therefore, the purpose of the present study was to examine for toxic side effects of the chronic weekly intravenous administration of plasmid DNA delivered with a nonviral gene transfer method using pegylated immunoliposomes (PIL).

Methods. A 7-kb expression plasmid encoding for rat tyrosine hydroxylase (TH) was encapsulated in PILs targeted with either the murine OX26 monoclonal antibody (MAb) to the rat transferrin receptor (TfR) or with the mouse IgG2a isotype control antibody. Rats were treated with weekly intravenous injections of 5 μg/rat/week of the TH expression plasmid DNA encapsulated in either the TfRMAb-targeted PIL or the mouse IgG2a-targeted PIL for a total period of 6 weeks. A third control group of rats was treated with saline.

Results. The animals treated with either saline, the TfRMAb-PIL, or the mouse IgG2a-PIL had no measurable differences with respect to body weights, 14 serum chemistries, or organ histology of brain, liver, spleen, kidney, heart, or lung. Immunocytochemistry showed no evidence of inflammation in brain. The delivery to brain of the TH expression plasmid was confirmed with Southern blotting.

Conclusions. The PIL nonviral gene transfer method causes no toxic side effects following chronic weekly intravenous administration in rats.

KEY WORDS: gene therapy; brain; liposomes; nonviral gene transfer; inflammation.

INTRODUCTION

An important issue with either viral or nonviral gene delivery systems is organ toxicity associated with the delivery vector (1). In the case of either adenovirus or Herpes simplex virus, the preexisting immunity to these viruses causes an inflammatory reaction (2,3). A single injection of either adenovirus or Herpes simplex virus into the brain causes inflammation leading to demyelination (4,5). More than 90% of the human population has a preexisting immunity to adeno-associated virus (6). Therefore, there is a need to establish nonviral gene transfer technology with minimal toxicity. The principal forms of nonviral gene transfer include the use of complexes of DNA/cationic polymers or the hydrodynamic injection method. Cationic polyplexes have a relatively narrow therapeutic index. A nitrogen/phosphate (N/P) ratio of 6–10 is necessary for gene expression in the lung following the intravenous injection of the cationic polymer/plasmid DNA complexes, whereas an N/P ratio >20 is lethal (7). The hydrodynamic method involves the rapid intravenous injection of a volume of saline greater than the existing blood volume of the animal. This results in transitory right heart failure and hepatic congestion causing a selective expression of plasmid DNA in the liver (8). This gene delivery method results in an increase in liver enzymes, and the mortality with this method can be as high as 40% depending on the salt solution injected (8).

An alternative form of nonviral gene transfer involves the use of pegylated immunoliposomes (PIL). In this formulation, the nonviral plasmid DNA is encapsulated in the interior of an 85-nm liposome that has a net anionic charge (9). The surface of the liposome is pegylated with several thousand strands of 2000-Da polyethyleneglycol (PEG). The pegylated liposome is then targeted to distant sites by conjugating a transporting ligand to the tips of 1–2% of the PEG strands. Peptidomimetic monoclonal antibodies (MAb) to either the transferrin receptor (TfR) or the insulin receptor (IR) have been used to target PILs carrying expression plasmids to distant sites following intravenous injection (9,10). The PILs do not aggregate in saline and have prolonged blood residence times (11). PILs have been administered intravenously to mice on a weekly basis for the treatment of brain cancer (12), and PILs have been given to rats for the treatment of experimental Parkinson's disease (13). PILs targeted with the TfRMAb have been used to deliver nonviral plasmid DNA to brain. Because of the expression of the TfR on both the blood–brain barrier (BBB) and the neuronal plasma membrane, the TfRMAb-targeted PIL delivers the plasmid DNA to brain as well as other organs rich in TfR, such as liver and spleen (9,14). However, to date, there has been no evaluation of the potential toxicity of repeat intravenous administration of PILs.

