Reducible cationic lipids for gene transfer

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One of the main challenges of gene therapy remains the increase of gene delivery into eukaryotic cells. We tested whether intracellular DNA release, an essential step for gene transfer, could be facilitated by using reducible cationic DNA-delivery vectors. For this purpose, plasmid DNA was complexed with cationic lipids bearing a disulphide bond. This reduction-sensitive linker is expected to be reduced and cleaved in the reducing milieu of the cytoplasm, thus potentially improving DNA release and consequently transfection. The DNA–disulphide-lipid complexation was monitored by ethidium bromide exclusion, and the size of complexes was determined by dynamic light scattering. It was found that the reduction kinetics of disulphide groups in DNA–lipid complexes depended on the position of the disulphide linker within the lipid molecule. Furthermore, the internal structure of DNA–lipid particles was examined by small-angle

X-ray scattering before and after lipid reduction. DNA release from lipid complexes was observed after the reduction of disulphide bonds of several lipids. Cell-transfection experiments suggested that complexes formed with selected reducible lipids resulted in up to 1000-fold higher reporter-gene activity, when compared with their analogues without disulphide bonds. In conclusion, reduction-sensitive groups introduced into cationic lipid backbones potentially allow enhanced DNA release from DNA–lipid complexes after intracellular reduction and represent a tool for improved vectorization.

Key words: cationic lipid transfection, disulphide-bond-bearing lipid, gene therapy, non-viral gene transfer, reducible DNA–lipid complexes.

INTRODUCTION

Cationic DNA-binding compounds are applied as vectors for gene transfer into mammalian cells. These systems currently show lower efficiency *in vivo* than viral vectors, but exhibit advantages over the latter in terms of immunogenicity, safety and production. For efficient gene transfer, four major criteria have to be ensured: (i) DNA–lipid complex entry into the cell, (ii) escape from the endosome before lysis in the lysosome, for endocytotic pathways, (iii) nuclear import, in non-dividing cells, and finally (iv) DNA release from complexes either before or after transport into the nucleus [1,2].

In non-viral gene transfer, cationic compounds are used to complex DNA. Cationic lipids are widely applied, as they provide a positively charged molecular structure that binds DNA, as well as a hydrophobic moiety which participates in the formation of highly ordered, compacted lipid–DNA particles. This hydrophobic entity has also been suggested to be involved in cell-membrane interaction, membrane passage and escape from the endosome. The positively charged moiety of cationic lipids generally consists of either quarternary ammonium ions [dioctadecyldimethylammonium bromide ('DODAB'), dioctadecyltrimethylammonium propane ('DOTAP')], or polycations [dioctadecylamidoglycyl spermine ('DOGS'), dioleoyloxypropyl-dimethylammonium amidospemine ('DOSPA')], which are often derived from natural DNA-interacting agents such as spermine [3–5]. Besides this group of cationic lipids, alternative cationic polymeric compounds such as polyethylenimine, poly-lysine or their derivatives have been reported to be successful for non-viral gene delivery [1,6–10]. One frequently

used non-ionic 'helper' lipid for gene-transfer formulations is dioleoylphosphatidylethanolamine (DOPE). This lipid allows the generation of liposomes with non-liposome-forming cationic lipids such as cholesteryl *N*-(dimethylaminoethyl)carbamate (DC-Chol), and it facilitates membrane fusion due to its molecular structure and its ability to form inverted hexagonal lipidic phases [11].

The aim of the present work was to enhance gene delivery by cationic lipids that additionally allow intracellular DNA–lipid complex destabilization and subsequent DNA release. Recent analysis of systems using pH-sensitive lipids has led to promising transfection efficiency while fulfilling the requirements mentioned above [12]. As an alternative, we have used a panel of recently developed reducible cationic lipids (RCLs) [13,14]. These new compounds are lipopolyamines that bear a disulphide linker at different positions within the molecule. The disulphide bonds are incorporated either between the hydrophilic and hydrophobic part of the molecule or within an alkyl chain. During completion of this work, the use of disulphide-bond-bearing lipids for gene delivery has also been presented by Tang and colleagues [15–17], who complexed plasmid DNA with dioleoylglycerolsuccinyl-2-hydroxyethyl-disulphide-ornithine or cholesteryl hemidithiodiglycolyl-tris(aminoethyl)amine, a disulphide analogue of DC-Chol. Alternatively, disulphide linkers have been used to stabilize lipid–DNA particles, which are formed by a cysteine-bearing detergent [18], to link oligonucleotides to lipids [19] or to graft polymers to lipids [20].

