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P. Delépine,[2,3,4†*] C. Guillaume,[2†] T. Montier,[2,4] J. C. Clément,[5] J. J. Yaouanc,[5] H. Des Abbayes,[5] F. Berthou,[6] A. Le Pape,[7] C. Férec[1,2,3,4]

[1]*EFS-Bretagne, 46, rue Félix Le Dantec, BP 454, 29275 Brest, France*

[2]*Inserm EMI-U 01-15: "génétique moléculaire et épidémiologie génétique", 46 rue Félix Le Dantec, BP 454, 29275 Brest, France*

[3]*Laboratoire de biologie moléculaire et d'histocompatibilité, CHU Morvan, 5 avenue Foch, 29609 Brest, France*

[4]*Laboratoire de génétique somatique et constitutionnelle-transfert de gènes, Université de Bretagne Occidentale, Brest, France*

[5]*Laboratoire de Chimie Organique, UBO, UMR CNRS 6521, UFR Sciences, BP 809, 29285 Brest, France*

[6]*Laboratoire de Biochimie, Faculté de Médecine, 2, avenue Camille Desmoulin, 29200 Brest, France*

[7]*Laboratoire d'imagerie de ciblage et de vectorisation, Faculté de médecine, 37200 Tours, France*

*Correspondence to: P. Delépine, Inserm EMI-U 01-15, 46 rue Félix Le Dantec, BP 454, 29275 Brest Cedex, France. E-mail: pascal.delepine@univ-brest.fr

Abstract

Background A multitude of cationic lipids have been synthesized since they were first proposed for use in gene therapy. Cationic lipids are able to efficiently transfect cells both *in vitro* and *in vivo*. Whereas most research groups have focused their investigations on the toxicity of these molecules, and on the location of expression of the DNA transferred by these vectors, little has been done to determine their biodistribution and elimination pathways. Our group has developed a family of cationic lipids termed phosphonolipids. Following a large *in vitro* screening experiment, we have selected several molecules for *in vivo* testing, with some of these phosphonolipids forming lipoplexes efficient in transfecting mouse lungs. It was thus of interest to study their fate after intravenous injection.

Methods The respective biodistributions of both the GLB43 phosphonolipid and plasmid DNA were investigated and compared with DNA expression sites. Using the optimal conditions determined for phosphonolipids, we followed the gene transfer agent and plasmid DNA distributions versus time by radiolabeling them with ^{14}C and ^{32}P, respectively. Otherwise, we performed imaging by radiolabeling plasmid DNA with ^{99m}Tc.

Results The lipoplexes appear to be directly located in the lung after administration. Secondly, the plasmid is released mainly into the lungs and the phosphonolipid vector is rapidly degraded. The hydrophilic moiety of the phosphonolipid is eliminated in the urine, as is the free plasmid.

Conclusions This study reveals that there are slight differences in the observed results depending on the technique used to label the DNA; secondly, results show that the residence time of phosphonolipids in the mouse body is related to the DNA binding time. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords lipoplexes; phosphonolipids; biodistribution; imaging; small animal model

Introduction

The concept of gene therapy consists of the transfer of a gene into a cell with the aim of treating hereditary or acquired diseases. Because of electrostatic repulsion between DNA and cell surfaces, naked DNA is effective only in transfecting cells of particular tissues, for example, muscle [1–3]. Thus,

strategies for assisting DNA transfer have been developed. A variety of vectors including liposomes, viruses and cationic lipids have been tested [4,5] but, at present, no one vector can be considered as the perfect gene transfer agent. Although gene transfer using viruses occurs at high levels, viruses also induce inflammatory and immunological responses that, following repeated administration, decrease transfection efficiency [5,6]. Transfection levels with cationic lipids are lower than those obtained with viruses; however, they are well tolerated and no decreases in transfection efficiency have been reported after repeated administration. All of the above-mentioned vectors have been tested for gene therapy in various diseases, with some methods used in clinical trials.

Since the CFTR gene was initially cloned, cystic fibrosis (CF) has become an ideal candidate disease for the use of genetic therapy. As such, various research groups have tried to develop and adapt these vectors to achieve a sufficient level of CFTR gene transfection in the lung to correct the CF disease. It is now clear that the type of vector required is dependent on the disease being treated, and thus on the type of tissue and cells to be transfected [7]. Two major kinds of vectors are used in the treatment of CF: (1) viruses, most notably adenoviruses and adeno-associated viruses, because of their natural respiratory tract specificity, and (2) cationic lipids.