The purpose of the present study was to examine the potential toxicity of repeat weekly intravenous administration of PIL-encapsulated plasmid DNA that was targeted to tissues in the rat with either the murine OX26 MAb to the rat TfR, or PILs targeted with the corresponding mouse IgG2a isotype control antibody. The plasmid DNA used in the present studies is the clone 877 DNA, which encodes for rat tyrosine hydroxylase (TH), as described previously (13). The delivery of the TH expression plasmid to brain with the TfRMAb-targeted PIL results in a normalization of striatal TH enzyme activity in brain of rats lesioned with a neurotoxin (13). For the present toxicity study, the TH expression plasmid DNA was encapsulated in either the TfRMAb-PIL or the mIgG2a-targeted PIL and was injected weekly for 6 weeks at a dose of 5 μg/rat of PIL-encapsulated plasmid DNA. Body weights of the animals were determined during the treatment period, and at the end of the 6-week treatment, blood was obtained for measurement of 14 parameters of serum chemistry reflecting liver and renal function. Major organs were removed at the end of the treatment period for pathologic analysis. In addition, brain was examined in detail with immunocytochemistry using antibodies to multiple antigens that reflect underlying tissue inflammation. Immunocytochemistry of brain was performed with the mouse OX1 MAb to rat leukocytes, the mouse OX2 MAb to the rat class II multiple histocompatibility complex (MHC) antigen, the mouse OX18

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MAb to the rat class I MHC antigen, the mouse OX35 MAb to the rat lymphocyte CD4 receptor, and the mouse OX42 MAb to the rat macrophage. Finally, the present studies used Southern blotting to confirm distribution of the TH expression plasmid in brain following targeting with the TfRMAb-PIL.

METHODS

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and didodecyldimethylammonium bromide (DDAB) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL). Distearoylphosphatidylethanolamine (DSPE)-PEG^{2000} was obtained from Shearwater Polymers (Huntsville, AL), where PEG^{2000} is polyethylene glycol (PEG) of 2000 Daltons. DSPE-PEG^{2000}-maleimide was custom-synthesized by Shearwater Polymers. The LiposoFAST-Basic extruder and polycarbonate filters were from Avestin (Ottawa, Canada). [α-^{32}P]dCTP (3000 Ci/mmol) was from NEN Life Science Products Inc. (Boston, MA). *N*-Succinimidyl[2,3-^{3}H]propionate ([^{3}H]NSP, 101 Ci/mmol), Sepharose CL-4B, and Protein G-Sepharose CL-4B were from Amersham Pharmacia Biotech (Arlington Heights, IL). The nick translation system was purchased from Invitrogen Life Technologies (Carlsbad, CA). Exonuclease III was purchased from Promega (Madison, WI); 2-iminothiolane (Traut's reagent) was obtained from Pierce Chemical Co. (Rockford, IL). Mouse myeloma ascites containing IgG2a (κ) (mIgG2a), pancreatic DNase I with a specific activity of 2000 Kunitz units/mg, horse serum, mouse IgG1 isotype, mouse anti–glia fibrillary acidic protein (GFAP) monoclonal antibody (MAb), and glycerol-gelatin were from Sigma Chemical Co. (St. Louis, MO). The antitransferrin receptor monoclonal antibody (TfRMAb) used in these studies is the murine OX26 MAb to the rat TfR, which is a mouse IgG2a. TfRMAb and mIgG2a were individually purified by protein G affinity chromatography from hybridoma-generated ascites. The biotinylated horse anti-mouse IgG, Vectastain ABC kit, and 3-amino-9-ethylcarbazole (AEC) substrate kit were purchased from Vector Laboratories (Burlingame, CA). Mouse antirat class I multiple histocompatibility complex (MHC) monoclonal antibody (OX18), mouse antirat leukocyte CD45 (OX-1), mouse antirat lymphocyte CD4 (OX-35), mouse antirat class II MHC (OX-6), and mouse antirat macrophage CD11b (OX42) were purchased from Serotec (Raleigh, NC). Optimal cutting temperature (O.C.T.) compound (Tissue-Tek) was purchased from Sakura FineTek (Torrance, CA). Adult male Sprague-Dawley rats (weighing from 180–220 g) were obtained from Harlan Breeders (Indianapolis, IN).