Our work reports on the complexation of DNA by an extended family of reducible lipopolyamines. We have studied their reduction within DNA complexes (RCL complexes, RCLCs),

Abbreviations used: DOPE, dioleoylphosphatidylethanolamine; DC-Chol, cholesteryl *N*-(dimethylaminoethyl)carbamate; RCL, reducible cationic lipid; RCLC, RCL complex; EtBr, ethidium bromide; DTT, dithiothreitol; SAXS, small-angle X-ray scattering; RLU, relative light units.

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leading to DNA release. To evaluate the effect of these disulphide linkers, we have compared gene-transfer efficiency of lipid–DNA complexes formed by these novel reducible lipids with that of complexes formed by non-reducible lipid analogues.

MATERIALS AND METHODS

Cationic lipids, micellar solutions and liposomes

Two groups of cationic lipids bearing a disulphide bond have been synthesized (Figure 1) and characterized as described in [13,14]. In the first group (RPR 128522 and RPR 132621), a disulphide bond links the hydrophilic moiety to the hydrophobic part of the lipid molecule. In the second group (RPR 132775 and RPR 202059), the disulphide link has been introduced into the hydrophobic alkyl chains. A third group of analogous cationic lipids that do not bear a disulphide bond (RPR 120535, RPR 203769 and RPR 228091, see Figure 1), were used as a control. Lipids of all three groups contain a spermine-derived

amino acid linked to the various hydrophobic entities. The synthesis of RPR 120535 was performed according to [21]. RPR 203769 and RPR 228091 were prepared using the same procedures with the following modification: glycyloctadecylamide was coupled to the cationic part of the molecule to obtain RPR 228091. For the synthesis of RPR 203769, octadecylamine and pentamine were bound to aspartic acid, and the resulting hydrophobic entity was coupled to the hydrophilic head of the compound.

Aqueous micellar solutions (10 mM) of lipids were prepared by sonication for 10 min (115 V, 50–60 Hz; Laboratory Supplies Co, Hicksville, NY, U.S.A.) and subsequent heating for 30 min at 50 °C [22]. Micellar solutions were diluted to form 1 mM and 0.1 mM stock solutions.

Liposomes of RPR 128522 were formed with DOPE (Avanti Polar Lipids, Alabaster, AL, U.S.A.) at a molar ratio of 1:1, as described in [23].

DNA

A 4.5 kb plasmid (pCOR plasmid pCMV-Luc), containing the firefly luciferase gene driven by a cytomegalovirus promoter, was isolated and purified as described in [24]. Lyophilized DNA was dissolved in 150 mM NaCl at 20 µg/ml and used for DNA–lipid complex formation and DNA-transfection experiments.

DNA–lipid complex formation

DNA–lipid complexes were formed by mixing equal volumes of a constant DNA concentration and a varying cationic lipid concentration, which led to different charge ratios (+/–). Charge ratios are expressed as mol of positive charges/mol of negative charges, assuming that the spermine lipids bear three positive charges at neutral pH (as suggested by NMR and microanalysis). Since 1 µg of DNA corresponds to 3 nmol of negatively charged phosphate, the number of nmol of cationic lipid per µg of DNA is equivalent to the charge ratio (+/–). As an exception, RPR 132621 bears two spermine moieties per molecule and thus presents six positive charges at neutral pH. Consequently, 0.5 nmol of RPR 132621/µg of DNA corresponds to a charge ratio (+/–) of 1.

The final DNA concentration was 10 µg/ml, and the final NaCl concentration was 75 mM.

The compaction of DNA by cationic lipids was monitored by the exclusion of ethidium bromide (EtBr) molecules (Euromedex, Strasbourg, France) from DNA after lipid–DNA association. EtBr (4 µg) was added to DNA–lipid complexes (1 ml) in 75 mM NaCl. The residual EtBr fluorescence was assayed using a Jobin-Yvon Spex Fluoromax-2 spectrofluorimeter (Instruments SA, Edison, NJ, U.S.A.) at 20 °C with a DNA concentration of 10 µg/ml (excitation, 260 nm; emission, 560 nm) [22]. Values are expressed in arbitrary fluorescence units. Molecules that resulted from dithiothreitol (DTT; Sigma) reduction of the cationic lipids RPR 128522, RPR 202059 and RPR 132621, and spermine (Fluka) were also tested for DNA complexation.

