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Biochimica et Biophysica Acta 1368 (1998) 276–288

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes

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Received 2 June 1997; revised 31 July 1997; accepted 7 August 1997

Abstract

Most of the cationic lipids used for gene transfer experiments drastically lose their efficiency in the presence of serum. We used a cationic lipid with a spermine head group and its fluorescent analog to study the cellular uptake and the intracellular fate of lipoplexes in the presence and absence of serum. We found that the amount of DNA and lipid taken up by the cells was not related to the efficacy of the gene transfer. When the lipofection was performed in the presence of serum, lipoplexes were contained within small intracellular vesicles. In the absence of serum, the vesicles were larger and heterogeneous in size and shape. By analysis of their size distribution, we showed that lipoplexes preformed in the absence of serum tended to aggregate. This aggregation was inhibited in the presence of serum. We used a carbonate formulation that led to the preformation of large particles: those large particles gave a high lipofection efficiency in the presence of serum and their intracellular distribution was identical to that observed in the absence of serum. © 1998 Elsevier Science B.V.

Keywords: Cationic lipid; DNA; Gene transfer; Serum; Cellular uptake

1. Introduction

There is growing evidence that gene therapy will play a role in the future for the treatment of genetic disorders and acquired diseases [1]. However, in order for gene therapy to be effective, it is necessary to develop vectors that transport and introduce DNA into the target cells, leading to the expression of the therapeutic gene. Practically, an ideal gene delivery vector should have the following characteristics: protecting and delivering DNA efficiently; non-toxic and non-immunogenic; and easy to produce in large quantities.

Compared with viral transfection systems, cationic lipids represent one of the few examples that can meet these requirements [2]. For this reason, cationic lipids have been used to transfect cells both in in vitro and in vivo experiments (reviewed in [3]). Several formulations containing cationic lipid are being used in clinical trials in cases of cystic fibrosis [4] or

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DOPE, dioleoyl phosphatidylethanolamine; FBS, fetal bovine serum; lipofection, cationic lipid-mediated transfection; lipoplexes, cationic lipid/DNA complexes; MEM, minimum essential medium; PBS, phosphate-buffered saline, pH 7.4

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PII S0005-2736(97)00194-6

cancer [5].

Formulation of DNA with cationic lipids, often in combination with a neutral phospholipid such as dioleoyl phosphatidylethanolamine (DOPE), results in condensed particles, termed as lipoplexes [6], formed by ionic interaction between the cationic lipid and negatively charged DNA and subsequent hydrophobic interactions between the lipid moieties [7]. The DNA in these formulations is protected from the environment and exhibits increased resistance to nucleases [8–10]. By condensing the DNA and generating a particle with specific properties (size, charge surface characteristics), cationic lipids may also cause the particle to interact with the surface of the cell and induce its uptake. A net positive charge, and therefore a cationic lipid excess, has been observed for optimal in vitro lipofection efficiency, likely because of the net negative charge exhibited by the plasma membrane [8,11,12]. Although much attention has been focused on elucidating the mechanism by which the DNA is introduced into the cells, a common and generally accepted mechanism has not been identified. Most of the authors suggest that intracellular delivery of the DNA occurs via endocytosis [13–15], whereas others tend to believe that fusion events at the plasma membrane are involved in the uptake of DNA [16]. Phagocytosis, pinocytosis [17] or pore formation mechanism [8] have also been proposed to account for the internalization process of lipoplexes. Membrane-associated proteoglycans could mediate the binding and delivery of lipoplexes into cells [18].

The effectiveness of gene delivery in vivo is poorly predicted by in vitro results [19] because the various biological barriers and the interaction of lipoplexes with blood elements are not reflected in in vitro systems. For instance, many formulations leading to efficient in vitro gene transfer in the absence of serum are not effective in systems that contain as low as 5–10% serum [12,20,21], an observation that has obvious shortcomings for potential in vivo applications.

We have synthesized a series of cationic lipids bearing a spermine head group, a lipophilic tail and, for one of them, a side chain harboring a rhodamine moiety [22,23] which displays a good lipofection efficiency of cultured cells, in the absence or presence of serum. However, in some conditions of formulation, the lipofection efficiency of these cationic lipids was much lower in serum-containing medium. In this paper, these transfecting agents were used in these serum-sensitive conditions for studying the cellular uptake of lipoplexes and their intracellular fate when lipofection was performed in the presence or absence of serum. Our results suggest that the main effect of serum is to interact with preformed lipoplexes, leading to a modification of their intracellular traffic.

2. Materials and methods

2.1. Cell culture

Two fibroblast cell lines were used: CV-1, derived from African green monkey kidney, and NIH 3T3 derived from NIH Swiss mouse embryo cultures. The CV-1 cell line was obtained from Dr. S. Koutouzov (INSERM U25, Paris). NIH 3T3 cells were obtained from the American Type Culture Collection. The cells were grown in Minimum Essential Medium (MEM, Gibco) for CV-1, or Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 4.5 g/l glucose for NIH 3T3, supplemented with 2 mM glutamine, penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively), and 10% (v/v) FBS. Cultures were maintained at 37°C in a 5% CO_2/air incubator.