The use of cationic lipids is of interest because they have few side effects and are highly efficacious in transfecting the lung cells of rodents when delivered via intravenous injection [3,8,9]. However, this method of delivery can potentially result in systemic, rather than localized, cell transfection. To this end it is important to study plasmid DNA and lipid vector distribution. Although some groups have already reported such investigations, very few studies have been focused on the biodistribution of both components simultaneously.

Our group has previously shown the efficacy of some cationic phosphonolipids in the transfection of mice lungs via intravenous injection [10] and has studied the plasmid DNA distribution after lipoplex administration [11]. This report deals with the biodistribution of a lipoplex solution composed of GLB43, the most efficient of our ammonium polar head phosphonolipids, after systemic administration. To perform these experiments, both plasmid and lipid were radiolabeled with ^{32}P and ^{14}C, respectively; their localization was observed at various times after injection to determine which organs had been reached by the lipoplexes. In parallel, the plasmid was radiolabeled by chelation with ^{99m}Tc and an imaging sequence was performed to follow the plasmid distribution. We report here results that show the fast biodegradation of the lipoplexes and elimination of the split transfer agent following the release of the plasmid, mainly in the lungs.

Materials and methods

Cationic phosphonolipids

The phosphonolipid GLB43 (Figure 1) was synthesized by our group as previously described [12]. GLB43 was ^{14}C-radiolabeled by methylation with ^{14}C-methyl iodide (specific activity 55 mCi/mmol; Amersham, UK) during the final step of synthesis. The output of the last stage of the synthesis is 100%, giving GLB43 a specific activity of 55 mCi/mmol.

Plasmid DNA

Two different plasmids were used: pCMVLacZ and pCMVLuc, which encode β-galactosidase and luciferase proteins, respectively. Plasmids were produced in our laboratory and were transformed into the DH 5α strain of *E. coli*. DNA was isolated from bacterial culture using a Qiagen endofree GigaKit and its concentration determined by measurement at 260 nm on a spectrophotometer. The purity was checked using the A260/A280 ratio. Only the plasmids with a ratio value between 1.8 and 2.00 were used.

Radiolabeling of the plasmid DNA

The pCMV LacZ plasmid DNA was labeled with [α-P32] dCTP using a nick translation technique [13]. Residual unincorporated nucleotides were removed using a G50 Sephadex nick-column (Amersham Pharmacia). The specific activity of the probes was 20 µCi/µg. The [α-P32] plasmid was used within 24 h of labeling.

For imaging studies, only pCMVLuc was labeled by chelation with ^{99m}Tc. Its specific activity was 5 µCi/µg.

Lipoplex preparation

Cationic phosphonolipid was dissolved in chloroform in glass vials. To inject 5 µCi of ^{14}C per mouse, the solution of radiolabeled GLB43 was diluted with unlabeled solution to give a final concentration of 17.5% of ^{14}C-labeled GLB43. Chloroform was then evaporated by vacuum, resulting in a dry lipid film. Sterile pyrogen-free distilled water was added in appropriate amounts to the lipid. The vials were then sealed and stored overnight at 4 °C. The resulting solution was sonicated for 10 min in a bath sonicator (Prolabo, Paris, France). The plasmid and lipid were then combined and incubated for 30 min at room temperature to form the lipoplexes. The lipid-to-DNA charge ratio used was 4 as this ratio had been previously shown to be the optimal for GLB43 [9]. Each mouse received 50 µg of plasmid DNA, with 5 µCi of each component (lipid and plasmid) in the preparation.

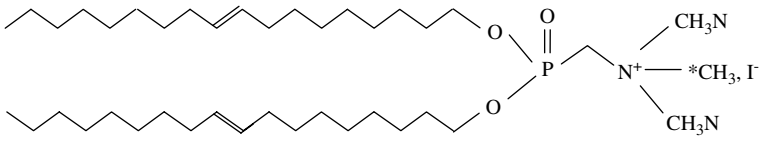

Figure 1. Structure of GLB43. *C indicates the labeled carbon atom when the final synthesis step used ^{14}C-methyl iodide

Lipid stability

The phosphonolipids were expected to degrade at low temperatures due to their thermal sensitivity. In addition, lipid stability at animal body temperature was also assessed. Thin-layer chromatography (TLC) on silica gel (Merck, Germany) was performed after various temperature treatments to evaluate the stability of GLB43. Untreated GLB43 powder was dissolved in chloroform and applied as the first spot to provide a reference, as this is the usual way the phosphonolipids are prepared. The second spot corresponded to a sample of the unlabeled sonicated lipid solution used for animal testing. For the third spot we applied the ^{14}C-GLB43 sonicated solution. The synthesis intermediates obtained just before the adjunction of methyl iodide were also applied to the plate (fourth spot), and, finally, sonicated lipid solution that had been incubated for 15 min at 40 °C (slightly over mouse body temperature) constituted the fifth spot.

