Receptor-Mediated Gene Targeting to Tissues *In Vivo* Following Intravenous Administration of Pegylated Immunoliposomes

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Purpose. Gene therapy has been limited by the immunogenicity of viral vectors, by the inefficiency of cationic liposomes, and by the rapid degradation *in vivo* following the injection of naked DNA. The present work describes a new approach that enables the non-invasive, non-viral gene therapy of the brain and peripheral organs following an intravenous injection.

Methods. The plasmid DNA encoding β-galactosidase is packaged in the interior of neutral liposomes, which are stabilized for *in vivo* use by surface conjugation with polyethyleglycol (PEG). The tips of about 1% of the PEG strands are attached to a targeting monoclonal antibody (MAb), which acts as a "molecular Trojan Horse" to ferry the liposome carrying the gene across the biological barriers of the brain and other organs. The MAb targets the transferrin receptor, which is enriched at both the blood-brain barrier (BBB), and in peripheral tissues, such as liver and spleen.

Results. Expression of the exogenous gene in brain, liver, and spleen was demonstrated with β-galactosidase histochemistry, which showed persistence of gene expression for at least 6 days after a single intravenous injection of the pegylated immunoliposomes. The persistence of the transgene was confirmed by Southern blot analysis.

Conclusions. Widespread expression of an exogenous gene in brain and peripheral tissues is induced with a single intravenous administration of plasmid DNA packaged in the interior of pegylated immunoliposomes. The liposomes are formulated to target specific receptor systems that enable receptor-mediated endocytosis of the complex into cells *in vivo*. This approach allows for non-invasive, non-viral gene therapy of the brain.

KEY WORDS: gene delivery; transferrin receptor; blood-brain barrier; β-galactosidase.

INTRODUCTION

Current approaches to gene delivery *in vivo* use either viral vectors (1,2) or cationic liposome/DNA formulations (3,4). In a third approach, plasmid DNA is attached to a receptor ligand to trigger receptor-mediated endocytosis into the target cell. In the receptor-mediated approach, the plasmid DNA is complexed to the targeting ligand with a polylysine bridge and this method has worked in cell culture and in some *in vivo* applications (5–7). However, the DNA is attached to the conjugate of polylysine and the targeting ligand only by electrostatic interactions and these DNA/polycation interactions may be disrupted in the circulation immediately after injection into the bloodstream. In addition, the plasmid DNA is unprotected and is subject to degradation by endonucleases, which are not found in tissue culture medium, but which are ubiquitous *in vivo* (8). In contrast, viral vector systems house the exogenous gene in the interior of the viral capsid, which protects the DNA from endonucleases *in vivo*.

In the present approach to targeting an exogenous gene to tissues *in vivo,* a molecular formulation was designed to keep the advantages of both viral vectors and receptor-mediated gene targeting. As shown in Fig. 1A, the supercoiled non-viral plasmid DNA is packaged in the *interior* of a liposome (9). This formulation makes the plasmid DNA resistant to endonucleases *in vivo* (10), and should be contrasted with conventional cationic liposome/DNA mixtures, where the DNA is exteriorized. Liposomes are immediately coated on the surface by serum proteins and this triggers rapid uptake by cells lining the reticuloendothelial system (RES) in the body *in vivo*. This rapid uptake by the RES can be reduced by conjugation of polymeric strands to the surface of the liposome (11). In the present formulation, the surface of a 75 nm liposome is conjugated with approximately 3000 strands of polyethyleneglycol (PEG) of 2000 Dalton molecular weight, designated PEG^{2000} (Fig. 1A). Pegylated liposomes have prolonged circulation times *in vivo* compared to conventional liposomes (11). However, pegylated liposomes are relatively inert and are not targeted specifically to any tissue. Tissue targeting can be achieved by the conjugation of the tips of the PEG^{2000} strands with a targeting ligand (12). The targeting ligand may be either an endogenous peptide or a peptidomimetic monoclonal antibody (MAb) that undergoes receptor-mediated endocytosis into cells. In the present formulation, the murine OX26 MAb to the rat transferrin receptor (TfR) is used to target the pegylated immunoliposome carrying the plasmid DNA to tissues *in vivo* that express the TfR (9).