2.2. Plasmids

Plasmid pCMVLuc carries a cassette containing the enhancer–promoter from the immediate-early gene of cytomegalovirus (CMV), the gene coding for the luciferase of *Photinus pyralis* and the polyadenylation site of SV40 [24]. Plasmid was grown using standard techniques and was purified using Wizard Megaprep kit (Promega) according to the supplier's recommendations. Radiolabeled plasmid was prepared by random priming using ^{35}S-dATPαS (> 1000 Ci/mmol, Amersham) and Random Primed DNA Labeling Kit (Boehringer, Mannheim, Germany). Fluorescent labeled plasmid was prepared by nick translation [25]. Two nmol of FluoroRed Rhodamine 4-dUTP (Amersham) were incorporated into 30 μg of plasmid DNA by nick translation using 100 pg of DNase I (Appligene Oncor), 20 U of DNA Poly-

merase I (Appligene Oncor) and 60 nmol of dATP, dCTP and dGTP, for 2 h at 16°C, in 50 mM Tris HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM DTT and 50 μg/ml Bovine Serum Albumin. Unincorporated nucleotides were separated by spin column chromatography (MicroSpin S-400 HR, Pharmacia). Nick translation was optimized to produce plasmid DNA modification at one fluorescent residue in 1000 base pairs.

2.3. Cationic lipids preparation and lipoplexes formation

The lipopolyamines used in this study, RPR 120535 and RPR 121653, were synthesized and characterized as described [22,23]. Stock solutions were prepared by dissolving lyophilized cationic lipids in distilled water at 25 mM. Cationic lipids were mixed and diluted in 150 mM NaCl, or in 150 mM NaCl/20 mM $NaHCO_3$. The plasmid pCMVLuc encoding the luciferase gene was diluted in 150 mM NaCl or in 150 mM NaCl/20 mM $NaHCO_3$, and mixed with an equal volume of the cationic lipids solution. At this stage, the RPR 120535 and RPR 121653 concentrations were 60 and 10 μM respectively, and the plasmid concentration was 10 μg/ml. These conditions corresponded to a theoretical charge ratio of 9.0, taking into account four possible positive charges on the cationic lipids. The final DNA/cationic lipids mixture was vortexed, left for 10 min at room temperature, diluted in culture medium (DMEM or MEM) in the absence or presence of 10% FBS and then added to the cells.

For lipofections using extracted plasmid, the same procedure was used except for the following modifications: after quantification of total DNA using Picogreen™ dsDNA quantitation reagent (Molecular Probes), the extracted plasmid was mixed with a carrier DNA, namely a plasmid without luciferase gene, diluted in 150 mM NaCl before mixing with an equal volume of RPR 120535. The lipofection was performed in the following conditions: 1 μg of DNA (carrier DNA + total extracted DNA) and 6 nmol of RPR 120535 per culture well (charge ratio of 7.7), diluted in serum-free medium.

2.4. Lipofection

The day before the experiment, NIH 3T3 cells were seeded into 6-well culture plates on the basis of 200 000 cells per well. Just before lipofection, cells were washed twice with fresh medium with or without FBS. The lipoplexes solution prepared as described above (Section 2.3) was added to the cells (6 nmol of RPR 120535, 1 nmol of RPR 121653 and 1 μg of plasmid per well, corresponding to a charge ratio of 9.0). 10% (v/v) of FBS was added to the culture wells containing serum-free lipofection medium after 2 h at 37°C. The cells were incubated for 24 h at 37°C in the presence of 5% CO_2. CV-1 were transfected with the same procedure, except that they were seeded two days before the experiment on the basis of 100 000 cells per well.

2.5. Luciferase assay

The transfected cells were washed twice with PBS, and lysed with 200 μl cell culture lysis reagent (Promega). Luciferase expression was quantified on 5 μl of centrifuged lysate supernatant, using a luciferase assay kit (Promega). Light emission was measured by integration over 10 s at 25°C using a Lumat LB9501 luminometer (EG and G, Berthold, Evry, France). Relative light units (RLU) were calculated versus background activity. Light emission was normalized to the protein concentration of each sample, determined using the Pierce BCA assay (Interchim).

2.6. Plasmid extraction and quantification

The transfected cells were trypsinized and washed once with PBS. Selective plasmid extraction was performed according to Hirt's protocol [26] with the following modifications: cells were resuspended in buffer A (25 mM Tris-HCl, pH 8; 10 mM EDTA, 50 mM glucose), then lysed with buffer B (0.2 M NaOH, 1% SDS). Most of the cellular DNA and proteins were removed by adding buffer C (3 M potassium acetate, pH 4.8; 2 M acetic acid) and centrifugation at 14 000 g for 10 min at room temperature. A volume ratio of 1/2/1.5 was used for the three buffers (A/B/C, respectively). After a phenol/chloroform extraction step, plasmid was then concentrated by ethanol precipitation and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The amount of extracted plasmid was then quantified by dot-blot analysis. The sample to analyze was heated at 100°C for 10 min then chilled on ice, and

applied onto a Hybond™-N membrane (Amersham) prewetted with 10 × SSC (1.5 M NaCl, 0.15 M Na_3 citrate). The membrane was placed for 5 min in denaturing solution (1.5 M NaCl, 0.5 M NaOH) then for 1 min in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2; 1 mM EDTA). The DNA was linked to the membrane by baking in an oven at 80°C for 2 h. The membrane was prehybridized using prehybridization solution (GIBCO) containing 6 × SSC, 5 × Denhardt's solution, 0.5% (w/v) SDS, 100 μg/ml sheared, denatured salmon sperm DNA, for 1 h at 65°C, and hybridized overnight at 65°C with 0.2 pmol of ^{35}S-labeled plasmid DNA. After several washes, the membrane was cut and radioactivity was counted by liquid scintillation. Serial dilutions of pCMVLuc in supernatants of non-transfected cells were used to generate a calibration curve with a linear regression coefficient higher than 0.98. The amount of extracted plasmid was determined by comparison with this calibration curve. Treatment of the plasmid by DNase I completely abolished the signal.