The chromatogram was developed to 10 cm with ethanol. For visualization, the plate was sprayed with sulfuric acid, dried, then sprayed with absolute ethanol and immediately burnt.

Distribution of the lipoplexes in mice

All the animal experiments were conducted in accordance with the "Principles of laboratory animal care" (NIH publication #85-23 revised 1985) and with the agreement of the regional veterinary services (authorization A29019-3, April 28th, 2000). Five-week-old female Swiss mice were used. Once anesthetized by ether inhalation, each mouse received 200 µl of the lipoplex solution as an intravenous bolus in the tail vein. Mice were then transferred to metabolism cages. For these experiments, the ^{32}P-labeled plasmid was diluted with cold plasmid solution and then mixed with the phosphonolipid. Five µCi of each component (lipid and plasmid) were injected into each mouse.

Sample collection

A time-course experiment (5, 15 and 30 min, 1, 6 and 24 h) was performed using 2–5 animals, which were anesthetized and euthanized by exsanguination (cardiac puncture). It should be noted that at 6 and 24 h, the animals received only ^{14}C-radiolabeled lipoplexes. Their lungs, liver, spleen, heart, kidney, brain, skin, viscera, tail and carcass were then harvested. Urine was collected in the metabolism cages. Each organ and the carcass were then crushed in mortars cooled by liquid nitrogen. The total radioactivity of each pulverized organ was measured by liquid scintillation in a 1600 CA Tricarb liquid scintillation analyzer (Packard). The ^{32}P measurements were performed without scintillation liquid, the ^{14}C measurements after addition of scintillation liquid.

Imaging studies

Six-week-old female Swiss mice were anesthetized by ether inhalation. Mice then received 200 µl of saline solution (NaCl 0.9%) containing either a lipoplex solution or plasmid solution that corresponded to 50 µg of ^{99m}Tc-radiolabeled plasmid DNA via the tail vein. Each preparation was tested once on five mice. The distribution of radioactivity was evaluated by means of a specially designed high-resolution imaging device (Gamma imager, Biospace; France). Images were recorded just after injection and then 30 min, 3 and 6 h post-injection. The scintigraphic technique used was based on the one described by Lerondel *et al*. [14].

Luciferase expression

We decided to explore gene expression in the three organs that exhibited the highest levels of radioactivity: the lungs, the liver and the spleen. Mice were injected intravenously as described above with unlabeled lipoplexes containing 50 µg of plasmid. Luciferase activity was assayed at 24, 48, 72 h and 7 days post-administration using a 'luciferase assay system' (Promega). Five mice were used for each time point. After collection, each tissue was crushed in a mortar cooled by liquid nitrogen and incubated with lysis buffer for 30 min (Promega). Centrifugation at 10 000 *g* was performed and supernatant luciferase activity was assayed using an MLX microtiter plate luminometer (Dynex Technologies). The RLU values were converted into picogrammes of luciferase by means of a standard curve established for each type of tissue. The total protein concentration of these supernatants was measured with a Coomassie Plus protein assay kit (Pierce). Results are also expressed in picogrammes of luciferase per mg of total proteins.

Results and discussion

In a previous study we investigated the ability of some cationic phosphonolipids to efficiently transfect mouse

lung. Using our best phosphonolipid, GLB43, the optimal conditions for lung transfection via intravenous injection were obtained with a lipid-to-DNA charge ratio of 4 without co-lipid. Under these experimental conditions, 24 h after injection, the maximum level of expression is 500-fold higher than that obtained with naked DNA [9]. Moreover, we have shown that this kind of lipoplex is able to transfect lung epithelial cells (particularly type I pneumocytes) after an intratracheal administration [15]. At this stage of the work, we felt that it was important to determine the biodistribution of the lipoplexes in the mice.

Expression levels

We performed an expression study in the lungs, the liver and the spleen of the mice. The lungs were chosen because they concentrate the main part of the radioactivity and because they are our organs of interest. Liver and spleen were chosen because they are major organs of the reticulo-endothelial system (RES) and they may capture lipoplexes.