Plasmid DNA Preparation and Radiolabeling

The tyrosine hydroxylase expression plasmid, driven by the SV40 promotor and designated clone 877, was constructed as described previously (13). Clone 877 plasmid DNA was purified from *E. coli* with the Plasmid Maxi Kit and desalted per the manufacturer's instructions (Qiagen, Chatsworth, CA). The size of the DNA was confirmed by 0.8% agarose gel electrophoresis. DNA was labeled with [^{32}P]dCTP using nick translation. The specific activity of [^{32}P]DNA was 15–20 μCi/μg. The trichloroacetic acid precipitability was 99%.

PEGylated Liposome Synthesis and Plasmid Encapsulation

POPC (18.8 μmol), DDAB (0.6 μmol), DSPE-PEG^{2000} (0.6 μmol), and DSPE-PEG^{2000}-maleimide (0.2 μmol) were dissolved in chloroform, followed by evaporation, as described previously (14). The lipids were dispersed in 0.2 ml of 0.05 M Tris-HCl buffer (pH 7.0) and vortexed for 1 min, followed by 2 min of bath sonication. Supercoiled DNA (200 μg) and 1 μCi of [^{32}P]DNA were added to the lipids. The dispersion was frozen in ethanol/dry ice for 5 min and thawed at room temperature for 25 min, and this freeze–thaw cycle was repeated five times to produce large vesicles with the DNA loosely entrapped inside. The large vesicles were converted into small (85-nm-diameter) liposomes by extrusion. The liposome dispersion was diluted to a lipid concentration of 40 mM, followed by extrusion five times each through two stacks each of 200- and 100-nm pore size polycarbonate membranes with a hand-held LiposoFAST-Basic extruder as described previously (11). The mean vesicle diameters were determined by quasielastic light scattering using a Microtrac Ultrafine Particle Analyzer (Leeds-Northrup, St. Petersburg, FL) as described previously (11).

The plasmid adsorbed to the exterior of the liposomes was removed by nuclease digestion, and 6 U of pancreatic endonuclease I and 33 U of exonuclease III were added in 5 mM $MgCl_2$ to the liposome/DNA mixture after extrusion. After incubation at 37°C for 1 h, the reaction was stopped by adding 20 mM EDTA. The nuclease digestion removed any exteriorized plasmid DNA, as demonstrated by agarose gel electrophoresis and ethidium bromide staining of aliquots taken before and after nuclease treatment, as described previously (11). The formulation before antibody conjugation is designated a pegylated liposome (PL), and the formulation after antibody conjugation is called a pegylated immunoliposome (PIL).

MAb Conjugation to the PEGylated Liposome Encapsulated with DNA

TfRMAb or mIgG2a was thiolated and individually conjugated to the maleimide moiety of the PEGylated liposome to produce the PIL with the desired receptor specificity. PIL conjugated with the OX26 MAb is designated TfRMAb-PIL, and PIL conjugated with the mIgG2a isotype control is designated mIgG2a-PIL. Either MAb or mIgG2a was radiolabeled with [^{3}H]NSP as described previously (15). [^{3}H]MAb had a specific activity of >0.11 μCi/μg and a TCA precipitability of >97%. The MAb (3.0 mg, 20 nmol) was thiolated with 40:1 molar excess of 2-iminothiolane (Traut's reagent), as described previously (15). The thiolated MAb, which contained a trace amount of ^{3}H-labeled MAb, was conjugated overnight to the PEGylated liposome with encapsulated plasmid DNA containing a trace amount of [^{32}P]DNA. The unconjugated MAb and the oligonucleotides produced by nuclease treatment were separated from the PIL by Sepharose CL-4B column chromatography as described previously (11). The number of MAb molecules conjugated per liposome was calculated from the total ^{3}H-labeled MAb radioactivity in the liposome pool and the specific activity of the labeled MAb,

assuming 100,000 lipid molecules per liposome, as described previously (15). The average number of MAb molecules conjugated per liposome was 57 ± 12 (mean ± SD, n = 4 syntheses). The final percentage entrapment of 200 μg of plasmid DNA in the liposome preparation was computed from the ^{32}P radioactivity and was 30 ± 2% (mean ± SD, n = 4 syntheses), or 60 μg of plasmid DNA.