2.7. Cationic lipid quantification

RPR 121653 is a derivative of RPR 120535, which bears a covalently linked rhodamine moiety. It can therefore be easily quantified by measuring its fluorescence emission. The transfected cells were washed twice with PBS, and lysed with 200 μl cell culture lysis reagent (Promega). A 100 μl-aliquot of the centrifuged supernatant lysate was used to measure fluorescence emission at 590 nm (excitation 544 nm) in a Titertek® Fluoroskan II. The amount of cationic lipid RPR 121653 contained in the lysate, referred to as the amount of internalized cationic lipid, was determined by comparison with the fluorescent signal obtained with serial dilutions of known amounts of the cationic lipid in the same buffer.

2.8. Fluorescence microscopy

Cells were seeded on glass slides and transfected as indicated above. After 24 h, cells were rinsed three times with PBS, then fixed for 20 min in 3% (w/v) paraformaldehyde in PBS, washed, and mounted in Mowiol for examination. Slides were then analyzed with a Zeiss Axiophot microscope equipped with a Zeiss Neofluar 100 X objective lens.

2.9. Size distribution of lipoplexes

The size distribution of the lipoplexes was determined by dynamic light scattering in a Coulter N4 Plus particle analyzer. Unimodal fit yielded the mean hydrodynamic particle diameter. Samples were prepared as described above (Section 2.3), except that solutions were ten-fold more concentrated in order to obtain a signal in light scattering. The size distribution of the complexes was assayed in the initial incubation medium (150 mM NaCl or 150 mM NaCl plus 20 mM $NaHCO_3$) after 10 min or 2 h of incubation, or after dilution of the preformed complexes in serum-free or serum-containing DMEM and subsequent incubation for 10 min or 2 h.

3. Results

Most of the cationic lipids currently used for in vitro gene transfer are much less efficient in the presence of serum [12,20,21]. We synthesized a cationic lipid with a spermine head group, referred to as RPR 120535 [22]. Preparation and characterization of this cationic lipid, as well as its lipofection efficiency, have been described elsewhere [22,23,27]. It was shown that RPR 120535 has a high lipofection efficiency, both in vitro in the presence and absence of serum, and in vivo. However, in some conditions of formulation, the lipofection efficiency of RPR 120535 is reduced in the presence of serum. In this work, we have used two fibroblastic cell lines, NIH 3T3 and CV-1, with different lipofection abilities: NIH 3T3 are known to be readily transfectable by cationic lipids, while CV-1 cells display a lower lipofection capacity. These cells were transfected for 2 h with RPR 120535/pCMVLuc complexes in the presence or absence of serum, followed by 24 h of incubation in the presence of serum. Lipofection was performed in serum-sensitive conditions of formulation, namely preformation of lipoplexes in 150 mM NaCl, at a charge ratio of 9.0. As shown in Fig. 1, the luciferase activity was 100-fold higher in the serum-free medium in both cell lines. We first asked whether this decrease of lipofection efficiency originated from a decreased uptake of lipoplexes by cells as a result of the presence of the serum in the medium.

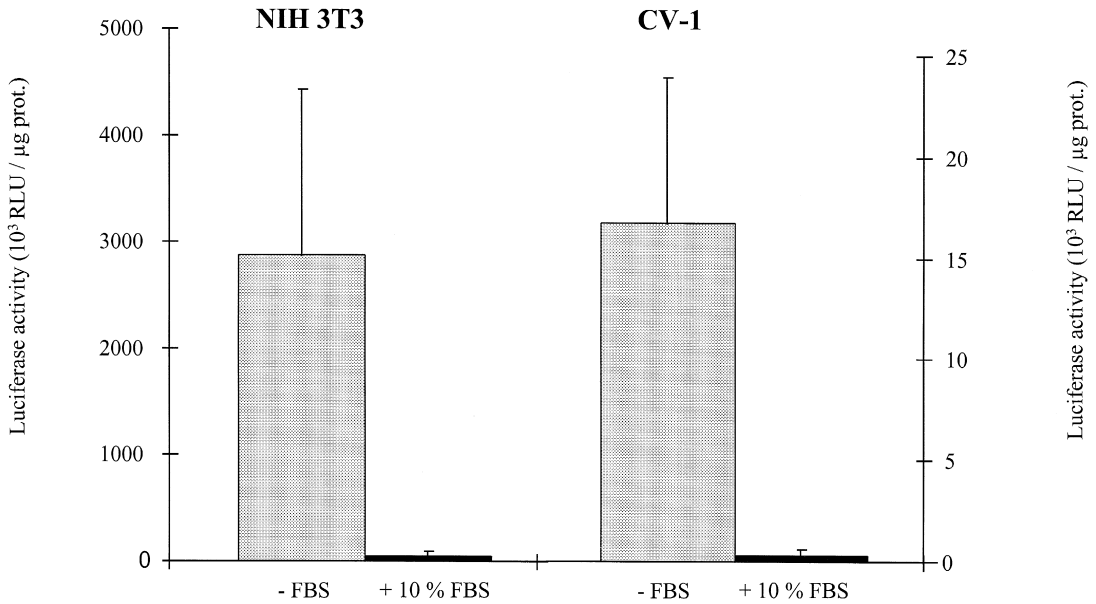

Fig. 1. Lipofection by lipopolyamine RPR 120535 in the presence or absence of serum. NIH 3T3 and CV-1 cells were transfected for 2 h with pCMVLuc complexed with RPR 120535 (6 nmol of cationic lipid per µg of plasmid, theoretical charge ratio of 7.7) in the absence or presence of 10% FBS. 10% of FBS was added in the culture wells containing lipofection medium without FBS after 2 h at 37°C. The cells were then incubated for 24 h before assay of luciferase expression. Values represent means ± SD ($n = 6$).

