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IN VIVO DELIVERY OF HUMAN α 1-ANTITRYPSIN GENE TO MOUSE HEPATOCYTES BY LIPOSOMES

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The pTG7101 plasmid containing the full length human α1-Antitrypsin was encapsulated in large (142±15 nm of diameter) and small (54±11 nm of diameter) liposomes and administered i.v. to mice (80 ng/mouse). Control animals were treated with empty (small and large) liposomes plus free DNA and with the liposome solvent buffer. The immunohistochemical results on liver criosections and cytophotometric analysis of hepatocyte chromophore absorbance, after peroxidase reaction, indicated that significant presence of immunoreactive human α 1-antitrypsin was present 7 days after mice treatment with encapsulated DNA in small liposomes but not when large liposomes were used. This effect was observed in a great number of liver parenchymal cells. These results agree with the observation that only small liposomes have easy access to hepatocytes and support the idea that small liposomes are appropiate vehicles for *in vivo* delivery of specific genetic material to liver parenchymal cells, with high efficiency.

Many techniques have been developed for introducing genetic material into cells *in vitro*, such as calcium phosphate precipitation (9,13), microinjection (7), electroporation (26,27), liposomes (12), and viral vectors (25, 15). Gene transfer in live animals has also been attempted using vehicle mediated procedures and the most promising methods appear to involve the use of retroviral and liposome vectors (for review 10, 17). Liposomes have showed to be efficient vehicles for many *in vitro* (22) and *in vivo* (21,4) applications. Since small liposomes can deliver the encapsulated material to liver parenchimal cells (28), we think that they could be an appropriate carrier system for *in vivo* delivery of foreign DNA to hepatocytes. In the present work we study the ability of small liposomes to encapsulate a plasmid (pTG

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7101) containing the full length gene encoding human α1-Antitrypsin (α 1-AT) and their efficiency to in vivo deliver the functioning gene to mouse hepatocytes.

MATERIAL AND METHODS

Mice. 8-10 week old Swiss mice were purchased from Iffa-Credo Laboratories (France). They were maintained under standard laboratory conditions and housed four to seven per cage.

Materials. Egg phosphatidylcholine (PC) and brain Phosphatidylserine (PS) were purchased from Lipid Products (S. Nutfield, U.K.); Cholesterol (CH) and Diaminobenzidine from Sigma Chem. Corp.; the antibodies rabbit IgG anti human α1-Antitrypsin and goat antirabbit IgG peroxidase labelled from Sigma Chemical Co.; Polycarbonate filters from Nuclepore; 5(6) carboxyfluorescein (CF) from Eastman Kodak Co. The pTG7101 plasmid was a generous gift from Dr. P. Meulien (Transgène S.A., Strasbourg). It contains the ampicillin and neomycin resistant genes and the full length of a human α1-Antitrypsin gene with the ability to express the protein in mammalian cells. E. coli strain a DH5 rec- containing the pTG 7101 plasmid were grown in LB medium (Tryptone 1%, Yeast Extract 0.5%, NaCl 1%, pH 7.5) with ampicillin (100μg/ml). The plasmid isolation and purification for liposome encapsulation was performed using procedures previously described (23).

Preparation of liposomes. A Benzene:Methanol (9:1) lipid mixture (60 μmol) of CH:PC:PS (5:4:1) was dried to form a thin film around the wall of a round-bottomed tube and then lyophilized for a minimum of 3 h. Liposomes were prepared by dehydration-rehydration method (19) and then extruded through polycarbonate filters of 400 and 200 nm pore diameter, as described previously (3). The 400 nm filtered liposomes were ultracentrifuged (10^5 x g, 40 min) and only the supernatant fraction was used. The liposomes were separated from non encapsulated material using a Sepharose 4B column. Before purification, liposomes entrapping DNA were incubated (1 h at 37 °C) with DNase I (Sigma, 50 μg/ml in 5 mM $MgCl_2$) to remove the material associated from the liposome surface.