Chronic Intravenous Administration of PIL-Encapsulated DNA

Adult male Sprague-Dawley rats weighing 200–220 g were anesthetized with ketamine (50 mg/kg) and xylazine (4 mg/kg) intraperitoneally. Animals were divided into three groups. PIL or saline was injected i.v. via femoral vein with a 30-g needle. The first group was injected with TfRMAb-PIL carrying clone 877 plasmid DNA at a dose of 5 μg per rat. The second group was injected with mIgG2a-PIL carrying clone 877 plasmid DNA at a dose of 5 μg per rat. The third group was injected with saline. The average intravenous injection volume for all treatments was 300 μL. These intravenous treatments were given once a week for 6 consecutive weeks. Each week before injection, the body weight for each rat was measured. At 3 days following the sixth injection, the rats were anesthetized, and blood was collected from the vena cava. Serum was stored at –20°C for serum chemistry measurements by autoanalyzer in the UCLA Medical Center Clinical Laboratory. The rats were then sacrificed, and organs were removed for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was performed by the avidin–biotin complex (ABC) immunoperoxidase method (Vector Laboratories). Brains were removed immediately after sacrifice, and cut into three sagittal slabs. One slab was immersion fixed in cold 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) for 24 h at 4°C. The second slab was fixed in cold 100% methanol for 24 h at –20°C. These slabs were cryoprotected in 20% sucrose in 0.1 M phosphate-buffered water, pH 7.4 (PBW), for 24 h at 4°C, and 30% sucrose in PBW for 24 h at 4°C. Brains were embedded in O.C.T. medium and frozen in dry ice powder. Frozen sections (20 μm) of rat brain were cut on a Mikron HM505E cryostat. Endogenous peroxidase was blocked with 0.3% H_2O_2 in 0.3% horse serum–phosphate-buffered saline (PBS) for 30 min. Nonspecific binding of proteins was blocked with 10% horse serum in PBS for 30 min. Sections were then incubated in primary antibodies overnight at 4°C. Based on either results provided by the manufacturer or on pilot studies, the fixative (methanol or paraformaldehyde) was chosen to preserve the target antigenicity in the fixed tissue. For methanol-fixed brain sections, OX1 (5 μg/ml), OX18 (5 μg/ml), or OX35 (5 μg/ml) was used as the primary antibody; for paraformaldehyde-fixed brain sections, OX6 (5 μg/ml), OX42 (5 μg/ml), or mouse anti-GFAP MAb (1 μg/ml) was used as the primary antibody. Identical concentrations of isotype control antibody were also used as primary antibody. Mouse IgG1 was used as the isotype control antibody for OX18, OX1, OX6, and GFAP, and mouse IgG2a was used as the isotype control antibody for OX35 and OX42. After incubation and wash in PBS, sections were incubated in biotinylated horse antimouse IgG for 30 min. After development in AEC, sections were mounted with glycerol-gelatin and examined by light microscopy.

Hematoxylin and Eosin Staining of Rat Organs

The third sagittal slab of brain, as well as liver, spleen, kidney, heart, and lung of each rat were removed and immersion fixed in 10% formalin in 0.1 M phosphate buffer for 48 h at 4°C. The fixed organs were embedded in paraffin and stained with hematoxylin and eosin and examined by light microscopy.