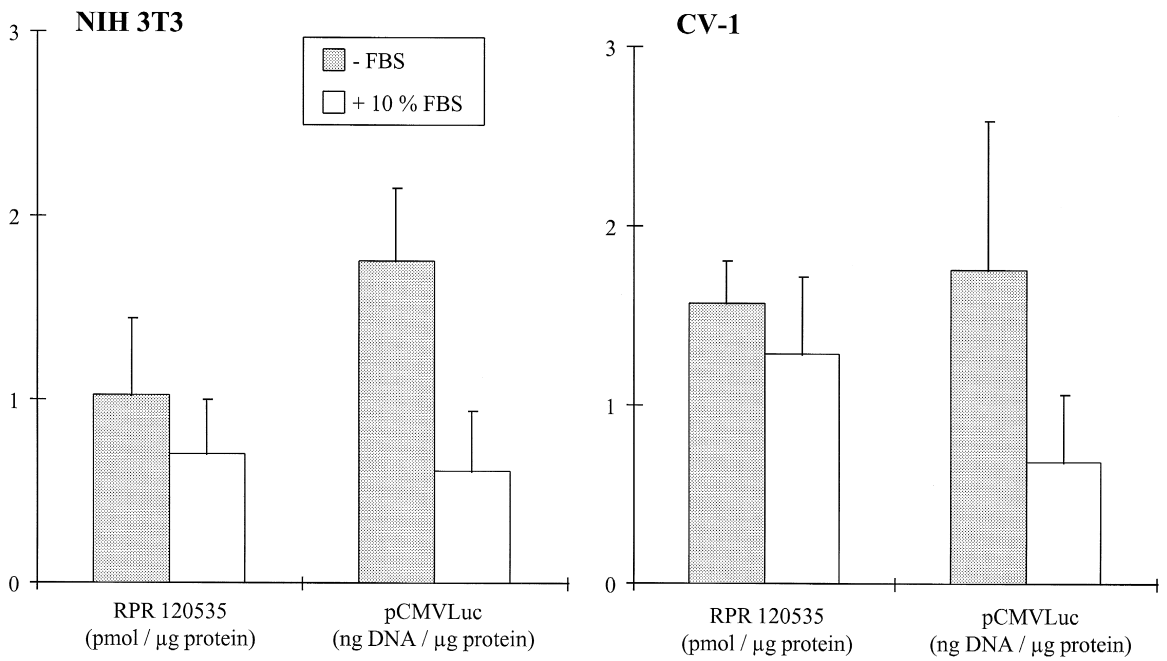

Fig. 2. Uptake of lipoplexes in the presence or absence of serum. NIH 3T3 and CV-1 were transfected for 2 h with pCMVLuc complexed with cationic lipids (6 nmol of RPR 120535 and 1 nmol of RPR 121653 per µg of plasmid; theoretical charge ratio of 9.0) in the absence or presence of 10% FBS. 10% of FBS was added in culture wells containing lipofection medium without FBS after 2 h at 37°C. The cells were then incubated for 24 h before assay of DNA uptake (dot-blot) and cationic lipid uptake (fluorescence) as described in Section 2. Values represent means ± SD ($n = 4$). The difference in the amount of DNA uptake between the two conditions of lipofection was significant for both cell lines ($p < 0.05$).

3.1. Uptake of lipoplexes

We quantified the amount of plasmid DNA and cationic lipid recovered in transfected cells 24 h after lipofection. We took advantage of a covalently rhodamine-labeled cationic lipid, analog to RPR 120535, referred to as RPR 121653, to assay the amount of internalized cationic lipid. As indicated in Fig. 2, for both cell lines, there was no sharp difference in the amount of internalized cationic lipid in the presence or absence of serum. The ratio of internalized cationic lipid to the initial amount used for lipofection was around 12% and 20% for CV-1 and NIH 3T3 cells, respectively. We used dot-blot analysis of clear lysate supernatants to estimate the amount of internalized plasmid. In the absence of serum, CV-1 and NIH 3T3 cells took up respectively 14% and 25% of the initial amount of plasmid DNA used for the lipofection. In the presence of serum, cells internalized two to three times less plasmid than in the absence of serum (Fig. 2). We therefore concluded that serum has little or no effect on the uptake of the lipoplexes.

3.2. Effect of the serum on degradation or inactivation of the transfected plasmid

Serum is known to exhibit an intrinsic DNase activity [9,28]. We first examined by agarose gel electrophoresis the plasmid extracted from the transfected cells and purified by phenol/chloroform extraction followed by ethanol precipitation. As shown in Fig. 3, the majority of the extracted plasmid was in a supercoiled or a relaxed form, whether the lipofection was performed in the presence or absence of serum. It was verified by a restriction analysis and comparison with the native plasmid, that the two DNA bands, the supercoiled and relaxed forms, actually corresponded to the plasmid pCMVLuc (data not shown). In the presence of serum however, the amount of supercoiled and relaxed plasmid was decreased. Neither linear plasmid nor fragments were visible. An additional band, which migrated between the relaxed and the supercoiled form of the plasmid, was also present in the negative controls and most probably originated from genomic DNA extracted from the cells.