Gene targeting to brain and peripheral organs with pegylated immunoliposomes has been reported using luciferase as the exogenous gene (9). However, prior work with luciferase demonstrated a low level of persistence of the transgene *in vivo* with a peak of gene expression at 48 h, followed by return to baseline activity at 72 h after intravenous injection of pegylated immunoliposomes (9). The luciferase expression vector used in prior work contained a heterologous intron in the 3'-untranslated region (UTR), and such inserts can decrease gene expression *in vivo* (3). Therefore, the present studies examine β-galactosidase gene expression in brain and peripheral tissues following the administration of pegylated immunoliposomes carrying a β-galactosidase gene packaged in an expression plasmid with a short 3'-UTR lacking a heterologous intron (pSV-β-galactosidase, Promega). Gene expression is measured at 2, 4, and 6 days after a single intravenous administration using β-galactosidase histochemistry. The persistence of the transgene *in vivo* was confirmed with Southern blotting.

METHODS

Synthesis of Pegylated Immunoliposome with Encapsulated Gene

The liposome was formulated as described previously (9) with 19.2 μmol of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-

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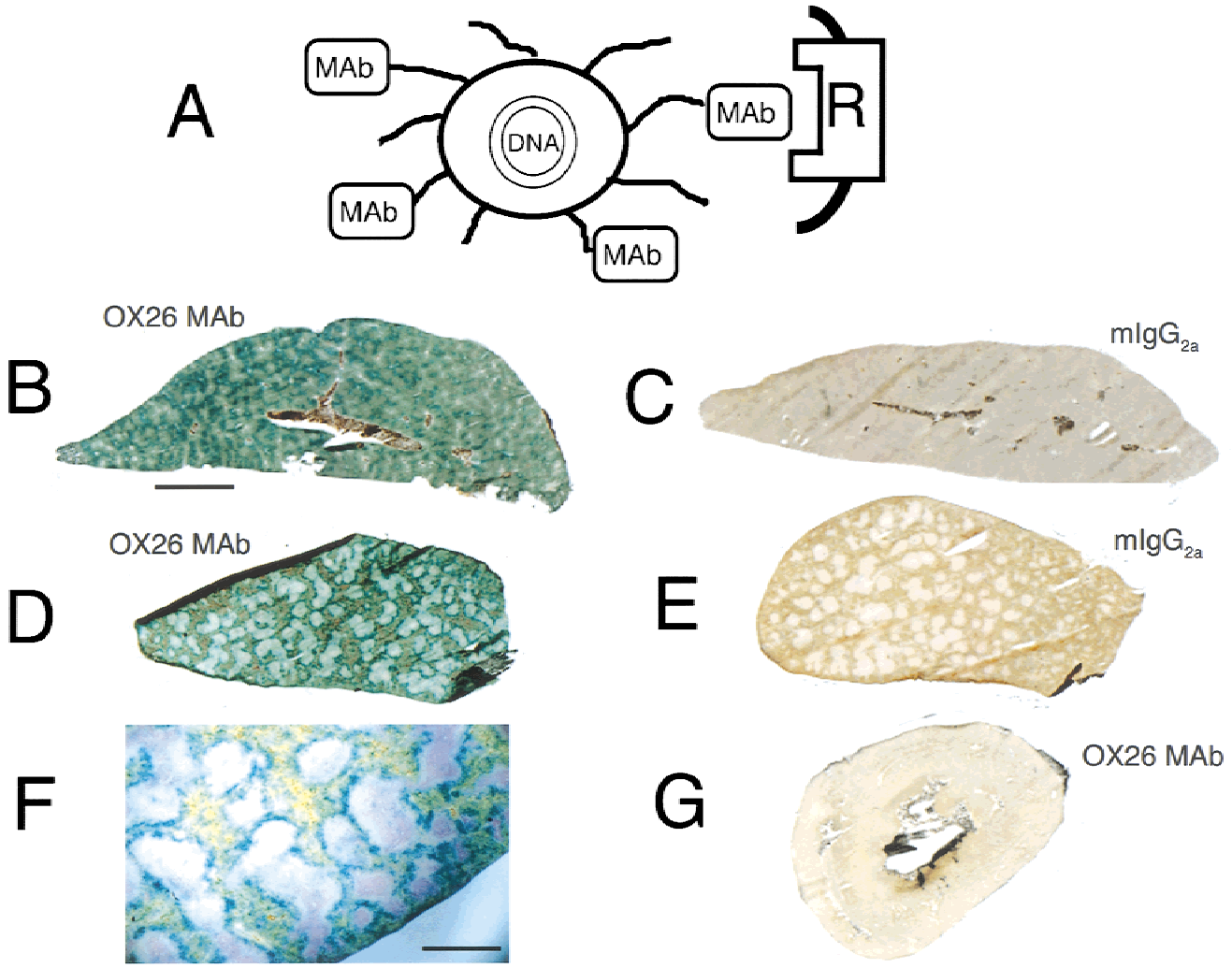