Southern Blotting

Plasmid DNA was isolated with the Hirt procedure (16) from rat brain 3 days following the intravenous injection of saline, clone 877 encapsulated in TfRMAb-PIL, or clone 877 encapsulated in mIgG2a-PIL. Rat brain (100 mg) was homogenized in 2 ml lysis buffer (20 mM Tris pH 7.5, 10 mM EDTA, 1% SDS) containing 15 μg/ml DNase-free RNase A using a Polytron PT-MR 3000 homogenizer (Littau, Switzerland) at full speed for 10 s Samples were incubated for 30 min at 37°C. Proteinase K was added to a final concentration of 1 mg/ml and samples incubated for 2 h at 37°C. Nuclear DNA was precipitated overnight at 4°C in the presence of 1.1 M NaCl. Samples were centrifuged at 14,000 rpm and 4°C for 30 min. Supernatants were extracted with phenol:chloroform, and plasmid DNA precipitated with ethanol in the presence of 10 μg glycogen carrier. Aliquots of precipitated material were resolved by gel electrophoresis in 0.8% agarose and blotted onto a GeneScreen Plus membrane (14). To prevent hybridization with the endogenous rat TH genomic DNA, membranes were hybridized with [^{32}P]pGL2 clone 734 (17), which contains the clone 877 backbone but without the rat TH cDNA insert (13). Southern blot hybridization was performed as previously reported (14). Autoradiograms were developed with Kodak X-Omat Blue film and intensifying screens for 24 h at –70°C. Films were scanned with a Umax PowerLook III scanner, and images imported and cropped in Adobe Photoshop 5.5 on a G4 Power Macintosh.

Statistical Analysis

Statistical differences at the $p < 0.05$ level among different groups were evaluated by analysis of variance with Bonferroni correction.

RESULTS

The animals were divided into three groups depending on whether the rat was treated with weekly intravenous injections of (a) saline, (b) the TH expression plasmid encapsulated in mouse IgG2a targeted PILs, or (c) the TH expression plasmid encapsulated in the OX26 TfRMAb-targeted PILs. The body weights of the animals in the three treatment groups are shown in Fig. 1, and there was no significant difference between the body weights of the animals in the three groups throughout the treatment period.

The results of the chemistry analysis of the serum taken 3 days after the sixth weekly injection are shown in Table I. There are no significant differences in any of the 14 different serum chemistries for any of the three treatment groups

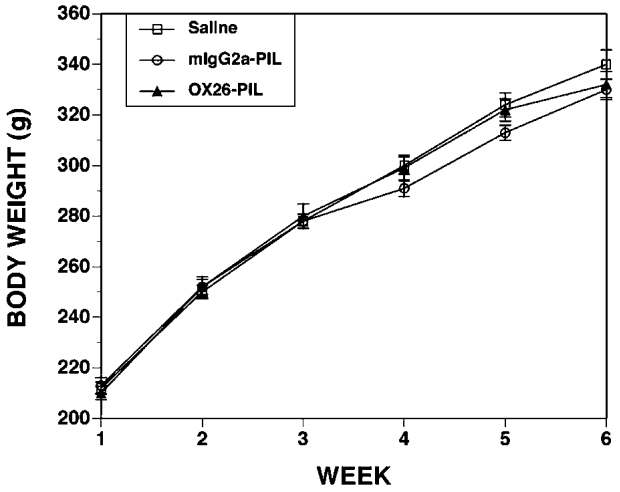

Fig. 1. The body weight of each rat in the three treatment groups was measured weekly during the course of treatment, and the mean ± SE (n = 6 rats per group) is shown. The OX26-PIL is the TfRMAb-targeted PIL, and the mIgG2a-PIL is the PIL targeted with the non-specific mouse IgG2a, which is the isotype control antibody for the OX26 MAb.