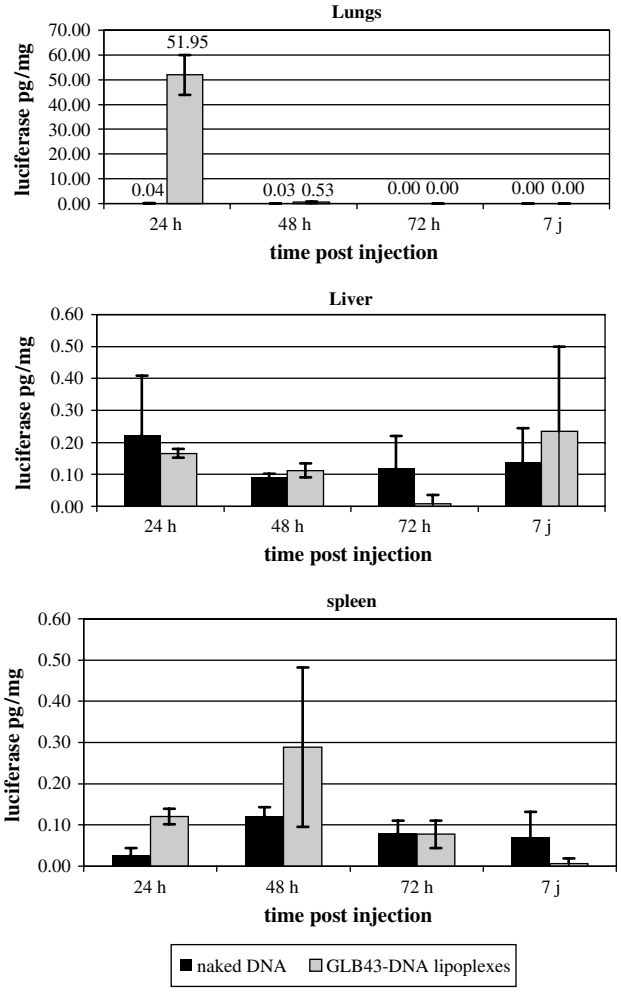

Figure 2. Luciferase expression levels in the lungs, liver and spleen, the three organs predominantly accumulating the plasmid DNA. Each animal received an intravenous injection of a preparation containing 50 μg of plasmid DNA. Evaluation of the transfection was performed 24, 48, 72 h and 7 days after injection. (Mean ± SD)

As depicted in Figure 2, the expression levels obtained under the above-mentioned conditions are notable only in the lungs. This organ specificity was noted, despite some liver localization, with gene expression driven by the CMV promoter expected to result in ubiquitous expression of the transgene. This expected ubiquitous expression can be discussed because some expression has been reported in the nose with a construction using a KRT18 promoter whereas the same construction with a CMV promoter did not exhibit any [16].

Osaka *et al*. have already reported such discordance (localization/expression) and correlated them with an accumulation of DNA essentially in the lungs and RES tissues [17]. In the investigations reported here we attempted to better understand this phenomenon by following the biodistribution of the lipoplex components, i.e. both the lipid and the DNA.

Thin-layer chromatography

In parallel to the experiments on biodistribution, we studied the stability of GLB43, which is expected to be thermosensitive. Confirmation of the identity of radiolabeled and unlabeled GLB43 as well as their lipid stability were checked by TLC. Figure 3 shows the chromatogram obtained after detection. One should note that the migration of the sonicated and unsonicated lipidic solutions is similar; therefore, sonication did not alter GLB43. The same observation was performed for ^{14}C-labeled GLB43, and unlabeled GLB43 spots. However, after migration, the occurrence of a second spot that did not exhibit any radioactivity is noticeable. This corresponds to about 5% of unlabeled lipid (synthesis intermediate).

This experiment also revealed that a 15-min incubation time at 40 °C caused a partial degradation of GLB43, which consisted of the loss of one acyl chain. Indeed, three spots corresponding to intact GLB43, GLB43 with only one acyl chain, and a free fatty acyl chain are also observed on the chromatogram.