To evaluate the encapsulation efficiency, the phospholipid concentration was determined by the ammonium ferrothiocyanate technique of Stewart (29). The entrapped CF was evaluated by fluorimetry (490 nm excitation and 520 nm emision wavelenght). The amounts of encapsulated PI and DNA were determined in the aqueous phase after liposome disruption by chloroform. PI was evaluated by spectrophotometry (288 nm wavelenght) and DNA by spectrofluorimetry using the DNA-binding dye Hoechst 33258, with a 365 nm excitation and 458 nm emission wavelength (20). Liposome diameter was evaluated by a transmission electron microscopy, using phosphotungstic acid negative stain.

Liposome stability in plasma. Liposomes encapsulating carboxyfluorescein were mixed with fresh mouse plasma in 1:10 volume ratio and were incubated at 37 °C at different intervals, aliquots of 10μl were collected at different time intervals and the fluorescence measured in absence (CFa) or presence (CFb) of Triton X-100 (0.1% final concentration) using 490 nm excitation and 520 nm emission wavelengths. Latent CF was determined from the following formula: (CFb-CFa)/CFa ; it was expressed as percentage of latency in respect to the liposomal preparation prior to plasma incubation.

Staining procedures. In order to identify the target cells for large and small liposomes in the liver, we have used a previously described procedure (1,5) based on the "in vivo" administration of liposomes encapsulating the fluorescent DNA binding dye, propidium iodide (PI, 10μg/mouse). After 1h of i.v. administration, the mice were killed and their liver removed and fixed in liquid nitrogen. The fluorescence in frozen sections was observed and photographed with an AH-2 Vanox-T Olympus microscope. For macrophage staining, mice received an i.v. injection (200 μl) of diluted iron dextran solution (1:50) 24 h before sacrifice. Paraffin sections of formalin-fixed liver were stained to identify macrophages using a two-step histochemical staining procedure as previously described (6).

Gene delivery. Swiss mice were i.v. treated with pTG 7101 plasmid (80 ng/mouse) encapsulated in both larger (n=4) and small liposomes (n=6), prepared as described in methods. In addition, other mice groups were treated with SSC buffer (150mM ClNa, 15 mM Natrium Citrate) (n=4), or 1 μmol of lipid in large empty-liposomes plus 80 ng of free plasmid (n=4) and 0.37 μmol of lipid in small liposomes plus 80 ng of free plasmid (n=4). After 7 days of treatment the mice were killed and their liver removed for immunohistochemical studies using antihuman α1-AT antibodies.

Immunohistochemy. Liver cryosections were fixed in ice-cold 100% methanol. To eliminate the endogenous peroxidase activity of the liver, sections were incubated with 0.03%

H_2O_2-100% methanol for 30 min. Then, they were washed with saline solution and treated with 1%Triton X100. The sections were blocked out afterwards with a 1% BSA (Bovine Serum Albumine) solution for 30 min. and then incubated with a 1:30 dilution of rabbit IgG anti-human α1-AT or normal rabbit serum for 90 min. at room temperature. After 3 to 5 washes with TBS (10mM Tris pH 7.5, 0.85% NaCl), the second incubation was carried out with a 1:60 dilution of Goat antirabbit IgG peroxidase conjugated. Immunoperoxidase staining was performed with 0.01% H_2O_2 - DAB 0.3 mg/ml, in complete darkness. The counterstain was hematoxilin.

Cytophotometry of immunoreactive α1-AT in the liver. After immunoperoxidase reaction, the chromophore amount per cell, was quantified by a microspectrometric technique on hepatocyte cells of liver sections. We used a Carl Zeiss microscope photometer with a fast scanning stage, motor controlled plate, connected on line to a Digital computer. The chromophore absorbance of hepatocytes was measured at a wavelength of 460 nm using a 100x objetive. Each cell was enclosed in a square area,where a 0.5 μm resolution scanning was made. The measurement of chromophore absorbance was carried out by the integration method of Casperson (8). The average of chromophore concentration in hepatocytes from each liver was obtained from the mean values of at least six different liver sections measurements. In all cases, measures were made at random and the mean value of each liver section was considered when its standard error was less than 10%.