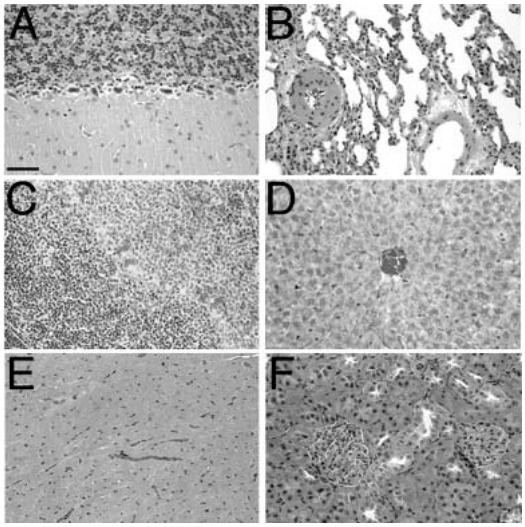

Fig. 2. Hematoxylin and eosin staining of formalin fixed cerebellum (A), lung (B), spleen (C), liver (D), heart (E), and kidney (F), removed 3 days after the sixth weekly intravenous injection of the TH expression plasmid encapsulated in the OX26 TfRMAb-targeted PIL. The magnification is the same in all panels, and the *magnification bar* in panel A is 37 μm.

(Table I). The organ histology in the rats sacrificed 3 days following the sixth weekly treatment is shown in Fig. 2 for brain cerebellum (Fig. 2A), lung (Fig. 2B), spleen (Fig. 2C), liver (Fig. 2D), heart (Fig. 2E), and kidney (Fig. 2F). The histology shown in Fig. 2 is for organs removed from rats treated with the TfRMAb-PIL. The organ histology of these animals was normal (Fig. 2) and was no different from the histology of organs taken from animals treated with either saline or the mIgG2a-PIL.

The results of the brain immunocytochemistry are given in Table II. No OX1-immunoreactive leukocytes were found in brain in any of the three treatment groups, although there was immunopositive choroidal endothelium staining in all groups (Table II). There was an occasional OX6-immunoreactive class II antigen-presenting cell in the meningial surface of all three treatment groups with no evidence of any parenchymal infiltration of class II immunopositive cells in any of the treatment groups (Table II). OX18 immunoreactivity indicative of the class I MHC antigen was found on capillary endothelium and in focal subependymal microglia, and the same staining pattern was found in all three treatment groups (Table II). OX35-immunoreactive CD4 lymphocytes were rare in brain with the same pattern in all three treatment groups (Table II). OX42-immunoreactive microglia were found diffusely in the parenchyma throughout the cerebrum and cerebellum, with an identical pattern in all treatment groups (Table II). Immunoreactive GFAP astrocytes were found diffusely throughout the cerebrum and cerebellum, with the same pattern in all three treatment groups (Table II). There was no immunoreactivity in brain with the nonspecific mouse IgG1 (mIgG1), which is the isotype control antibody for the OX1, OX18, OX6, and the GFAP antibodies (Table II). There was no immunocytochemical staining of brain using the nonspecific mouse IgG2a (mIgG2a), which is the isotype control antibody for the OX35 and OX42 antibodies (Table II).

Table I. Summary of Serum Chemistry

Assay	Units	Saline	mIgG2a-PIL	OX26-PIL
Sodium	mM	143 ± 1	142 ± 1	140 ± 1
Potassium	mM	4.4 ± 0.1	4.6 ± 0.1	4.6 ± 0.2
Chloride	mM	100 ± 1	100 ± 1	100 ± 1
CO_2	mM	29 ± 1	29 ± 1	27 ± 1
Glucose	mg/dl	168 ± 8	160 ± 6	163 ± 4
Creatinine	mg/dl	0.45 ± 0.03	0.40 ± 0.01	0.45 ± 0.02
Urea nitrogen	mg/dl	19 ± 1	21 ± 2	18 ± 1
Total protein	g/dl	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
Albumin	g/dl	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Bilirubin, total	mg/dl	0.35 ± 0.03	0.25 ± 0.05	0.33 ± 0.02
Alk phos	U/L	231 ± 27	212 ± 25	281 ± 11
AST (SGOT)	U/L	65 ± 5	59 ± 2	74 ± 6
ALT (SGPT)	U/L	54 ± 2	52 ± 3	59 ± 1
Calcium	mg/dl	9.4 ± 0.1	9.5 ± 0.2	9.2 ± 0.1

Data are mean ± SE (n = 6 rats in each of the three treatment groups).

