Biochemical Pharmacology, Vol. 51, pp. 1309–1314, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00038-X

Long-Term Expression of the Human α1-Antitrypsin Gene in Mice Employing Anionic and Cationic Liposome Vectors

*J. Crespo, C. Blaya, A. Crespo and S. F. Aliño**

DEPARTMENT OF PHARMACOLOGY, FACULTY OF MEDICINE AND DENTISTRY, UNIVERSITY OF VALENCIA, AVDA. BLASCO IBÁÑEZ, 15, 46010, VALENCIA, SPAIN

ABSTRACT. The efficiency of both anionic and cationic liposomes as vectors for *in vivo* human α1-antitrypsin (AAT) gene transfer was studied in mice with and without an associated partial hepatectomy. The pTG7101 plasmid, containing the full-length human AAT gene, was encapsulated in small liposomes bearing 10% of negatively (phosphatidylserine, PS) or positively (DOTAP) charged lipids. The results indicate that the DNA/lipid ratio was increased in cationic liposomes by inclusion of monosialoganglioside-G_{M1}. The expression of human protein after *in vivo* gene transfer was quantified in mouse plasma by an ELISA procedure, and revealed that both anionic and cationic liposomes mediated the presence of human protein in mouse plasma for 2–3 weeks. This effect was prolonged (>5 months) when a partial hepatectomy was performed after treatment. In addition, it was observed that the efficacy of liposome-mediated gene transfer was more limited when the plasmid was externally associated to cationic liposomes. BIOCHEM PHARMACOL 51;10:1309–1314, 1996.

KEY WORDS. gene therapy; α1-antitrypsin; liposome vector; *in vivo* gene transfer; liver; partial hepatectomy

The AAT† is a 52 kD serum antiprotease whose most important physiological role is to limit neutrophil elastase activity in the lung. This glycoprotein is highly polymorphic and several human phenotypes are associated with a low production of functioning protein. Inherited AAT deficiency occurs with high frequency (approximately 1 per 1000) in European [1] and American [2] populations.

Individuals with abnormally low levels of circulating AAT have been shown to be predisposed toward chronic obstructive lung disorders because antiprotease deficiency upsets the alveolar protease-antiprotease balance, leading to elastase-mediated tissue destruction and chronic pulmonary emphysema [3, 4]. The current approach to hAAT deficiency treatment involves exogenous i.v. administration of human protein; however, the procedure has serious limitations and the experimental approach to hAAT gene therapy calls for a major advance.

Several strategies have been developed to induce hAAT expression in hepatocytes, including retroviral vectors [5, 6] or anionic liposomes [7, 8] in *ex vivo* and *in vivo* experiments, respectively. However, the new encoded serum protein diminished after a limited period of hepatocellular transplantation of transferred cells or liposome-mediated *in vivo* gene delivery. Because cationic liposomes were able to offer some advantages as a gene transfer system [9] and subtotal hepatectomy can prolong the gene expression of DNA vectors [10], including the hAAT gene [11], in the present work we set out to study the efficiency of both types of liposomes as vehicles for *in vivo* hAAT gene transfer in the presence or absence of a partial hepatectomy.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old C57BL/6 male mice were purchased from B&K Universal Ltd. (England). They were maintained under standard laboratory conditions and housed 4 to 7 mice per cage.

Products

The lipids egg PC, brain PS, and CH, as well as bovine brain G_{M1} were obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.); the cationic lipid DOTAP and the hAAT were purchased from Boehringer Mannheim; goat anti-hAAT and goat anti-hAAT peroxidase conjugate were from Cappel, and the polycarbonate filters from Nuclepore. The pTG7101 plasmid was a generous gift from Dr. P. Meulien and Dr. J. P. Lecocq (Transgène, S.A., Strasbourg, France). It contains the ampicilin- and neomicin-resistant genes and the full length of a hAAT gene and has the

* Corresponding author. Tel. 34-6-3864621; FAX 34-6-3864173.

† *Abbreviations:* AAT, α1-antitrypsin; hAAT, human α1-antitrypsin; PC, phosphatidylcholine; PS, phosphatidylserine; CH, cholesterol; G_{M1}, monosialoganglioside-G_{M1}; Dt, DOTAP; Lp^-, anionic liposome; Lp^+, cationic liposome.