Biodistribution

Some biodistribution studies have already been performed but, in most cases, they have dealt only with the distribution of DNA [18–20] or lipid vectors alone [21,22]. The studies that deal with both factors at the same time are usually performed by labeling them with an external marker such as ^{3}H-cholesteryl hexadecyl ether for the lipid, or by using ^{14}C-inulin to represent the DNA [23]. Rhodamine was also used to follow either DNA or lipid distribution in the lungs [24]. These approaches may not reflect the true situation: most researchers in this field are indeed aware of the importance of co-lipid adjunction and the fact that the addition of products to the

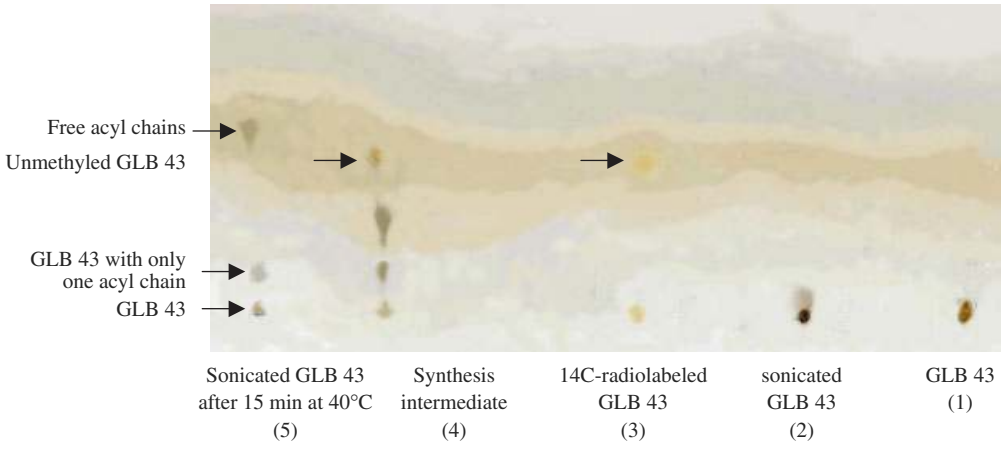

Figure 3. Chromatogram obtained after migration of GLB43 samples differentially treated. Each sample was applied onto a silica gel plate. The chromatogram was developed to 10 cm using ethanol, and then revealed

lipid formulation is likely to affect the lipoplex behavior. Moreover, products like inulin do not bear anionic charges like DNA, and thus the resulting complex may not have the same chemical properties with regard to interactions, stability and reactivity as those of the true lipoplex.

As such, we chose to follow plasmid DNA and GLB43 by radiolabeling them. This technique does not alter the integrity of the lipoplex, and thus neither its behavior nor fate. To observe the distribution of the lipoplexes, the release of the DNA from the lipoplexes and the elimination pathway of its different components, we radiolabeled DNA and GLB43 with two different markers: the lipid was labeled with ^{14}C and the plasmid with ^{32}P (Tables 1 and 2). Five minutes after injection of the GLB43/DNA lipoplex, radioactivity was no longer detectable in the blood. The radioactivity from ^{14}C and ^{32}P were mainly located in two organs: the lungs and the liver, and, to a lesser extent, in the urine. Some radioactivity was also found in the tail and likely resulted from the diffusion of the lipoplexes through the capillary endothelium during the injection. As shown in Tables 1 and 2, a slight increase is noted in the lung radioactivity values for the lipid and the plasmid at 15 min in comparison with the values at 5 min.

Beyond 15 min, ^{14}C-lipid distribution significantly changed: in parallel with a moderate rise in the liver and a large increase in the urine, a fast and significant ($p < 0.05$) decrease was observed in the lungs. At 30 min, the respective levels of radioactivity in the urine and the organs fell by 10–30% of the injected dose.

After 15 min the plasmid distribution [^{32}P] changed as seen for the lipid: a decrease was also observed in the lungs, which, however, remained the most radioactive organ. A significant ($p < 0.05$) rise in the plasmid content of the liver and a less marked one in the spleen were also observed. The radioactivity in the urine stayed low. Therefore, within 5–15 min after injection into the mice, the plasmid and the cationic lipid vector were co-located and mainly found in the lungs. After 15 min, the biodistributions of ^{32}P and ^{14}C components were the same. Later, one could note differences in that their biodistribution was no longer concomitant. This suggests that in the period between the injection time and 15 min, GLB43 phosphonolipid and plasmid are still associated and thus the integrity of the lipoplexes is conserved. They are mainly located in the lungs, which constitute the first microvascular bed encountered after a tail vein injection. These results are in agreement with those previously reported by Li *et al*. [25]. These authors showed that when lipoplexes are exposed to serum *in vitro*, they aggregate first and then release DNA and lipid, the latter being further degraded. With GLB43, the first step occurs in the first 15 min.