Fig. 1. (A) A double-stranded super-coiled plasmid DNA is packaged in the interior of an 75 nm liposome and the surface of the liposome is conjugated with approximately 3000 strands of polyethyleneglycol (PEG) of 2000 Dalton molecular weight (PEG^{2000}). About 1% of the PEG^{2000} strands are conjugated with a targeting ligand (9). In the present formulation, a monoclonal antibody (MAb) to the transferrin receptor (TfR) is used. (B) Liver histochemistry at 2 days after intravenous injection of OX26 pegylated immunoliposomes carrying the pSV-β-galactosidase plasmid. (C) Liver histochemistry at 2 days after intravenous injection of pegylated immunoliposomes carrying the pSV-β-galactosidase plasmid and conjugated with mouse (m) IgG_{2a}. (D) Spleen histochemistry at 2 days after intravenous injection of OX26 pegylated immunoliposomes carrying the pSV-β-galactosidase plasmid. (E) Spleen histochemistry at 2 days after intravenous injection of mouse IgG_{2a} pegylated immunoliposomes carrying the pSV-β-galactosidase plasmid. (F) High magnification of spleen β-galactosidase histochemistry at 2 days after intravenous injection of OX26 pegylated immunoliposomes encapsulated with the pSV-β-galactosidase plasmid. (G) Heart histochemistry at 2 days after intravenous injection of OX26 pegylated immunoliposomes carrying the pSV-β-galactosidase plasmid. The magnification in panels B, C, D, E, and G is the same and the magnification bar in panel B is 3.3 mm. The magnification bar in panel F is 0.94 mm. Only the specimen in panel F was counterstained whereas all other specimens were not counterstained.

phosphocholine (POPC), 0.2 μmol of didodecyldimethylammonium bromide (DDAB), and 0.6 μmol of distearoylphosphatidylethanolamine (DSPE)-PEG^{2000}. The DSPE-PEG^{2000} was comprised of DSPE-PEG^{2000} and DSPE-PEG^{2000}-maleimide in a ratio of 95:5 (9). The surface charge of the empty liposome was not measured directly, but would be expected to be negative, given the 3-fold molar excess of the anionic lipid, DSPE-PEG^{2000}, relative to the cationic lipid, DDAB. The bifunctional PEG derivative, DSPE-PEG^{2000}-maleimide, enabled conjugation of the thiolated MAb to the tip of the PEG strands (9). Following initial mixing of the lipids in chloroform, evaporation of chloroform, resuspension in 0.05 M Tris (pH = 8.0), and sonication, 100 μg of super-coiled plasmid and 1 μCi of nick-translated plasmid labeled with ^{32}P, were added to the lipids. The liposomes were formed by series of freeze/thaw cycles and extrusion through 400, 200, and 100 nm pore-sized polycarbonate membranes as described previously (9). Plasmid DNA bound to the exterior of the pegylated liposome was quantitatively removed by treatment with exonuclease III/DNase I (9). The thiolated OX26 MAb or thiolated mouse IgG_{2a}, which contained a trace amount of [^{125}I] labeled IgG, was conjugated to the pegylated liposome overnight and unconjugated MAb was separated by Sepharose CL4B gel filtration chromatography as described previously (9). The final percent entrapment of the DNA within the interior of the liposome was determined

by measuring the total ^{32}P radioactivity in the liposome peak, and this entrapment was typically 20% of the starting plasmid DNA, which included loss of material in the dead volume of the extruder. The number of MAb molecules conjugated to each liposome was computed as described previously (9,12), and ranged from 35-50 MAb molecules per individual liposome. The diameter of the pegylated immunoliposomes was measured by quasielastic light scattering as described previously (9,12) and ranged from 45 nm (10%) to 114 nm (90%) with a mean diameter of 73 nm in a unimodal pattern.