Received 9 August 1995; accepted 18 December 1995.

ability to express the protein in mammalian cells. The *E. coli* strain, a DH5 rac$^-$ containing the pTG7101 plasmid, was grown in LB medium [tryptone 1% (Gibco), yeast extract 0.5% (Gibco), NaCl 1% (Merck), pH 7.5] with ampicilin (50 μg/mL). The plasmid isolation and purification for liposome encapsulation were performed using previously described procedures [12].

Liposome Preparation

Lipids were dissolved in benzene-methanol (9:1), mixed in appropriate molar rates, and dried to form a thin film around the wall of a round-bottomed glass tube. Liposomes were prepared as previously described [13], although with slight modifications for anionic and cationic liposomes.

ANIONIC LIPOSOMES (LP$^-$). A dried lipid mixture (10 μmol) of PC:CH:PS (5:4:1, molar ratio) or PC:CH:PS:GM1 (4:4:1:1) was dispersed by sonication probe in distillate water. The liposome suspension was mixed with 50 μg of pTG7101 plasmid. The mixture was frozen in liquid nitrogen and lyophilized overnight.

CATIONIC LIPOSOMES (LP$^+$). DOTAP suspension was dispersed by sonication probe and mixed with the pTG7101 plasmid (100 μg plasmid/μmol DOTAP). The DOTAP-pTG7101 complex (Dt-pTG) was incubated for at least 30 min. Lipid mixtures from different compositions (PC:CH, 5:4; PC:CH:G_{M1}, 4:4:1) were dispersed by sonication probe. After sonication, the samples were centrifuged (20,000 × *g*, 20 min) to remove titanium particles released from the probe and large aggregates. Each preparation was mixed with the Dt-pTG complex, resulting in a final 10% DOTAP with respect to the total lipid mixture. The final mixtures of liposomes were frozen in liquid nitrogen and lyophilized overnight.

In both types of liposomes, the lyophilized samples were rehydrated with ultrapure water (4 μL/μmol of lipid). The volume was completed up to 600 μL with HEPES saline buffer and filtered (5 cycles) through consecutive polycarbonate filters of 400 and 50 nm pore diameter. Liposomes encapsulating pTG7101 were incubated (1 hr at 37°C) with DNase I (Sigma, 100 μg/mL in 5 mM $MgCl_2$) to degrade the nonencapsulated DNA. The nonentrapped material was separated from liposomes using a Sepharose 4B column (Pharmacia) and small liposomes encapsulating the pTG7101 plasmid were recovered from the supernatant fraction of the ultracentrifuged (50,000 × *g*, 45 min) liposome samples. In addition, the chromatographic pattern of these liposomes was studied on Sepharose 4B using a 1.5 × 22 cm column and the HEPES buffer as eluent at a ratio of 0.3 mL/min.

Encapsulation efficiency was evaluated as the DNA/phospholipid ratio. Phospholipid concentration was determined by the ammonium ferrothiocyanate technique [14]. The amount of encapsulated plasmid was determined in the aqueous phase after chloroform liposome disruption. The DNA was evaluated by fluorometry (Cytofluor 2350, Millipore) using the DNA-binding dye Hoechst 33258 (Sigma, Ex: 365 nm and Em: 458 nm wavelength).

Experimental Design

1. Single treatment/dose: Mice (n = 4 per group) were treated i.v. with the encapsulated pTG7101 plasmid in anionic and cationic liposomes or Dt-DNA complex (100 ng/mouse). Other groups treated with equivalent empty liposomes plus free plasmid or HEPES saline buffer were used as controls;
2. Single treatment/dose plus partial hepatectomy: In duplicate groups, as mentioned above, mice were treated i.v. with pTG7101 plasmid (100 ng/mouse), encapsulated in liposomes, or forming a Dt-DNA complex. Three hours later, hepatocyte cell division was induced by partial hepatectomy of superficially anaesthetized mice with ether;
3. Dose/dependence: Different groups of mice (n = 4 per group) were treated i.v. with 100, 200, or 500 ng of pTG7101 encapsulated in anionic liposomes [Lp$^-$(pTG)] and liver regeneration was induced 3 hr later by partial hepatectomy.