Concerning the lipoplex degradation, after 15 min, the amount of phosphonolipid released from the lungs is far greater than that of the plasmid. Three different hypotheses can be put forward to explain this phenomenon: (1) when lipoplexes are internalized via an endocytic pathway, the plasmid DNA is released from the lipoplexes and stays in the cell (cytosol or nucleus), while the phosphonolipid, or at least its hydrophilic moiety, is rapidly excreted from the cell; (2) the DNA alone penetrates the cell and the phosphonolipid is eroded by blood flux in the vessels; (3) lipoplex aggregates are progressively eroded, which results in the fast elimination of phosphonolipids, whereas the degradation of plasmid DNA is much slower. According to some preliminary studies conducted within our group, DNA decompaction *in vitro* takes place in the cell only after endocytosis (Montier *et al*., submitted). This observation supports the first hypothesis suggested above.

At the 30-min time point, ^{32}P and ^{14}C studies indicate that about 30% of the lipid and DNA are located in the liver. This suggests either a secondary aggregation after pulmonary release or a metabolic route for the lipoplexes. At the same time, a further third of the ^{14}C radioactivity is detected in the urine, and 1 h post-injection it accounts for about 70%. This suggests degradation of GLB43 and the elimination of its radiolabeled hydrophilic moiety via urinary excretion. This latter process takes place after the release of the DNA because no significant ^{32}P radioactivity is detected in the urine. The results obtained with GLB43

Table 1. Percentage of ^{14}C radioactivity present in organs of injected mice. ^{14}C-Radiolabeled lipoplexes were injected into the mice via the tail vein. Radioactivity in each organ was counted at different times after injection, as indicated. At 30 min and beyond, the total ^{14}C radioactivity was no longer 100% due to the collection difficulties after urinary elimination

	blood	brain	heart	lungs	liver	spleen	urine	viscera	carcass	tail	skin
5 min[a]	0.22 ± 0.18	0.04 ± 0.02	0.61 ± 0.18	52.9 ± 39.54	15.80 ± 10.71	1.38 ± 0.87	8.14 ± 5.40	1.87 ± 1.16	2.66 ± 1.69	15.12 ± 18.52	1.25 ± 1.17
15 min[a]	0.08 ± 0.08	0.04 ± 0.02	0.20 ± 0.04	69.27 ± 7.55	11.76 ± 1.49	0.65 ± 0.34	2.28 ± 0.56	1.40 ± 0.50	1.65 ± 0.76	12.09 ± 10.62	0.58 ± 0.28
30 min[c]	0.07 ± 0.06	0.05 ± 0.02	0.31 ± 0.28	9.42 ± 8.29	12.25 ± 12.45	2.05 ± 1.42	29.25 ± 4.26	2.81 ± 1.40	3.73 ± 0.25	3.91 ± 3.09	1.94 ± 1.63
1 h[c]	0.02 ± 0.01	0.05 ± 0.01	0.15 ± 0.06	2.00 ± 0.97	2.50 ± 1.60	1.78 ± 1.01	22.37 ± 3.54	0.58 ± 0.15	0.53 ± 0.26	5.29 ± 4.60	0.13 ± 0.05
6 h[b]	0.01 ± 0.00	–	0.02 ± 0.01	0.56 ± 0.26	2.14 ± 1.43	0.52 ± 0.22	–	–	–	–	–
24 h[a]	0.00	–	0.00	0.29 ± 0.27	0.76 ± 0.01	0.40 ± 0.12	–	–	–	–	–

[a] n = 3; [b] n = 6; [c] n = 7. (Mean ± SD).

Table 2. Percentage of ^{32}P radioactivity present in organs of injected mice. ^{32}P-Radiolabeled lipoplexes were injected into the mice via the tail vein. Radioactivity in each organ was counted at different times after injection, as shown. Values have been corrected for the radioactive decay of ^{32}P

	blood	brain	heart	lungs	liver	spleen	urine	viscera	carcass	tail	skin
5 min[a]	0.42 ± 0.05	0.10 ± 0.02	0.68 ± 0.13	52.28 ± 22.53	20.86 ± 3.40	4.09 ± 0.86	2.00 ± 0.38	1.16 ± 0.25	1.59 ± 0.37	16.41 ± 17.52	0.42 ± 0.27
15 min[a]	0.08 ± 0.10	0.03 ± 0.01	0.11 ± 0.07	76.14 ± 13.82	7.21 ± 0.42	0.75 ± 0.30	0.2 ± 0.04	0.62 ± 0.22	0.52 ± 0.10	14.16 ± 14.78	0.15 ± 0.15
30 min[a]	0.23 ± 0.05	0.08 ± 0.02	0.29 ± 0.03	43.20 ± 2.51	35.59 ± 5.16	8.35 ± 0.81	2.20 ± 0.07	2.85 ± 1.37	2.73 ± 0.62	3.77 ± 4.12	0.72 ± 0.56

[a] n = 3. (Mean ± SD).

also show that the release of the DNA in the lungs from the lipoplexes occurs in the first 60 min after injection, as already reported for other cationic lipids [26]. According to Osaka *et al*. [17], who used a different lipid vector, this release can occur later than 1 h.