RESULTS

Liposome characterization. Since liposome distribution in the body is greatly influenced by their size, we have prepared liposomes encapsulating DNA with two different sizes. The liposome size was determined by transmission electron microscopy after negative stain. The results are summarized in Table 1. The 200 nm filtered liposomes (large liposomes) were an homogeneous liposome suspension with an average diameter of 142.5±15.7 nm and they mainly showed a multilamellar organization. The suspension of 400 nm filtered liposomes was very heterogeneous. It was composed of large multilamellar liposomes (from 150 to >300 nm of diameter) and a great number of small unilamellar liposomes. Small liposomes were separated by ultracentrifugation (10^5 x g, 40 min) recovering in the supernatant an homogeneous suspension of small liposomes with an average diameter of 54.6±11.6 nm. They showed unilamellar structure and in addition they have higher DNA/lipid ratio than large liposomes.

Stability of liposomes in mouse plasma. The *in vivo* ability of liposomes to deliver the encapsulated material to target cells is greatly limited by their stability. The stability of liposomes in plasma was evaluated *in vitro*, using carboxyfluorescein (CF) as tracer (32). The results (Figure 1) indicate that both large and small liposomes were stable in mouse plasma. Thus, more than 80-90% of CF remained encapsulated after 3 h of plasma incubation at 37 °C and values of 80% were observed after 24 h of incubation.

Table 1. Liposome characteristics. Liposome size was evaluated by electron microscopy after negative staining with phosphotungstic acid. The amount of encapsulated DNA was determined by fluorimetry (using Hoechst 33258) in the aqueous phase after liposome disruption by chloroform. Phospholipid concentration was determined by the ammonium ferrothiocyanate technique. The data are expressed as mean values± standard error.

	SIZE (nm)	DNA/LIPID(ng/μmol)
Large liposomes	142,5 ± 15,7	833,1 ± 403,1
Small liposomes	54,6 ± 11,0	1973,9 ± 540,1

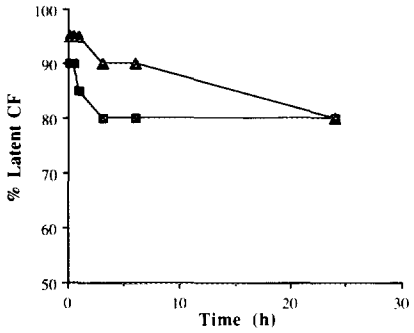

Figure 1. Liposome stability in mouse plasma. Large (■) and small (▲) liposomes encapsulating carboxyfluorescein (CF) were incubated in fresh mouse plasma (1:10 volume ratio) at 37 °C. Latent CF was measured at different time intervals, as described in methods. The values are expressed as percentage of latency before plasma incubation and represent the mean of two measurements which had differences not higher than 5%.

Liposome target cells in the liver. Liver macrophages were identified by histochemical procedures in the respective paraffin sections from iron-dextran injected mice. The stained macrophages were observed in the sinusoidal territories (figure 2A) with mainly a periportal distribution pattern. Liposome distribution in the liver as a function of their size was studied by i.v. administration of 10 μg of PI encapsulated in larger or small liposomes before sacrifice. Our results based on the fluorescent images from frozen liver sections show that fagocytic cells connected to liver sinusoids can be stained in vivo using large liposomes (figure 2B), but they have very limited access to hepatocytes. In contrast, small liposomes have great ability to deliver the entrapped material to liver parenchimal cells (figure 2C). In addition the observation of several liver sections from different lobules indicate that the greatest number of hepatocytes display nuclear fluorescence.