ELISA of hAAT in Mouse Plasma

Mice blood samples (200 μL) were taken before and after treatment at different timepoints from the tail vein, using heparinized glass capillaries. After centrifugation, plasma was recovered and a pool of 200 μL was obtained, mixing 50 μL from each animal in the group. The pooled plasma was inactivated at 55°C for 45 min and, after centrifugation (20,000 × *g*, 15 min), the samples were kept at −20°C until use for ELISA. The assay was performed in 96-well microtitration plates (CoStar), as previously described [8, 11]. Briefly, goat anti-hAAT and goat anti-hAAT peroxidase conjugate were used as capture and detecting antibodies, respectively. Capture antibody (1 μg/mL) diluted in adsorption buffer (carbonate 0.05 M, pH 9.6) was distributed (100 μL/well) in the microplate and incubated at 4°C overnight. After 3 washings (PBS-Tween 20 0.05%, 200 μL/well), wells were incubated for an additional 2 hr at 37°C with 1% bovine serum albumin (PBS-albumin, 200 μL/well) to block nonspecific binding sites. Then, the wells were rinsed 5 times and 100 μL of hAAT (from 0 to 30 ng/mL) or the mice plasma samples were added. After incubation (2 hr, 37°C) the wells were rinsed 5 times and incubated for an equivalent time period with goat anti-hAAT peroxidase conjugate (15 μg/mL, 100 μL/well) in PBS-albumin. After 3 washings, 150 μL of a solution containing o-phenylendiamine (0.4 mg/mL in citrate phosphate buffer, pH 5) plus H_2O_2 (1.5 μL/mL of a 30% solution) were added to each well. The reaction was stopped 2.5 min later by the addition of 50 μL/well of H_2SO_4 2 M.

RESULTS

Encapsulation Efficiency

The efficiency of anionic and cationic liposomes to entrap recombinant DNA was examined using the pTG7101 plas-

mid. The results are expressed as DNA/phospholipid ratio and represent the encapsulated fraction of plasmid DNA, because liposome suspension was previously digested with DNase I and the nonentrapped DNA was removed by liquid chromatography. For encapsulation efficiency, the liposomes entrapping pTG7101 plasmid were recovered from the supernatant fraction of the centrifuged sample (50,000 × *g*, 45 min). As shown in Table 1, the DNA/lipid ratio in liposome preparations was 3-fold lower in cationic liposomes (0.72 μg/μmol) than in anionic liposomes (2.31 μg/μmol). However, this ratio was reversed when G_{M1} was incorporated in the liposome preparation. In cationic G_{M1} liposomes (7.19 μg/μmol), the DNA/lipid ratio was increased 10-fold with respect to single cationic liposomes (0.72 μg/μmol). The incorporation of G_{M1} in other types of liposomes also increased the efficiency of DNA encapsulation, but to a lesser extent. Because cationic liposomes containing G_{M1} have a higher encapsulation efficiency than the same liposomes without G_{M1}, we used the former, cationic liposomes (Lp^+), for *in vivo* gene transfer. In contrast, anionic liposomes (Lp^-) without G_{M1} were used in *in vivo* experiments because the efficacy of encapsulation with and without G_{M1} was similar, based on our previous experience in the use of this type of liposome [7, 8, 11]. On the other hand, we also studied the chromatographic pattern (Fig. 1) of the different types of liposomes obtained from the supernatant fractions of centrifuged liposome preparations. The results show that a similar chromatographic pattern with a unique liposome peak may be observed in all cases.

In vivo *gene transfer efficiency*

We measured the plasma levels of hAAT after *in vivo* gene transfer of pTG7101 plasmid, using anionic [Lp^-(pTG)] and cationic [Lp^+(pTG)] liposomes in normal mice, and mice with a partial hepatectomy, 3 hours after i.v. liposome injection.

EFFICIENCY OF ANIONIC LIPOSOMES. We have previously [8] observed that a single i.v. dose of pTG7101 plasmid (100 ng/mouse) encapsulated in anionic small liposomes (but not free plasmid) induced the presence of hAAT in mouse plasma. In the present study, we found that the effect

TABLE 1. Liposome characteristics

Type of Liposome		DNA/PL (μg/μmol)	% Recovered DNA	% Recovered PL
Lp^-(pTG)	10 μmol	2.31	6.58	23.69
Lp^-G_{M1}(pTG)	10 μmol	2.66	4.32	16.20
Lp^+(pTG)	5 μmol	0.72	0.43	11.91
Lp^+G_{M1}(pTG)	5 μmol	7.19	4.13	14.36

The table shows the encapsulation efficiency (μg DNA/μmol of phospholipid) obtained from anionic (Lp^-) and cationic (Lp^+) liposomes encapsulating the pTG7101 plasmid, with and without G_{M1} inclusion. Also, the percentages of both recovered DNA and phospholipid (PL).