We next examined whether the extracted plasmid was still functional for lipofection. The plasmid extracted from transfected NIH 3T3 cells in the presence or absence of serum was used to transfect NIH 3T3 cells again. Since the amount of extracted plasmid was low, it was diluted with a carrier DNA before mixing with RPR 120535 and incubation with NIH 3T3 cells, in order to obtain properly formed lipoplexes. The luciferase activity obtained was com-

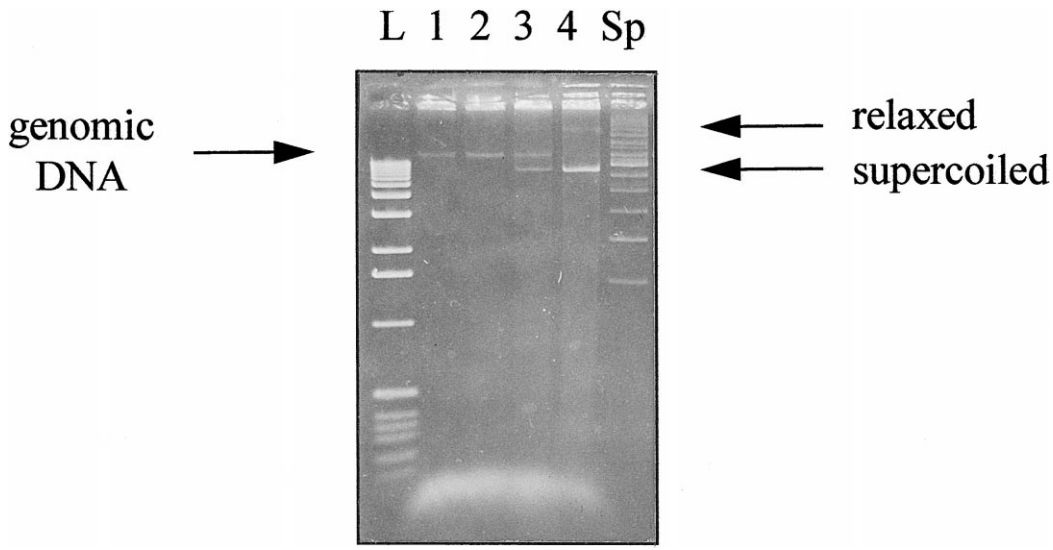

Fig. 3. Agarose gel electrophoresis analysis of plasmid extracted from transfected cells. DNA was extracted from CV-1 cells transfected with pCMVLuc complexed with RPR 120535 in the presence (3) or absence (4) of FBS, or from non transfected cells in the presence (1) or absence (2) of FBS. Electrophoresis was done on 0.8% agarose gel and DNA was stained with ethidium bromide. L: molecular weight marker for linear DNA fragments (1kb-ladder); Sp: molecular weight marker for supercoiled DNA fragments (1kb-ladder).

Table 1
Quantification of plasmid DNA extracted from transfected cells

Conditions of the first lipofection	[a]Amount of extracted pCMVLuc (ng) assayed by	
	Dot-blot analysis	Lipofection activity
+ 10% FBS	7.5	11.6
− FBS	17.0	15.7
Control (without plasmid)	n.d.[b]	n.d.[b]

[a] After 24 h-lipofection of NIH 3T3 in the presence or absence of serum, the plasmid was extracted from transfected cells. The amount of extracted plasmid was quantified either by dot-blot analysis or by measurement of the lipofection activity of NIH 3T3 cells as described in Section 2. The results are from one experiment that was a representative of one out of 3 experiments.
[b] n.d = non detectable.

pared with the luciferase expression obtained with known amounts of native pCMVLuc diluted in carrier DNA. These serial dilutions of pCMVLuc gave a linear relationship between the luciferase activity and the amount of DNA (regression coefficient higher than 0.98, data not shown). The amount of plasmid DNA calculated from the lipofection experiment was compared with the amount quantified by dot-blot analysis. As indicated in Table 1, the extracted plasmid has retained its functional integrity, whether the lipofection was performed in the presence or absence of serum. Moreover, the results obtained with the two methods were coherent. This indicates that the amount of plasmid DNA quantified by dot-blot corresponds to active plasmid DNA.

3.3. Modification of the intracellular traffic of lipoplexes by serum

We next investigated whether the serum has an effect on the intracellular pathway of the lipoplexes. The intracellular localization of the lipoplexes was analyzed by fluorescent microscopy, using the fluorescent cationic lipid RPR 121653 or a fluorescent nick-translated plasmid. It was observed that RPR 121653 was able to transfect both NIH 3T3 and CV-1 cells and that its lipofection efficiency was decreased by serum (data not shown). We found that plasmid DNA and cationic lipid were present in 100% of the cells, displaying a punctuate cytoplasmic staining, mostly in the perinuclear region (Fig. 4). However, a clear difference was observed depending on whether the serum was present or not, both in NIH 3T3 and CV-1 cells. When the lipofection was performed in a serum-free medium, the labeled cationic lipid appeared to be contained within vesicles that seemed very heterogeneous in size and shape (Fig. 4(B) and (D)). In the presence of serum, labeled vesicles were smaller and homogenous (Fig. 4(A) and (C)). The same pattern of fluorescence and the same difference in the presence or absence of serum were observed in cells transfected with RPR 120535 and a fluorescently labeled plasmid (Fig. 4(E) and (F)). When we exposed cells to fluorescently labeled dextran, a polymer which is endocytosed and delivered to the lysosomal compartment, we found a similar pattern of fluorescence as these observed with cells transfected in the presence of serum, i.e. small and homogenous intracellular vesicles (data not shown).

3.4. Modification of the size of the lipoplexes by serum

Since the main difference observed between the two conditions of lipofection was the size of the vesicles that contained the lipoplexes, we decided to

Fig. 4. Fluorescence microscopy of NIH 3T3 and CV-1 cells transfected with fluorescent cationic lipid or DNA. Cells were transfected for 2 h in the presence (Panels A, C, E and G) or absence (Panels B, D, F) of serum as described in the legend of Fig. 2, except that cells were seeded on slides. After 24 h of incubation, cells were fixed and mounted for observation. Panels A and B: NIH 3T3 cells transfected with labeled cationic lipid, panels C and D: CV-1 cells transfected with labeled cationic lipid. Panels E and F: CV-1 cells transfected with nick-translated plasmid. Panel G: CV-1 cells transfected with serum-insensitive particles (complexes of labeled cationic lipid and DNA preformed in the presence of $NaHCO_3$).