We have also observed that after 30 min about 30% of the DNA is located in the liver. However, despite gene control under a CMV promoter, no gene expression was seen in this organ (Figure 2). One can explain this observation as follows: (1) either the plasmid DNA is released from the lipoplexes in the lungs and partially degraded during its travel in blood stream before reaching the liver; or (2) some lipoplexes that have reached the liver after lung aggregation are caught by the RES cells before being directed mainly to the lysosomes.

Imaging studies

Through the use of specialized imaging techniques, we followed the distribution of the ^{99m}Tc-labeled plasmid. First, it is of note that the plasmid alone is directly eliminated in the urine (Figure 4a). This may explain why plasmid alone is not able to transfect mice when intravenously injected. When the complexes are administered intravenously, the radioactivity is immediately located to the lung and more generally in the chest (Figure 4b). At 30 min, the area showing radioactivity had increased and included the whole chest. A small amount is also visible in the liver. The most important change, however, is the large quantity of radioactivity seen in the bladder. At 1 h, the lung radioactivity is still predominant, but a slight increase in the liver signal is also noticed (data not shown). At 3 h, the signal intensity in the lungs progressively attenuates with the liver having a negligible radioactive signal. The radioactivity seen in the bladder also decreases by elimination in the urine. At 6 h, the residual signal remains essentially located in the lung. The liver radioactivity has also largely decreased with the radiolabeled elements eliminated in the urine.

This observation was not made in the ^{32}P study, probably because of the short time over which the experiment was conducted (30 min). Indeed, the use of a chelate formed by associating ^{99m}Tc and plasmid is a common technique [27]. So, whereas it has been shown that the nature of the chelator influences the biodistribution [28], the chelate is expected to be stable enough to remain intact in the blood stream and thus to reflect the plasmid DNA movement over the course of a few hours. After this time, the interaction between the chelates and albumin can interfere significantly with the results by exchange of ^{99m}Tc. This phenomenon impedes imaging beyond 6 h after intravenous administration.

Otherwise, during the first 30 min of the imaging study, no elimination of ^{99m}Tc nor of plasmid DNA occurred (the total radioactivity only decreased as a result of the short half-life of the technetium) but a large quantity is present in the bladder. Some signal is also present in