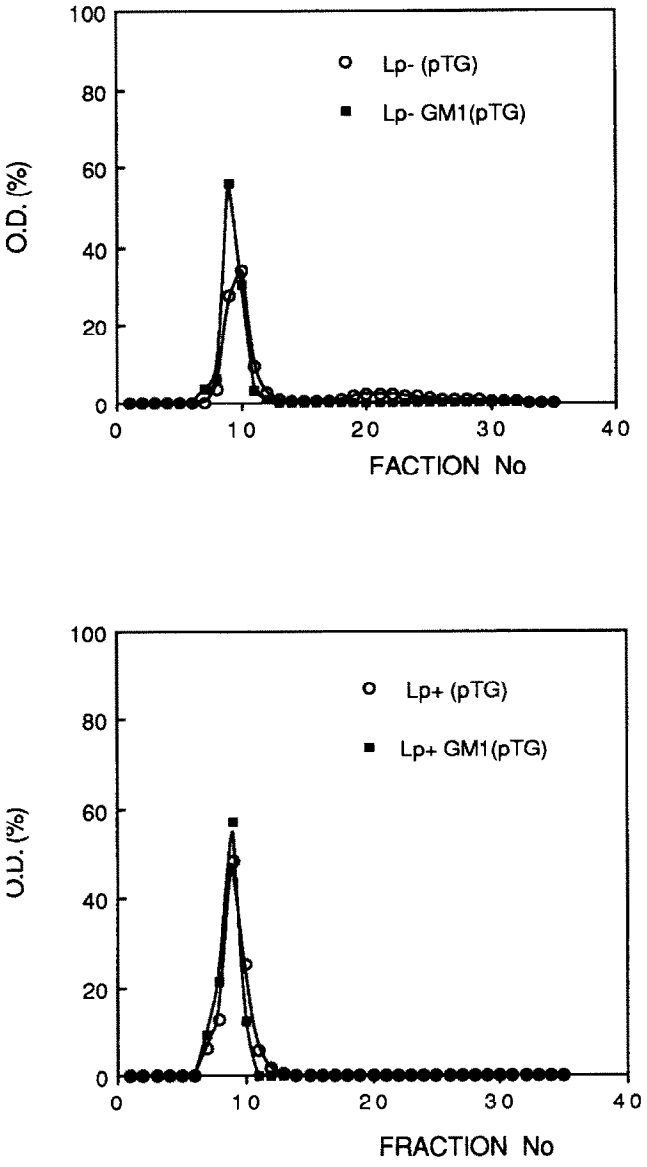

FIG. 1. Chromatographic pattern of liposomes. Anionic (upper panel) and cationic (lower panel) liposomes encapsulating the pTG7101 plasmid, with (filled squares) or without (open circles) G_{M1} ganglioside inclusion (as shown in Table 1) were chromatographied on Sepharose 4B using a 1.5 × 22 cm column. The elution solvent was HEPES buffer at a flow of 0.3 mL/min. Fractions were collected every 2 min and the optical density (O.D.) was measured at 260 nm by spectrophotometry. The greatest absolute value of O.D. in each chromatographic pattern was: 0.619 for Lp^-(pTG), 0.526 for Lp^- G_{M1}(pTG), 0.202 for Lp^+(pTG) and 0.247 for Lp^+G_{M1}(pTG). The data are expressed as O.D. percentage per fraction with respect to the total O.D. summation.

of this treatment diminished after a limited period (Fig. 2), but a prolonged gene expression (at least 3 months) of human protein was present in partially hepatectomized mice. In both cases (data not shown), no hAAT was detectable in the plasma of mice treated with HEPES buffer or free plasmid only. The results confirm our previous results, and support the notion that subtotal hepatectomy prolonged the gene expression of a DNA vector [10].