The delivery of the TH expression plasmid to brain was verified with Southern blotting as shown in Fig. 3 (Lane 3). No signal was detected in the saline-treated animals (Lane 1, Fig. 3) because these animals were not administered DNA. No hybridization signal was detected in the brain of animals treated with the expression plasmid encapsulated in the mIgG2a-PIL (Lane 2, Fig. 3) because this isotype control antibody was unable to target the PIL across the BBB and into brain cells.

DISCUSSION

These studies show that the repeat weekly intravenous administration of the PIL-based gene therapy in rats for 6 weeks causes no measurable toxicity in brain or peripheral tissues. In addition, these studies show that the chronic weekly intravenous administration of a TH expression plasmid encapsulated in TfRMAb-PILs causes no inflammation within the target organ, the central nervous system (CNS).

Table II. Summary of Immunocytochemistry

Antibody	Parameter	Fixative	Findings
OX1	Leukocytes	Methanol	Positive choroidal endothelium Same pattern in all 3 treatment groups
OX6	Class II MHC	Para.[a]	Occasional positive cell in meninges Same pattern in all 3 treatment groups
OX18	Class I MHC	Methanol	Weak staining of capillary endothelium Focal subependymal microglia Same pattern in all 3 treatment groups
OX35	CD4-lymphocytes	Methanol	Minimal staining of brain and equal to mouse IgG2a control Same pattern in all 3 treatment groups
OX42	Macrophages	Para.	Diffuse immunoreactive microglia throughout cerebrum and cerebellum Same pattern in all 3 treatment groups
GFAP	Astrocytes	Para.	Diffuse immunoreactive astrocytes throughout cerebrum and cerebellum Same pattern in all 3 treatment groups
Mouse IgG1	Control	Methanol	No reaction (control for OX1, OX18)
		Para.	No reaction (control for OX6, GFAP)
Mouse IgG2a	Control	Methanol, Para.	No reaction (control for OX35) No reaction (control for OX42)

Para., paraformaldehyde.

There is no general systemic toxicity following weekly PIL administration based on the observation that the body weights of the animals increase over the 6 week treatment period at the same rate for all 3 treatment groups (Fig. 1). The PIL targets the plasmid DNA to TfR-rich organs such as the brain, liver, or spleen (9,14). The serum chemistries show normal hepatic function tests and an absence of an increase in serum bilirubin or liver enzymes (Table I). In contrast, the intravenous injection of adenovirus in primates results in increased liver enzymes secondary to hepatic inflammation caused by reaction to the immunogenic viral vector (18). There is no change in serum electrolytes or other renal function tests (Table I). The normal serum chemistry is paralleled by the normal organ histology for liver, speen, kidney, heart, lung, and brain (Fig. 2). The serum chemistry and organ histology were examined at 3 days following the sixth weekly injection because prior work has shown the TH gene expression following PIL injection is maximal at this time (13).

The intracerebral injection of viral vectors, such as adenovirus or Herpes simplex virus, leads to inflammation of the brain, as evidenced by perivascular cuffing with lymphocytes and increased immunoreactivity for class I and class I MHC antigens in brain (2–5). Therefore, the present studies performed a detailed immunocytochemical analysis of brain to examine for any evidence of inflammation in the brain following the chronic delivery to brain of a TH expression plasmid encapsulated in a TfRMAb-targeted PIL. The brain immunocytochemistry of the animals treated weekly with the TfRMAb-targeted PIL was compared to that of control groups of rats treated weekly with either saline or with mIgG2a-targeted PILs. There is an identical pattern of immunoreactivity in rat brain using OX1, OX6, OX18, OX35, OX42, and GFAP antibodies in immunocytochemical analysis of brain for all three treatment groups (Table II). In these studies, the brain was fixed with either methanol or paraformaldehyde, depending on which was the optimal fixative for each antigen (Methods), to preserve antigen recognition in the fixed brain. Chronic delivery of TfRMAb-targeted PILs to brain caused (a) no elevations in parenchymal class I (OX18) or II MHC (OX6), (b) no elevations in parenchymal infiltration by lymphocytes (OX35), leukocytes (OX1), or macrophages (OX42), and (c) no elevations in parenchymal gliosis (GFAP).