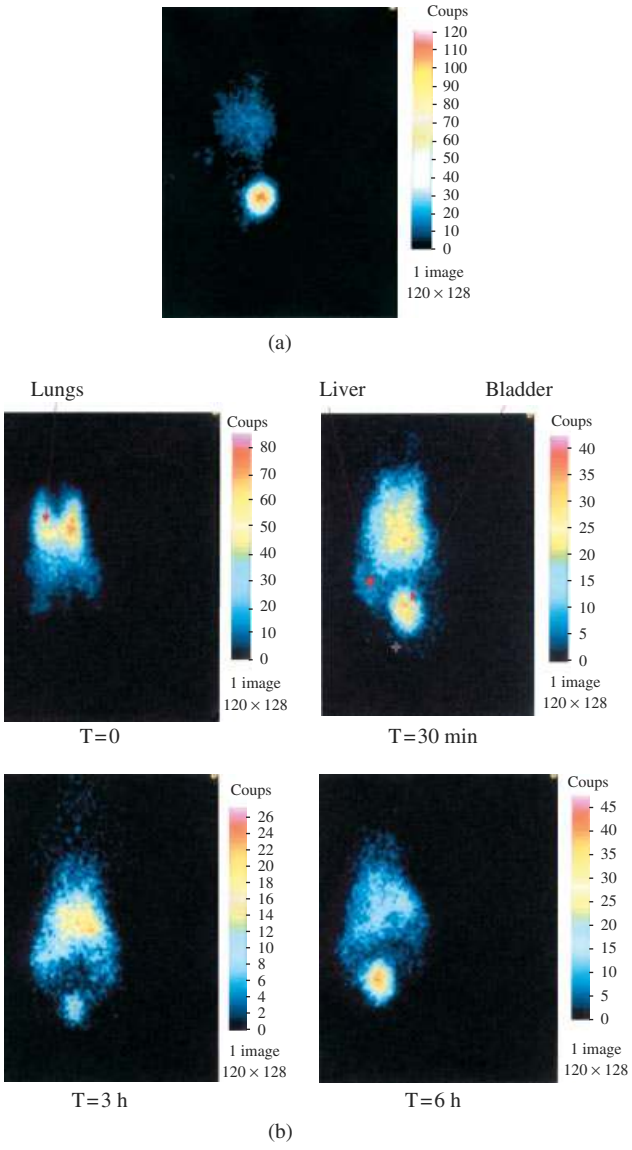

Figure 4. Kinetic study by scintigraphy. (a) Scintigraphic shot of a mouse 30 min after an intravenous injection of 50 µg of plasmid DNA labeled with ^{99m}Tc. (b) Mice were injected intravenously with a GLB43 lipoplex solution in which plasmid DNA was labeled with ^{99m}Tc (see Materials and Methods). Scintigraphy of each animal was performed at injection times, and at 30 min, 3 and 6 h post-injection

the liver. With these results, a difference was noticed between the two ways of labeling plasmid DNA. Indeed, the ^{32}P study revealed hardly any plasmid DNA presence in the urine 30 min after injection, contrary to the ^{99m}Tc imaging. This could suggest a degradation of the ^{99m}Tc-plasmid chelate followed by urinary elimination of the ^{99m}Tc. However, if this is the case, ^{99m}Tc would have been bound by serum albumin and its urinary presence is unjustified. This difference has not yet been explained. Surprisingly, this urinary location seen by imaging is also observed with free DNA chelates (not associated as a lipoplex) but, in this case, nearly all the signal is located in the bladder (Figure 4a). This eliminates the hypothesis of a poor chelating efficiency but reinforces

the idea of free plasmid that is not coupled in a lipoplex, despite the theoretical excess of phosphonolipids (charge ratio = ±4). The distribution also suggests a progressive release of DNA from the lipoplexes. The two hypotheses discussed above may also coexist with a primary tendency towards the release of DNA from lipoplexes, eliminated in the urine, and to a lesser extent a relocation of some lipoplexes from the lung to the liver. The signal trace in the liver provides proof for this hypothesis.

Conclusions

The use of two different techniques to follow lipoplex biodistribution reveals a slight difference in biodistribution pattern according to the method used, i.e. internal (^{32}P) or external (^{99m}Tc) labeling of the plasmid DNA. Thus, it demonstrates how the technique used to study the distribution influences the final result. Such differences have been reported by Bogdanov [19], who compared ^{32}P- and ^{99m}Tc-peptide-based labeling. Indeed, the slight modification in the composition has an important impact on the properties of the lipoplexes (size and zeta-potential importance).

Taking into account the desirability of our molecules compared with others, their fast elimination after DNA release is a major benefit. Whereas in mouse lungs the decrease in both DNA and other cationic lipids was similar [26], GLB43 levels decrease more rapidly than that of the plasmid DNA. Thus, it appears that the GLB43 in excess is quickly eliminated after DNA release. This is of great interest because it limits the damage due to an accumulation of exogene molecules. Moreover, whereas a rapid release from the lipoplexes is usually considered as inducing low efficiency, GLB43 results in attractive transfection levels in the lungs.

In conclusion, the structure of GLB43-plasmid DNA lipoplexes is maintained for approximately 15 min. After this time period, GLB43 is rapidly altered and its hydrophilic moiety, which is reactive, is eliminated in the urine. This is desirable because the shorter the amount of time this reactive moiety spends in the body after DNA release, the better it is for the organism. In addition, the lipidic component very likely has an hepatic metabolism similar to the natural fatty acyl chain. Thirty minutes after injection, following its release from the GLB43 complex, the plasmid DNA is still principally located in the target tissue, the lungs, and also in the liver. Therefore, the relative instability of GLB43 in the blood stream could be one of its main assets. These results are very informative but, although the levels of both DNA and lipid parts significantly differ within time when using ANOVA or Student's *t*-test, we have to keep in mind they were performed only on small size samples. Moreover, further studies dealing with DNA biodistribution should be carried out for longer periods of time. It would also be of interest to study the acyl chain distribution and to correlate it with the observed hepatic toxicity.

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