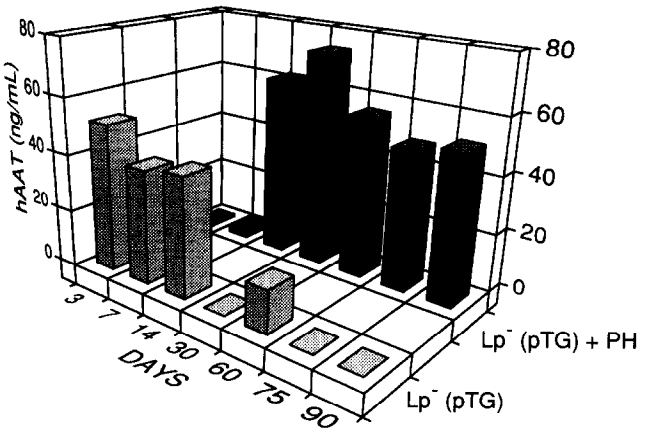

FIG. 2. Treatment with anionic liposomes. Two groups of mice (*n* = 5) were treated i.v. (day 0) with a single dose of 100 ng/mouse of pTG7101 plasmid encapsulated in anionic liposomes. In one group, hepatocyte division was induced 3 hr later by a partial hepatectomy [Lp⁻(pTG) + PH]. The AAT plasma levels were evaluated by an ELISA procedure. The data represent the mean of human protein levels on different days.

EFFICIENCY OF CATIONIC LIPOSOMES. Cationic lipids interact with negatively charged phosphate groups of DNA. Thereby, the pTG7101 plasmid could be encapsulated [Lp⁺(pTG)] or associated to the positively charged surface of liposomal membranes (Dt-pTG), forming a DOTAP-DNA complex. Both forms of liposome-associated plasmid were used for i.v. injection (100 ng pTG/mouse). The results (Fig. 3) indicated that hAAT in mouse plasma was several times greater using liposome-encapsulated plasmid as compared to the Dt-pTG complex. This increased effect was observed in both hepatectomized (3-fold) and nonhepatectomized (8-fold) mice groups. No detectable hAAT was found in plasma from control mice groups (data not shown). The human protein in plasma decreased after 2–4 weeks posttransfection but, as with anionic liposomes (see above), the partial hepatectomy increased and prolonged the gene expression of hAAT. In addition, the plasma levels of human protein from mice treated with encapsulated plasmid also remained several fold higher than levels induced by the Dt-pTG complex.

DOSE DEPENDENCE. These experiments are an extension of a previous study [11], in which we evaluated the effect of repeated i.v. application of Lp⁻(pTG) on hAAT gene expression. Here, we studied the effect of 3 different i.v. doses of plasmid (100, 200, and 500 ng/mouse) encapsulated in anionic liposomes on plasma levels of human protein from partially hepatectomized mice. A single dose of 100 ng of encapsulated plasmid induced a long-term expression (>5 months, Fig. 4). The efficiency of gene transfer was increased with the dose of 200 ng but no augmentation was observed with the 500 ng/mouse dose.

DISCUSSION

In the present work, we show that: 1. both anionic and cationic small liposomes can be used as vehicles for *in vivo* gene transfer of hAAT, but cationic liposomes could offer advantages due to their high encapsulation efficiency; 2. partial hepatectomy after liposome-mediated gene transfer increases the period of gene expression from 2–4 weeks to more than 5 months, and 3. the hAAT level in mouse plasma is a function of the encapsulated plasmid dose, but this effect might be limited at higher doses.

There are currently several methods of DNA transfer under investigation that utilize chemical, colloidal, or bio-

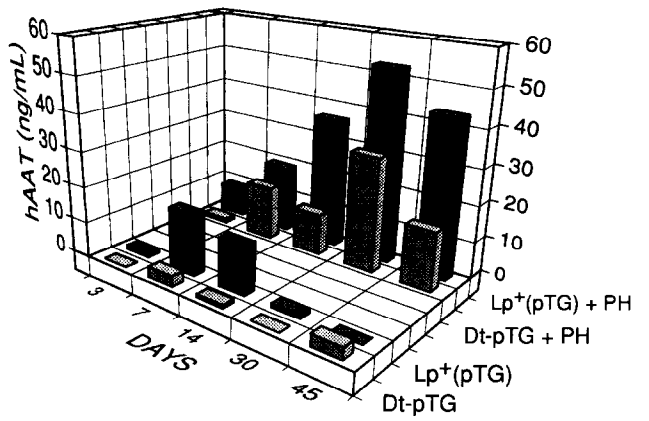

FIG. 3. Efficiency of cationic liposomes. Mice (*n* = 5 per group) were treated i.v. (day 0) with a single dose of 100 ng/mouse of pTG7101 plasmid, associated to cationic lipid DOTAP (Dt-pTG) or encapsulated in cationic liposomes [Lp⁺(pTG)]. Equivalent groups were treated and a partial hepatectomy performed 3 hr after the treatment to promote liver regeneration (Dt-pTG + PH and Lp⁺(pTG) + PH). The human protein levels were determined by an ELISA procedure. The data show means of AAT levels.