In Vivo Administration and Organ Histochemistry

The pegylated immunoliposomes carrying the DNA were injected intravenously into anesthetized adult male Sprague Dawley rats (250 g) at a dose of 10 μg of plasmid DNA per rat. The pharmacokinetics and organ uptake of these structures has been reported previously (9). Animals were sacrificed at 2, 4, or 6 days after intravenous injection and brain, heart, kidney, spleen, and liver were removed, and processed for β-galactosidase histochemistry with 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal, Promega) as described previously (9). The presence of endogenous β-galactosidase-like enzyme activity was examined in control tissues obtained from un-injected rats. These studies showed that brain, liver, spleen, and heart contain no endogenous β-galactosidase-like enzyme activity. However, there was abundant β-galactosidase-like activity in control rat kidney, and no further studies with kidney were performed. Slides were scanned with a 1200 dpi UMAX flatbed scanner with transilluminator and cropped with Adobe Photoshop 5.5 with a G4 Power Macintosh. Sections of organs from animals treated with either OX26 or mouse IgG_{2a} pegylated immunoliposomes were scanned simultaneously.

Southern Blotting

Genomic DNA was isolated from rat liver using the Genomic Isolation Kit (Qiagen) and the yield averaged 1.32 ± 0.17 μg DNA/mg tissue (mean ± SE, n = 4). The $OD_{260/280}$ was 1.7 for all samples. Ten μg DNA aliquots were digested with 15 U EcoRI for 1 h at 37°C, resolved by gel electrophoresis in 0.8 % agarose, blotted to a GeneScreen Plus membrane and hybridized with the ^{32}P-pSV-β-galactosidase DNA. Autoradiograms were performed with Kodak X-Omat Blue film for 3 h at 22°C. Ethidium bromide staining of the agarose gel before blotting showed the expected smear of partially digested genomic DNA ranging from 23-0.6 kb in all samples.

RESULTS

The 6.8 kb pSV-β-galactosidase plasmid (Promega) was incorporated in the interior of pegylated immunoliposomes that were conjugated with one of two different targeting ligands: either the OX26 MAb to the rat TfR or a mouse IgG_{2a} isotype control antibody (Fig. 1A). Organs were removed at 2, 4, or 6 days after administration for β-galactosidase histochemistry or Southern blot analysis. After administration of the mouse IgG_{2a} pegylated immunoliposomes, there was no measurable β-galactosidase gene expression in liver (Fig. 1C), spleen (Fig. 1E), brain (Fig. 3C), or heart (data not shown). No plasmid was detectable by Southern blot in liver at 48 h after intravenous administration of the mouse IgG_{2a} pegylated immunoliposomes (Fig. 2, lane 1). However, if the β-galactosidase expression plasmid was incorporated in the interior of pegylated immunoliposomes that were formulated with the OX26 MAb, there was abundant β-galactosidase gene expression in liver (Fig. 1B) or spleen (Fig. 1D) at 48 h after a single intravenous injection of the formulation. The exogenous plasmid DNA was also detected by Southern blot in liver at 2, 4, and 6 days after intravenous injection (Fig. 2). In contrast, there was no measurable β-galactosidase gene expression in heart (Fig. 1G) because of the insignificant expression of the TfR on endothelium comprising the continuous capillaries of the myocardium. The β-galactosidase histochemistry of liver (Fig. 1B) shows the gene is nearly evenly expressed throughout the hepatic lobule, which parallels the distribution of transferrin receptor in the normal rat liver (13). The high magnification of β-galactosidase histochemistry in spleen shows a prominent expression of the gene in the red pulp of spleen with less gene expression in splenic white pulp (Fig. 1F).

The pegylated immunoliposome formulation depicted in Fig. 1A enables widespread expression of an exogenous gene in the brain as shown in Figure 3. The expression of the β-galactosidase transgene in brain persists for at least 6 days after a single intravenous injection of the pegylated immunoliposome, as shown in Fig. 3B. There is also persistent expression of the β-galactosidase transgene in liver or spleen at 6 days after a single intravenous injection, and the histochemical pattern for liver or spleen at 6 days after injection was comparable to that shown for these organs at 2 days after injection (Fig. 1B,D). Measurements were also made at 4 days after intravenous injection and these histochemistry studies