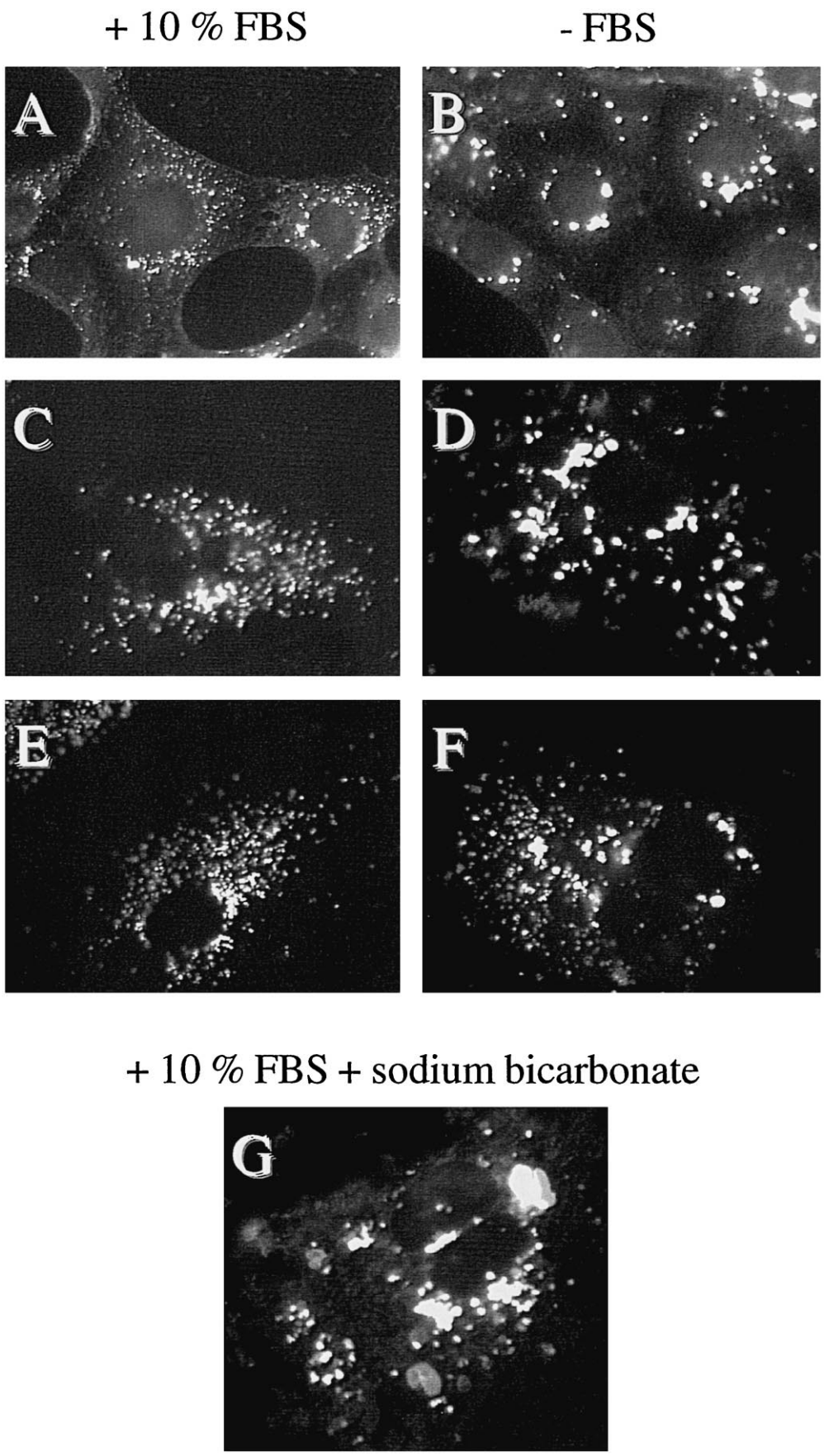
+ 10 % FBS
- FBS
A
B
C
D
E
F
+ 10 % FBS + sodium bicarbonate
G

Table 2
Size distribution of RPR 120535/RPR 121653/pCMVLuc complexes

Conditions of incubation	10 min Mean diameter ± SD (nm)	120 min
NaCl	135 ± 45	165 ± 61
NaCl (10 min) then DMEM + 10% FBS	532 ± 193	673 ± 239
NaCl (10 min) then DMEM − FBS	818 ± 365	1340 ± 603
NaCl/$NaHCO_3$	1717 ± 775	2903 ± 1131
NaCl/$NaHCO_3$ (10 min) then DMEM + 10% FBS	540 ± 218	1130 ± 535

Dilutions were prepared as described for the lipoplexes formation protocol (see Section 2.3), except that solutions were ten-fold more concentrated in order to obtain a signal in light scattering. The size distribution of the complexes was assayed in the initial incubation medium (150 mM NaCl or 150 mM NaCl plus 20 mM $NaHCO_3$) after 10 min or 2 h of incubation, or after dilution of the preformed complexes in serum-free or serum-containing medium and subsequent incubation for 10 min, or 2 h.

investigate the effect of serum on the size of the lipoplexes before incubation with cells. The size distribution of the lipoplexes was determined by dynamic light scattering. The assay was first performed in NaCl 150 mM (medium used for the initial formation of the lipoplexes). As indicated in Table 2, rather small and stable particles were obtained in the 100–200 nm diameter range. When these preformed small particles were diluted in DMEM, their size increased. In serum-containing DMEM, the particles reached a size of 500 nm that remained relatively stable up to 2 h. In contrast, in serum-free DMEM, after 2 h of incubation, particles were twice larger than in the presence of serum.