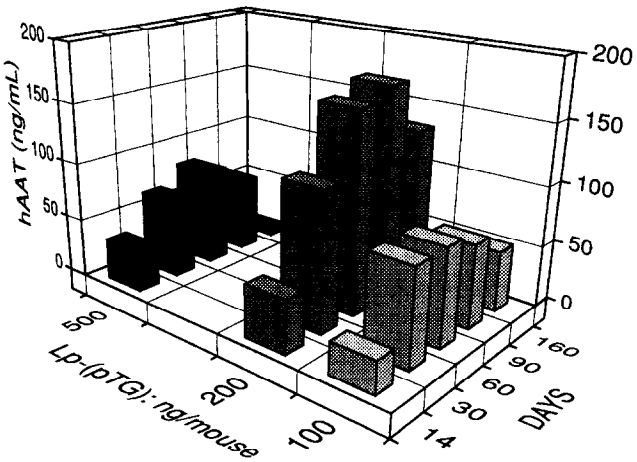

FIG. 4. Dose-dependence. Mice (*n* = 5 per group) were treated i.v. with a single dose of 100, 200, or 500 ng/mouse of pTG7101 plasmid encapsulated in anionic liposomes. Animals were partially hepatectomized 3 hr after treatment. The AAT plasma levels were evaluated by an ELISA procedure. The data represent means of human protein levels on different days.

logical means for gene transfection, but not all developed methods can be used for *in vivo* gene therapy strategies. The use of liposomes as a delivery system for gene transfer is a natural extension of their earlier applications as drug delivery agents [15, 16], where they demonstrated low toxicity. The gene transfer efficacy of liposomes appears to depend on several variables that probably reflect basic characteristics of the interactions between transfecting lipids, exogenous DNA, and the cellular type. In addition, the natural barriers of vascular endothelial cells also limit their applicability to *in vivo* therapy. Based on these considerations, the importance of liposome composition and structure in the efficacy of the overall process of gene transfection (from delivery to expression) remains a question worthy of further study. In the present study, we encapsulated the plasmid in anionic and cationic liposomes and the DNA/lipid ratio was evaluated in the liposome preparation. The results of the encapsulating efficiency showed that, whereas no relevant increases in DNA/lipid ratio were observed in anionic liposomes containing G_{M1}, the ratio was increased 10-fold when the ganglioside was present in cationic liposomes.

There are currently three models [17] of DNA-lipid interactions that either situate the DNA on the outer liposome surface, on the inner surface of the liposome, or coated by lipid modified to bind the DNA with high affinity. Our results suggest that the high efficiency of plasmid entrapment in cationic liposomes was due to electrostatic interactions between negatively charged phosphate groups of DNA and positively charged lipids. The G_{M1} inclusion in the lipid composition of cationic small liposomes must facilitate DNA interaction with the inner surface of cationic liposomal membranes. We used the DNase I digestion of liposome-encapsulated plasmid to determine whether or not the DNA is, indeed, contained only within liposomes, but we can not exclude that some DNA bound onto the outer surface of a liposome could remain protected from digestion. In this sense, our results (data not shown), based on the consecutive neuraminidase and DNase I digestion of cationic liposomes bearing G_{M1}, indicate that G_{M1} could limit nuclease access to DNA bound onto the liposome surface, but that the major part (86%) of liposome-associated DNA should be encapsulated because it remains resistant to nuclease digestion.