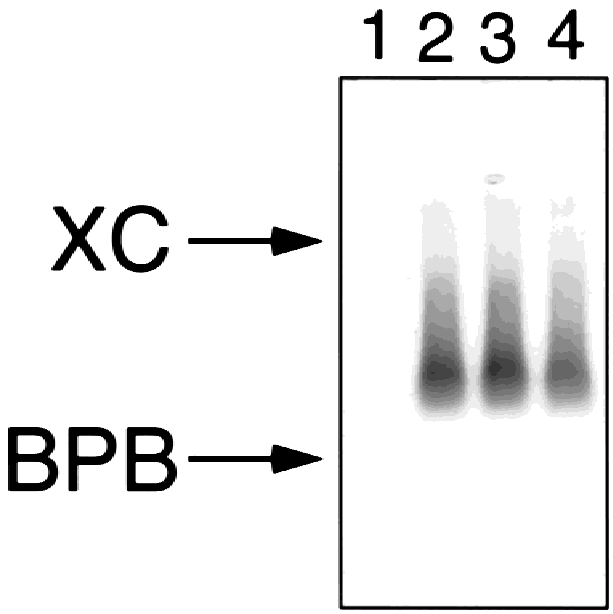

Fig. 2. Southern blot of rat liver with ^{32}P-pSV-β-gal DNA. Lane 1: liver at 2 days after intravenous administration of mouse IgG_{2a} pegylated immunoliposomes carrying the pSV-β-galactosidase plasmid. Lanes 2, 3, and 4: liver at 2, 4, and 6 days, respectively, after intravenous injection of OX26 MAb pegylated immunoliposomes carrying the pSV-β-galactosidase plasmid. The migration of the xylene cyanol (XC) and bromophenol blue (BPB) tracking dyes are indicated; the XC and the BPB dyes migrate near the 4.4 and 0.6 kb DNA sizing standards, respectively.

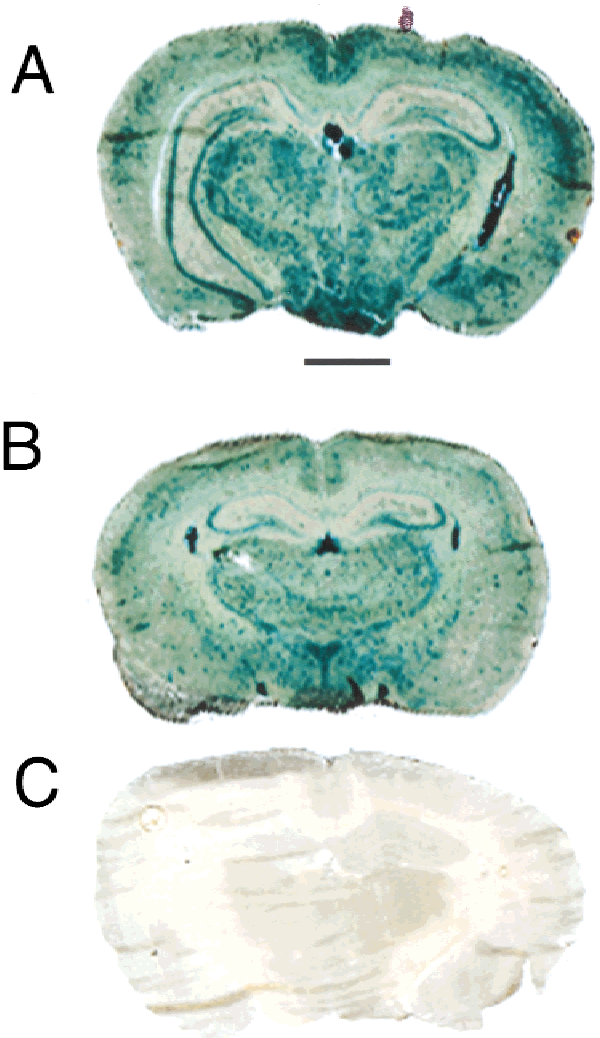

Fig. 3. Brain histochemistry showing β-galactosidase gene expression at either 2 days (panels A and C) or 6 days (panel B) after intravenous injection of pegylated immunoliposomes carrying the β-galactosidase plasmid and conjugated with either the OX26 MAb (panels A and B) or mouse IgG_{2a} isotype control (panel C). None of the specimens were counterstained. The magnification bar in panel A is 3.1 mm.

showed the level of β-galactosidase gene expression in liver, spleen, or brain was intermediate between the 2 day or 6 day activity (data not shown).