Immunohistochemy and cytophotometry. Immunoperoxidase reactions were performed on liver cryosections using rabbit IgG antihuman α1-AT or normal rabbit serum. We observe that only encapsulated plasmid in small liposomes was efficient to induce the presence of human immunoreactive α1-AT in liver parenchymal cells. In Figure 2, we show a different immunoperoxidase stain, using normal rabbit serum (D) and antihuman α1-AT (E and F), on liver sections of mice treated with pTG 7101 encapsulated in small liposomes. In the first case a very low reaction was observed whereas in the last case, a great α1-AT reactivity was observed in a majority of liver parenchymal cells.The cytophotometric analysis (figure 3) indicates that a similar amount of chromophore was obtained in hepatocytes from mice treated with free plasmid plus large or small empty-liposomes. In addition, no significant differences in the chromophore amount were observed between these groups and mice receiving only buffer. On the other hand clear differences were observed in the chromophore amount of hepatocytes from mice treated with encapsulated plasmid in small liposomes compared with control animals treated with SSC buffer and encapsulated plasmid in large liposomes.Whereas hepatocyte chromophore after immunoperoxidase reaction was greatly increased using encapsulated plasmid in small liposomes (138%), the effect was very limited and not

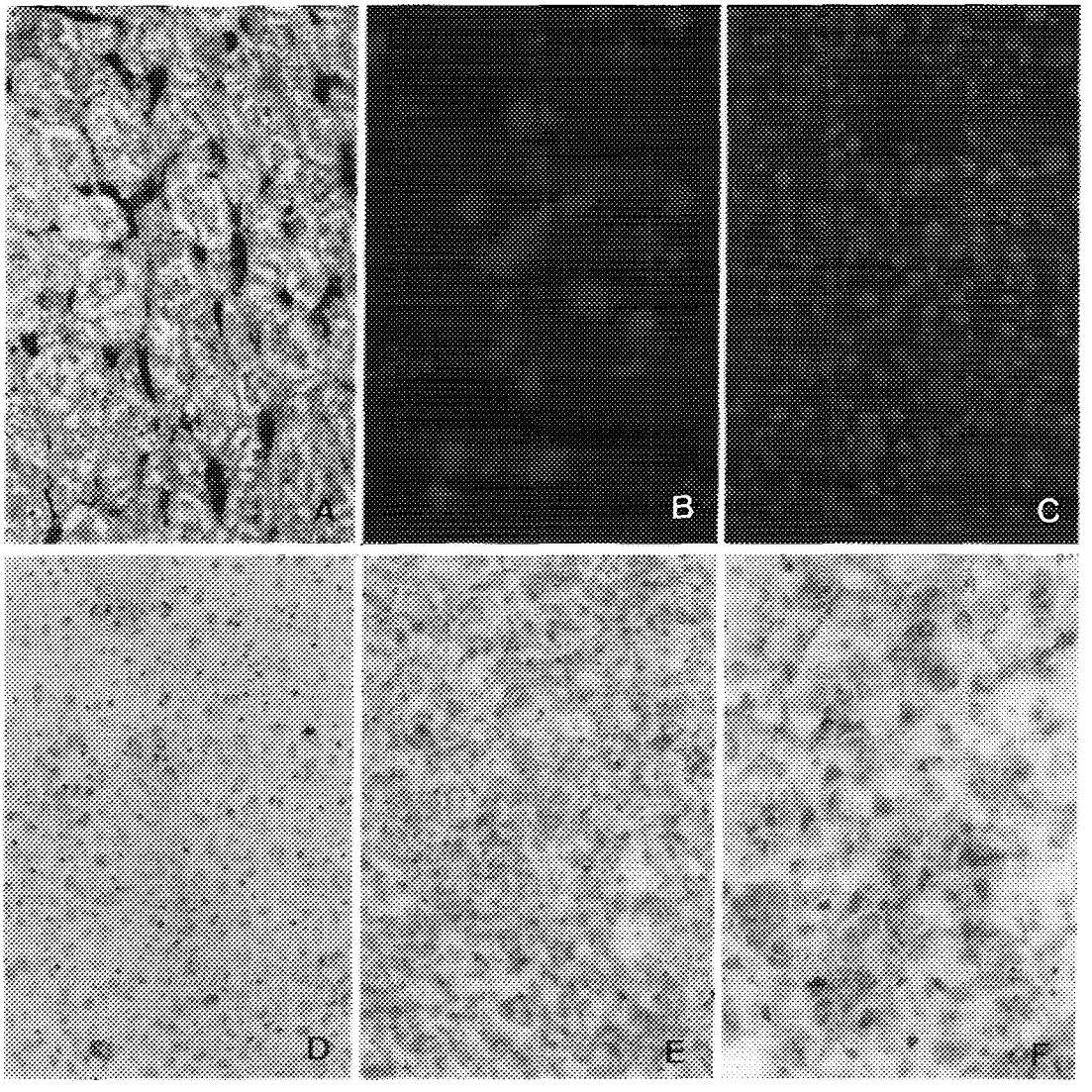

Figure 2. Liver micrographs. (A) Stain of liver macrophages by iron-dextran technique. The fluorescent images (B,C) show the differential liver uptake in vivo of liposomes encapsulating PI as a function of their size. Large liposomes (B) mainly display the nuclei of phagocytic cells, whereas small liposomes (C) display a very great number of hepatocyte nuclei. The immunohistochemical