Recently, we reported [7, 8] that small (but not large) liposomes are appropriate vehicles for *in vivo* hAAT gene transfer to mouse hepatocytes. Here, as in our previous work, we used the anionic small liposomes (PC:CH:PS) and the cationic small liposomes bearing the G_{M1} ganglioside (PC:CH:G_{M1}:DOTAP) for highly efficient encapsulation of the plasmid. We also used a DOTAP-DNA complex (Dt-pTG), in which the plasmid is externally associated to small liposomes. After i.v. injection of encapsulated plasmid in both anionic and cationic liposomes, we were able to detect the human protein for 2 weeks. However, the effect was very limited when the Dt-pTG complex was employed as vector for gene transfer. This shows that anionic and cationic small liposomes entrapping the plasmid were able to deliver the exogenous gene with similar potency. Because affinity is inversely correlated with the ED_{50} we propose that liposome-cell interaction must occur with high affinity because maximal response was observed at very low doses of encapsulated plasmid (approximately 0.2 μg/mouse), 3 orders of magnitude lower than the plasmid dose used by other authors [18].

On the other hand, it has been reported [10] that partial hepatectomy prolongs gene expression of a single dose of a DNA vector; we, thus, used this strategy to study the efficacy of anionic and cationic small liposomes as nonviral systems for gene transfer. When partial hepatectomy was combined with a single dose of encapsulated pTG7101 plasmid, the amount of hAAT in mouse plasma was increased and the period of gene expression was also prolonged (from 2–4 weeks to >5 months). The results agree with our previous observations [11] using anionic small liposomes, and show that a similar effect on hAAT gene expression was apparent in mice treated with encapsulated plasmid in both anionic and cationic liposomes. To our knowledge, this is the first study in which both anionic and cationic liposomes have been simultaneously employed to study the efficacy of liposome-mediated gene transfer. Under our experimental conditions, it seems that liposome size (but probably not liposome charge) influences the ability of small liposomes to deliver the encapsulated DNA to target cells, and that gene delivery could be mediated by a very active pinocytosis mechanism of the hepatocytes. However, the influence of liposome charge on gene transfer efficacy, involving receptor-mediated liposome-uptake mechanisms for gene delivery, remains to be elucidated.

The increased efficiency of liposome-mediated gene transfer after partial hepatectomy could be due to increased gene bioavailability because partial hepatectomy induces cellular division [19] and consequent alteration of DNA compartmentalization within the cell, leading to a prolonged foreign gene expression [20]. In addition, mechanisms involved in the reactivation of gene expression after cell division could favour foreign gene expression. The plasma levels of hAAT observed in our experiments were lower than those obtained by other authors [5, 21] using retroviral vectors in *ex vivo* experiments. However, it must be pointed out that the gene expression efficiency of bioavailable genes is an intrinsic property of the recombinant gene construction. Gene vectors containing viral or housekeeping promoters, enhancers, or other sequences can increase gene expression [21, 22]. However, although the cytomegalovirus promoter is extremely active in primary hepatocytes [22], it must be regulated differently *ex vivo* and *in vivo* [23], and the serum levels of hAAT fell after a short period of hepatocellular transplant of transduced cells [6, 23], probably due to inactivation of the cytomegalovirus promoter. We suggest that gene construction under control of natural promoters active in normal liver cells very likely offer advantages, such as high efficacy and long-term gene expression. Moreover, the encapsulation of recombinant

genes in small liposomes with fusogenic properties could increase gene bioavailability, resulting in an increased efficacy of liposome-mediated gene transfer.

We thank Prof. C. Coutelle (Dept. Biochem. and Mol. Genet., St. Mary's Hospital, London) for his valuable suggestions and revision of the manuscript and Dr. P. Meulien (Pasteur-Merieux, Marcy-L'Etoile, Lyon) and Transgène S.A. for the generous gift of pTG7101 plasmid. This work was partially supported by the CICYT PB92-0877 project.