3.5. Formation of lipoplexes insensitive to the presence of serum

Our results when taken together suggested that the larger particles ($> 1\,\mu m$) observed in serum-free DMEM, were more efficient for the lipofection of cultured cells than the smaller ones. The aggregating potency of DMEM could be attributed to its content in sodium bicarbonate, since 20 mM $NaHCO_3$ was able to lead to large lipoplexes by itself (Table 2). We then examined the lipofection of CV-1 and NIH 3T3 cells with these large particles, preformed in sodium bicarbonate, in the presence of serum: as shown in Table 3, the luciferase expression was of the same level as that obtained with particles, preformed in NaCl, in the absence of serum. The amount of plasmid DNA and cationic lipid taken up by the cells was similar in all conditions tested (Table 3). When the intracellular traffic of these serum-insensitive particles, preformed in sodium bicarbonate, was followed, the internalized labeled cationic lipid was localized within vesicles that seemed very heterogeneous in size and shape, as observed above for lipofection with particles preformed in NaCl and diluted in serum-free medium (Fig. 4(G)).

Table 3
Luciferase expression and uptake of lipoplexes in the presence or absence of serum

Conditions of complex formation Conditions of lipofection		NaCl DMEM − FBS	DMEM + 10% FBS	NaCl/$NaHCO_3$ DMEM + 10% FBS
Luciferase activity	NIH 3T3($\times 10^6$)	2.6 ± 1.3	0.1 ± 0.1	2.0 ± 1.5
(RLU/μg protein)	CV-1($\times 10^4$)	1.5 ± 0.8	0.02 ± 0.02	1.8 ± 0.7
Internalized RPR 121653	NIH 3T3	1.03 ± 0.42	0.70 ± 0.30	0.64 ± 0.14
(pmol/μg protein)	CV-1	1.57 ± 0.24	1.29 ± 0.43	1.60 ± 0.38
Internalized pCMVLuc plasmid	NIH 3T3	1.76 ± 0.39	0.61 ± 0.33	0.71 ± 0.38
(ng DNA/μg protein)	CV-1	1.76 ± 0.83	0.68 ± 0.38	2.05 ± 1.07

NIH 3T3 and CV-1 cells were transfected for 2 h with pCMVLuc complexed with cationic lipids (6 nmol of RPR 120535 and 1 nmol of RPR 121653 per μg of plasmid; theoretical charge ratio of 9) in the absence or presence of 10% FBS. Lipoplexes were preformed in 150 mM NaCl or in 150 mM NaCl/20 mM $NaHCO_3$, then diluted in DMEM with or without FBS. 10% of FBS was added in culture wells containing lipofection medium without FBS after 2 h at 37°C. The cells were then incubated for 24 h before assay of DNA uptake, cationic lipid uptake and luciferase expression (as described in Section 2). Values represent means ± SD ($n = 4$).

4. Discussion

Cationic lipids are now widely used as effective tools for delivering DNA into mammalian cells [3]. Yet little is known about the mechanism that governs cationic lipid mediated DNA uptake. While it seems clear that a transient membrane destabilization event must occur, the detailed mechanism of this event and its cellular localization, namely at the plasma membrane or at the endosomal membrane, are still debated questions [13,14,16,17]. Hence, the processes which result in the intracellular DNA delivery might vary depending not only on the compound and formulation tested, but also on the cell type. The ability of the serum to inhibit lipofection is an often described phenomenon [12,20,21]. We therefore investigated the mechanism underlying such an inhibitory effect, that represents a major drawback of this non viral method of gene transfer, especially in vivo.

First, we precisely quantified the amount of internalized plasmid by a dot-blot analysis of the clear lysate obtained from transfected cells. A 2- to 3-fold decrease in the amount of internalized plasmid was found in the presence of serum. In the same way, we analyzed the cationic lipid uptake and found no significant difference between the two conditions of lipofection. This was in sharp contrast with the two log collapse in transgene expression. We then examined the effect of serum on the degradation of the plasmid. When complexed with a cationic lipid, plasmid DNA exhibits increased resistance to nucleases [8,9]. However, besides nucleases, serum contains various negatively charged proteins that could bind to lipoplexes and modify their structural characteristics, making DNA accessible to nucleases. If serum had a pronounced DNA degradation effect, we expected that neither circular nor functional plasmid DNA could be extracted from cells transfected in the presence of serum. The purified plasmid DNA, extracted from transfected cells, was analyzed by two different methods. First, by gel electrophoresis analysis, we detected only supercoiled and relaxed plasmid but no linear DNA either in the absence or presence of serum. Second, the amount of functional plasmid was estimated from its capacity to transfect NIH 3T3 cells. It appeared that this extracted plasmid was still functional and we only observed a difference in the amount of extractable plasmid, between the two conditions of lipofection. It must be noted that phenol/chloroform extraction and ethanol precipitation are not very reproducible methods. Nevertheless, these results show that the plasmid recovered from the cells and quantified by dot-blot analysis is a functional, either supercoiled or relaxed plasmid. Our results also suggest that plasmid DNA was probably more degraded by nucleases in the presence of serum, but this effect was not sufficient to account for the hundred-fold decrease in transgene expression observed.