DISCUSSION

These studies demonstrate the widespread expression of an exogenous gene following intravenous administration of pegylated immunoliposomes carrying a non-viral plasmid gene packaged in the *interior* of the liposome (Fig. 1A). The exogenous gene is expressed deep within the parenchyma of organs targeted by the anti-Tfr MAb, such as liver, spleen, and brain (Fig. 1 and 3). Gene expression persists for at least 6 days after a single intravenous injection of the pegylated immunoliposome (Fig. 3). The β-galactosidase histochemistry is confirmed by Southern blot analysis (Fig. 2), which indicates the persistence of β-galactosidase enzyme activity in the tissues arises from the persistence of the trans-gene *in vivo*.

The packaging of the exogenous gene in the interior of the pegylated immunoliposome protects the gene against the ubiquitous endonucleases that exist *in vivo* (8), which is analogous to packaging an exogenous gene in the interior of a virus. However, unlike viruses, which express immunogenic coat proteins, the immunogenicity of the targeting MAb can be eliminated by "humanization" and genetic engineering of the MAb (14). The pegylated immunoliposome formulation shown in Fig. 1A also contrasts with cationic liposomes, which form a complex with DNA that is exposed to plasma. These structures rapidly aggregate *in vivo* (15) leading to deposition at the pulmonary microvasculature following intravenous administration (16). In the formulation used in the present studies, the surface of the liposome is pegylated, which stabilizes the structure in the blood, minimizes rapid uptake by the reticuloendothelial system, and enables a prolonged plasma residence time (9).

The specificity of the pegylated immunoliposome is a function of the MAb attached to the tips of the PEG strands (Fig. 1A). The gene expression in liver and spleen is due to the high expression of TfR on parenchymal cells in these tissues, and is not a non-specific result of clearance of particulate liposomes by the reticuloendothelial system. This is demonstrated by the observation that substitution of the anti-TfR MAb with the mouse IgG_{2a} isotype control leads to a loss of β-galactosidase gene expression in liver and spleen (Fig. 1C,E). Similarly, prior work with a luciferase reporter plasmid showed the absence of gene expression with mouse IgG_{2a} pegylated immunoliposomes in multiple organs in the rat (9). The anti-TfR MAb targets liver, spleen, and brain, but not heart (Fig. 1). Targeting exogenous genes to organs such as liver or spleen is a "one-barrier" gene delivery problem. Owing to the high porosity of the sinusoids perfusing either liver or the red pulp of spleen, 75 nm structures such as pegylated immunoliposomes freely gain access to the extravascular space in these tissues (17). Once in the extravascular compartment, the liposomes carrying the targeting ligand may then bind to the TfR on the parenchymal cells of liver or spleen.

Gene targeting to organs such as brain, which have continuous capillaries of restricted permeability, is a "two-barrier" gene targeting problem. The pegylated immunoliposome must first traverse the microvascular endothelial barrier, and then must traverse the plasma membrane of the target cell within the organ (9). The expression of the TfR on both barriers in brain enables gene expression in neurons (9). Prior work using high magnification microscopy demonstrated widespread expression of the β-galactosidase gene in neurons in the brain (9). The pegylated immunoliposome first undergoes receptor-mediated transcytosis across the BBB *in vivo,* owing to expression of the TfR on the BBB (18), and then undergoes receptor-mediated endocytosis into neurons within brain, owing to expression of the TfR on the neuronal plasma membrane (19). The BBB TfR is a bidirectional transcytosis system (20), and mediates the movement of the pegylated immunoliposomes through the endothelial barrier into brain interstitial space. The phospholipids forming the liposome fuse with the intracellular endosomal membrane subsequent to endocytosis and release the plasmid DNA into the cytosol of the target cell (21). Once inside the cytosol, the plasmid may diffuse to the nucleus for transcription. These studies show a persistence of gene expression in brain and

other organs *in vivo* for at least 6 days following a single intravenous injection of the plasmid.

In summary, persistent expression of an exogenous gene in tissues *in vivo* may be achieved with a non-invasive, non-viral approach to gene targeting *in vivo* (Fig. 1A). The targeting specificity of the formulation is a function of the targeting ligand that is tethered to the tips of the polyethyleneglycol strands on the surface of the liposome. Multiple ligands could be conjugated to the surface of the pegylated liposome to enable tissue-specific targeting of exogenous genes *in vivo*.

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