References

1. Fagerhol MK, The Pi-system genetic variants of serum alpha 1-antitrypsin. *Ser Haematol* **1:** 153–161, 1968.
2. Savransky E, Hytiroglou P, Harpaz N, Thung SN and Johnson EM, Correcting the PiZ defect in the α1-antitrypsin gene of human cells by targeted homologous recombination. *Lab Invest* **70:** 676–683, 1984.
3. Travis J and Salvesen GS, Human plasma proteinase inhibitors. *Ann Rev Biochem* **52:** 655–709, 1983.
4. Gadek JE, Fells GS, Zimmerman RL and Crystal RG, Antielastases of the human alveolar structures. Implications for the protease-antiprotease theory of emphisema. *J Clin Invest* **68:** 889–898, 1981.
5. Ponder KP, Gupta S, Leland F, Darlington G, Finegold M, Demayo J, Ledley FD, Chowdhury JR and Woo SLC, Mouse hepatocytes migrated to liver parenchyma and function indefinitely after intrasplenic transplantation. *Proc Nat Acad Sci USA* **88:** 1217–1221, 1991.
6. Kay MA, Baley P, Rothenberg S, Leland F, Flaming L, Ponder KP, Liu TJ, Finegold M, Darlington G, Pokorny W and Woo SLC, Expression of α1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. *Proc Nat Acad Sci USA* **89:** 89–93, 1992.
7. Aliño SF, Bobadilla M, García-Sanz M, Lejarreta M, Unda F and Hilario E, In vivo delivery of human α1-antitrypsin gene to mouse hepatocytes by liposomes. *Biochem Biophys Res Commun* **192:** 174–181, 1993.
8. Aliño SF, Bobadilla M, Crespo J and Lejarreta M, Human α1-antitrypsin gene transfer to in vivo mouse hepatocytes. *Hum Gen Ther* **7:** 531–536, 1996.
9. Yagi K, Noda H, Kurono M and Ohishi N, Efficient gene transfer with less cytotoxicity by means of cationic multilamellar liposomes. *Biochem Biophys Res Commun* **196:** 1042–1048, 1993.
10. Wu GY, Wilson JM, Shalaby F, Grossman M, Shafritz DA and Wu CH, Receptor-mediated gene delivery in vivo. Partial correction of genetic analbuminemia in nagase rats. *J Biol Chem* **266:** 14388–14342, 1991.
11. Aliño SF, Crespo J, Bobadilla M, Lejarreta M, Blaya C and Crespo A, Expression of human α1-antitrypsin in mouse after in vivo gene transfer to hepatocytes by small liposomes. *Biochem Biophys Res Commun* **204:** 1023–1030, 1994.
12. Maniatis T, Fritsch EF and Sambrook J, *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, New York, 1982.
13. Aliño SF, García-Sanz M, Iruarrizaga A, Alfaro J and Hernández J, High encapsulation efficiencies in sized liposomes produced by extrusion of dehydration-rehydration vesicles. *J Microencapsulation* **7:** 479–503, 1990.
14. Stewart JCM, Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal Biochem* **104:** 10–14, 1980.
15. Machy P, Arnold B, Aliño SF and Leserman LD, Interferon sensitive and insensitive MHC variants of a murine thymoma differentially resistant to methotrexate-containing antibody-directed liposomes and immunotoxin. *J Immunol* **136:** 3110–3115, 1986.
16. Aliño SF, Unda FJ, Iruarrizaga A, Alfaro J, Hilario E, Pérez-Yarza G, Bobadilla M and Lejarreta M, Efficacy of liposome-encapsulated indomethacin in response against metastatic 3LL and B16F1 cells. *Lab Invest* **66:** 671–679, 1992.
17. Smith JC, Walzen RL and German JB, Liposomes as agents of DNA transfer. *Biochim Biophys Acta* **1154:** 327–340, 1993.
18. Perales JC, Ferkol T, Beegen H, Ratnoff OD and Hanson RW, Gene transfer in vivo: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. *Proc Nat Acad Sci USA* **91:** 4086–4090, 1994.
19. Johansson S and Andersson G, Similar induction of the hepatic EGF receptor in vivo by EGF and partial hepatectomy. *Biochem Biophys Res Commun* **166:** 661–666, 1990.
20. Wilson JM, Grossman M, Cabrera JA, Wu CH and Wu GY, A novel mechanism for achieving transgene persistence in vivo after somatic gene transfer into hepatocytes. *J Biol Chem* **267:** 11483–11489, 1992.
21. Armentano D, Thompson AR, Darlington G and Woo SLC, Expression of human factor IX in rabbit hepatocytes by retrovirus-mediated gene transfer: potential for gene therapy of hemophilia B. *Proc Nat Acad Sci USA* **87:** 6141–6145, 1990.
22. Ponder KP, Dunbar RP, Wilson DR, Darlington GJ and Woo SLC, Evaluation of relative promoter strength in primary hepatocytes using optimized lipofection. *Hum Gen Ther* **2:** 41–52, 1991.
23. Sharfmann R, Axelrod JM and Verma IM, Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc Nat Acad Sci USA* **88:** 4626–4630, 1991.