staining was made in frozen liver sections, from mice previously treated (7 days) with encapsulated pTG7101 plasmid (80 ng) in small liposomes. Liver cryosections were fixed in ice-cold 100% methanol and incubated with normal rabbit serum (D) or rabbit IgG anti-human α1-AT (E, F)and peroxidase labelled goat antirabbit IgG. Immunoperoxidase staining was performed using diaminobenzidine method. The counterstain was hematoxilin. Micrographs were made with: (A, B, F) x40, (C, D, E) x20 objectives.

significant when the injected plasmid was entrapped in large liposomes (12%). In order to find out the rate of non-specific immunoreactivity, additional immunohistochemical reactions were performed using normal rabbit serum, as a first step. The cytophotometric results indicate that low non-specific reaction was present in all cases.

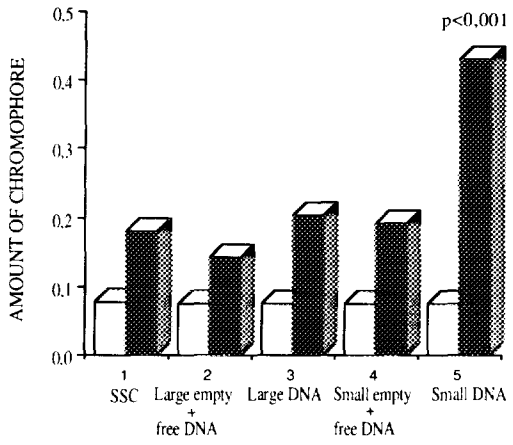

Figure 3. Cytophotometry of immunoperoxidase stain. Immunohistochemical stain in liver cryosections was performed using (□) normal rabbit serum and (■) rabbit IgG anti-human α 1-AT, as described in methods. The chromophore absorbance of hepatocytes at 460 nm wavelength was measured using a 100x objective of a microscope photometer with a fast scanning stage, motor controlled plate. The average concentration of hepatocyte chromophore in each liver was obtained from at least six different liver sections. Measurements were performed from liver sections of mice previously treated (7 days) with 100 µl of SSC buffer, large and small empty liposomes plus free pTG7101 plasmid (80 ng/mouse) and encapsulated pTG7101 plasmid (80 ng/mouse) in large and small liposomes.The results are expressed as the mean ± SE of hepatocyte chromophore concentration per mice group. The statistical significance of the data compared with buffer treated group was evaluated by Student t test.