In summary, these studies demonstrate that nonviral expression plasmids can be delivered to organs with the PIL gene transfer method without toxic side effects when administered at a PIL-encapsulated plasmid DNA dose of 25 μg/kg. The chronic weekly intravenous administration of this dose of plasmid DNA encoding for rat TH and encapsulated in TfRMAb-targeted PILs causes no evidence of toxicity in ei-

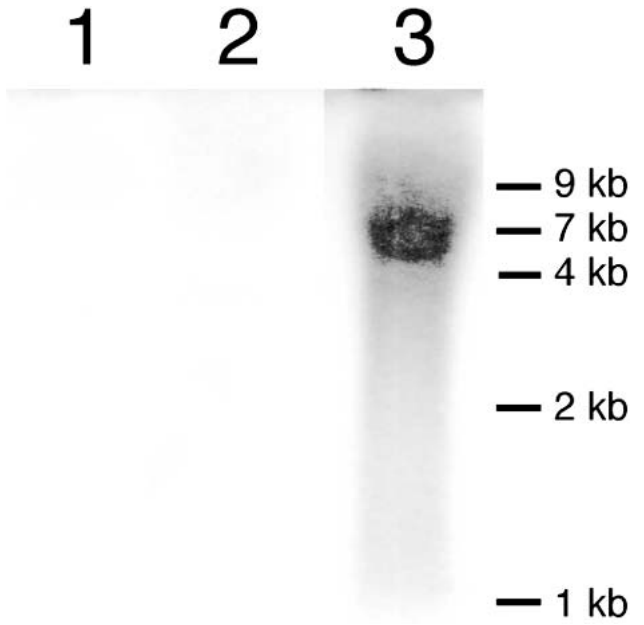

Fig. 3. Southern blot analysis of rat brain with [^{32}P]pGL2 clone 734. Lane 1, brain isolated from a saline-treated rat; lane 2, brain isolated from a rat injected with the TH expression plasmid encapsulated in the mIgG2a-targeted PIL; and lane 3, brain isolated from a rat injected with the TH expression plasmid encapsulated in the TfRMAb-targeted PIL. The migration of the DNA standards is indicated in the figure. The expected ~7-kb plasmid DNA corresponding to the size of the TH expression plasmid is seen only in the brain of the rats treated with the TfRMab-targeted PIL (lane 3).

ther the target organ, brain, or in peripheral tissues, such as liver, spleen, kidney, heart, or lung. It is possible that toxic effects may be observed at higher doses, but the dose used in this study in rats was chosen because this dose is therapeutic in rats (13). Moreover, a much higher dose, 200 μg/kg, of PIL-encapsulated plasmid DNA has been administered weekly to mice without evidence of toxicity (12). The need for high dosing of plasmid DNA with the PIL gene-targeting method is unlikely because a dose of 12 μg/kg of PIL-encapsulated plasmid DNA in adult primates results in levels of gene expression that are 50-fold higher than in rodents (10). The finding of a lack of toxicity following chronic PIL administration is important because the PIL gene transfer method delivers to the target organ a nonviral plasmid that directs gene expression for only a finite duration (12,13). The expression plasmid is transcribed episomally and is not permanently or randomly integrated into the host genome. Therefore, in order to sustain a pharmacologic effect with plasmid DNA-based gene therapy, it is necessary to administer the gene medicine on a chronic basis. The frequency of the administration is a function of the persistence of plasmid expression in the target organ. Long-term gene expression is possible with viral vectors that permanently integrate into the host genome, but this approach is associated with the risk of insertional mutagenesis (1). An alternative approach to gene therapy is chronic treatment with episomal-based plasmid DNA that is formulated in such a way that the DNA is able to target distant sites following intravenous administration. Prior work has shown that the PIL gene-targeting method enables widespread expression of the exogenous gene in distant sites such as brain in mice, rats, and rhesus monkeys (9–11). The present studies show that PIL-based gene therapy can be given chronically without the development of tissue toxicity in either the target organ, brain, or in peripheral tissues.

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