We next investigated the intracellular pathway of the lipoplexes. This has been previously studied with fluorescent lipids that were mixed with the transfecting agent [16,21,29]. In the present work, we have used a cationic lipid bearing a rhodamine moiety, that ensured us that the fluorescent pattern we observed corresponded to the cationic lipid. We made the assumption that the intracellular particles observed were lipoplexes-containing vesicles as suggested by electronic microscopy data [14,15]. We found a clear difference in the intracellular traffic in cells transfected in the presence or absence of serum. When the lipofection was performed in the presence of serum, the labeled cationic lipid was contained within small vesicles that looked like endocytic vesicles. In the absence of serum, cationic lipid containing vesicles appeared larger and heterogeneous in size and shape. The same observations were made while using a fluorescent plasmid. The presence of large vesicles has also been reported by Zabner et al. with complexes which contained either isatoic ester-labeled *N*-[1-(2,3-dimyristyloxy)propyl]-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide (DMRIE) or 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled DOPE [14]. These large particles may play an active role in the internalization of DNA into the cell, possibly by destabilizing the vesicles and releasing the DNA or lipoplexes into the cytoplasm. Lipopolyamines, unlike most cationic lipids, provide effective in vitro gene transfer even in the absence of a colipid, such as DOPE. It has been hypothesized that the addition of the colipid leads to fusion between the lipids in the complex and the endosomal membrane, destabilizing the endosome and releasing the DNA or lipoplexes into the body of the cell [30]. The ability of lipopolyamines to give rise to large intracellular vesicles might explain why they promote

efficient gene transfer without formulation with a colipid.

We next asked whether these large vesicles arose from the fusion and coalescence of endosomes or from the formation of large complexes prior to internalization. Analysis of the size distribution of the lipoplexes by dynamic light scattering showed that preformation of complexes between plasmid DNA and RPR 120535 in 150 mM NaCl led to stable particles in the 100–200 nm range. This result is in agreement with previous results concerning the structural characteristics of these lipoplexes [27]. When these preformed lipoplexes were diluted in culture medium (DMEM with or without serum), their size increased. In the presence of serum, the complexes remained relatively stable with diameter below 700 nm. On the contrary, in a serum-free lipofection medium, particles increased in size and became very large (diameter $> 1\,\mu m$). However, in this size range, dynamic light scattering is no longer an accurate method to determine size distribution. We therefore confirmed our size measurements by examining our lipoplexes using fluorescence microscopy and comparing them with size-calibrating fluorescent latex beads (FluoSpheres®, Molecular Probes). We observed that in the absence of serum, lipoplexes were twice larger than in the presence of serum (data not shown). These results indicate that lipoplexes preformed in NaCl tend to aggregate when diluted in a culture medium like DMEM. However, if serum is present in the medium this aggregation is inhibited. According to our results, large aggregated complexes are more potent transfecting agents than small ones, which is consistent with other reports [31,32]. This assumption is further substantiated by the serum-resistance of the large cationic lipid/DNA particles formed in sodium bicarbonate (Table 3).

The lipofection efficiency of lipoplexes could be also related to their structure, as reported by Boukhnikachvili et al. [33]. They compared the structure of various lipoplexes, composed of the lipospermine DOGS (dioctadecylamidoglycyl-spermine), displaying variable lipofection efficiencies in the presence of FBS. They showed that serum-sensitive lipoplexes were micellar complexes whereas serum-insensitive lipoplexes were of the lamellar type. In the absence of serum, micellar complexes could become lamellar and then efficient for gene transfer, whereas in the presence of serum, this evolution would be hindered. The interaction between lipoplexes and serum is probably due to negatively charged proteins present in the serum. The cationic/anionic charge ratio, which is an essential parameter for efficient lipofection, is also probably decisive for binding of serum components on lipoplexes.

The ability of large complexes to mediate gene transfer could question the involvement of endocytosis in the uptake of the complexes in the absence of serum. Indeed, both type of endocytosis, via coated pit and non-coated pathways, are limited in the size of the particle that can be taken up. Thus, particles greater than 200 nm in diameter are not efficiently taken up by the coated pit pathway. On the other hand, fibroblasts are not phagocytic cells, but some cultured cells have the capability to take up particles of much larger size by a phagocytic process [17]. The mechanism by which DNA is taken up by cells might be multiple and vary with cell lines, formulation and lipofection conditions used.

Lipofection studies which led to efficient in vitro gene transfer in the presence of serum arose from improved conditions of formulations ([20–22,33–35]): for instance, formation of lipoplexes in the presence of detergent and subsequent removal of the detergent by dialysis [34], precondensation of plasmid DNA with polylysine before formation of the lipoplexes [21], or sequential addition of the lipid to plasmid DNA [35]. These formulations have to be tested in in vivo experiments to determine if they offer a potential for their use in in vivo applications of gene transfer. Actually, besides interaction with serum proteins, interaction with complement [36] or with polyanions, and low diffusibility through the extracellular space are as many obstacles to a successful in vivo gene transfer. Particularly, a low net positive charge could be necessary for an easy diffusion of lipoplexes within the extracellular compartments. For instance, non viral gene transfer into the newborn mouse brain was enhanced by using a low charge ratio for lipospermine lipoplexes [37].

In conclusion, the structural characteristics of a lipoplex interacting with the cell membrane might particularly influence the way it will be taken up and determine its intracellular fate. In the present work, the comparison of serum-insensitive and serum-sensi-

tive particles showed that the amount of DNA and lipid taken up by the cells was not related to the efficacy of gene transfer. We have also shown that, in our conditions, gene transfer was more efficient when cells took up large particles, leading to the formation of large intracellular vesicles. These large vesicles would be more easily disrupted, thus releasing DNA into the cytoplasm. Strategies to overcome the entry barrier represented by plasma membrane might require improvements of our knowledge in structural characteristics and stability of the lipoplexes.

Acknowledgements

This work was supported by the CNRS and Rhône-Poulenc Rorer, and a grant from La Ligue Nationale contre le Cancer. The project was done as part of the Bio Avenir program supported by Rhône-Poulenc with the participation of the French Ministry of Research and the French Ministry of Industry. We thank Bruno Pitard for helpful discussions and advice in the use of dynamic light scattering.

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