DISCUSSION

In the present work we show that i.v. mouse injection of pTG 7101 plasmid, containing a human α1-AT gene, encapsulated in small liposomes is an efficient procedure for gene transfer to liver parenchymal cells. This procedure induced hepatocyte production of antiprotease α1-AT, as evaluated by immunohistochemical and cytophotometrical methods.In contrast, very low efficiencies were observed with equivalent doses of DNA encapsulated in large liposomes.

A wide variety of liposomes (22,31,2,3) related to the lipid composition and preparation method, can be prepared. We have used liposomes containing PS because negatively charged liposomes may spontaneously bind to plasma membrane and then are taken up more avidly than neutral liposomes (11,12). In addition, a number of early reports have indicated that the inclusion of cholesterol reduces the serum induced leakage of liposome contents and increases the clearance time of intact liposomes in the circulation as well as their efficacy as drug carriers (16,14,24). Our results indicate that CH:PC:PS liposomes are stable in plasma. Using encapsulated CF as a tracer we can observe that CF latency was 80-90% after 3h of liposome incubation, suggesting that they can be appropriate vehicles for i.v. *in vivo* use. However, a question concerning the efficiency of this procedure is the liposome ability to deliver the entrapped material to specific cells. In the present work we show that these liposomes are differently distributed in the liver as a function of their size. Thus, using PI encapsulated liposomes, we observe that large liposomes are mainly taken up by fagocytic cells (e.g. Kupffer cells) connected to the blood circulation, whereas small liposomes can escape from the blood stream through liver sinusoid pores and deliver the encapsulated material to

hepatocytes. These results agree with previous reports (28,1) and indicate that the small liposomes containing PS used in the present study have an easy acces to liver parenchymal cells. In addition, we have evidence that the supernatant fraction of ultracentrifuged 400 nm filtered liposomes yields a very homogeneous suspension of small liposomes with higher DNA/lipid ratio. Therefore, we can expect that these liposomes may be an appropriate carrier system for *in vivo* transfection of recombinant DNA to hepatocytes.

The introduction of genetic material into specific tissues, such as parenchymal cells, contributes to the development of gene therapy models and in particular to find new treatment strategies of hepatocyte related inherited diseases. Certain forms of gene deficiency have been regarded as candidates for gene therapy, but several of them would require very precise gene regulation. In contrast, deficiencies in circulating proteins such as α1-AT may not require tightly controlled levels of expression. For this reason we regarded the α1-AT gene as an appropriate candidate for specific gene therapy assay. To evaluate this possibility, mice were i.v. treated with small liposomes entrapping the pTG 7101 plasmid containing the full length of a human α1-AT gene. The results, based on immunohistochemical procedures on liver sections, indicate that a significant increase of α1-AT reactivity was observed in liver parenchymal cells, compared with liver sections from mice treated with free plasmid plus empty liposomes. In addition, no increased reactivity was observed when the immunohistochemy was made using normal rabbit serum as first antibody. Since low doses of DNA (80 ng/mouse) were required to induce this effect, the results support the idea that these small liposomes are efficient carriers to deliver functioning recombinant genes to hepatocytes. In contrast, when equivalent doses of DNA encapsulated in larger liposomes (>100 nm of diameter) were i.v. administered, the efficiency to increase the immunoreactive α1-AT into hepatocytes was largely limited. Other procedures have also been assayed for α1- AT gene therapy, such as autologous transplantation of *in vitro* transduced hepatocytes (18) but these procedures yielded a very low number of transduced cells, respect to the liver mass. Our results contribute to find new treatment strategies for hepatocyte related inherited diseases since small liposomes have the ability to deliver encapsulated DNA to the greatest number of hepatocytes and therefore the procedure offers the oportunity to obtain the highest efficiency for *in vivo* gene transfer to liver parenchymal cells, without surgical trauma. Unfortunately there are no specific animal models for α1-AT deficiency but it will be interesting to find out whether hepatocyte-transferred material using this procedure can contribute to increase the α1-AT plasma levels with functioning activity.

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