Dynamic light scattering

The particle size of DNA–lipid complexes was determined by dynamic light scattering using a Coulter N4 Plus particle analyser (Coulter, Amherst, MA, U.S.A.). Measurements were performed at 20 °C with a DNA concentration of 10 µg/ml and at an angle of 90°. The auto-correlation function of scattered-light intensity was used to calculate the mean particle size to give unimodal analysis results.

Group 1: disulfide bond within the hydrophilic moiety

RPR 128522, symmetric disulfide lipid

RPR 132621, gemini-like disulfide lipid

Group 2: disulfide bond within the hydrophobic chain

RPR 132775, asymmetric disulfide lipid

RPR 202059, asymmetric disulfide lipid

Group 3: control lipids without disulfide bond

RPR 120535, standard lipid

RPR 203769, non-reducible asymmetric lipid

RPR 228091, monoalkyl lipid

Figure 1 Overview of the lipids used in this study

Group 1 consists of the symmetric disulphide lipid RPR 128522 and the gemini-like lipid RPR 132621, which bear the disulphide bond within the hydrophilic part of the molecule. Group 2 is the two asymmetric lipids RPR 132775 and RPR 202059, which bear the disulphide bond within the hydrophobic chain. Group 3 contains the standard lipid RPR 120535, the non-reducible asymmetric lipid RPR 203769 and the monoalkyl lipid RPR 228091, which do not bear disulphide bonds and were applied as control lipids.

Small-angle X-ray scattering (SAXS)

DNA–lipid complexes were formed at variable charge ratios (+/–) and final DNA concentrations, as indicated in Figure 3 (see below). Precipitated lipid–DNA complexes were centrifuged at 7200 *g* (Qualitron Inc., Holland, OH, U.S.A.) for 20 s. Pellets were injected into capillaries of 1.5 mm diameter. In order to prepare reduced complexes, DTT (final concentration 100 mM) was added to DNA–lipid complexes, and the resulting suspensions were concentrated 20× by ultrafiltration in Eppendorf filtron 300 K devices for 10 min at 20500 *g*. Additionally, DTT was added to RPR 132621 before complex formation, and DNA complexes were generated from the reduced lipid. X-ray measurements were carried out with synchrotron radiation sources of either the European Synchrotron Radiation Facility ('ESRF') beam-line ID2 (Grenoble, France) with a 10% beam-line intensity and a sample–detector distance of 1.5 m, or at the Laboratoire pour l'utilisation du rayonnement synchrotron ('LURE') beam-line D43 (Orsay, France) at a sample–detector distance of 375 mm. The momentum transfer $q = (4\pi/\lambda)\sin\theta/2$, with the scattering angle, θ , from 0.05 to 0.25 nm^{–1} and an X-ray wavelength, λ , of 0.99 Å at the ESRF and of 1.43 Å at the LURE. Results are expressed in arbitrary units as a function of q . SAXS experiments were performed only once, but results were always compared with our internal standard RPR 120535, studied extensively by SAXS previously (e.g. [22]).

Cationic lipid reduction and HPLC

HPLC was carried out on aquapore butyl 7 µm columns with a linear gradient of water (with 0.1% trifluoroacetic acid) to acetonitrile (with 0.08% trifluoroacetic acid). This gradient was performed with a variance of 80%/20% to 0%/100% (v/v), as described previously [21]. Cationic lipids were detected with a UV spectrophotometer tuned to 220 nm. A volume of 1 ml of either pure lipid solution or an aqueous suspension of DNA–lipid complexes [charge ratios (+/–) of 0.7 or 4.0] was mixed with 50 µl of 100 mM DTT. The lipid concentration was 1 mg/ml in both cases. The HPLC analysis of these mixtures was performed before, 1 min after and 1 h after the addition of DTT.

DNA release

DNA–lipid complexes were formed as described above, and DTT was added to reach a final concentration of 5 mM. Selected samples were incubated at 37 °C for up to 24 h. Cysteine and glutathione (both from Sigma) were used as alternative reducing agents at the same concentration; the pH value of glutathione solutions was adjusted to 6.0–6.5. DNA release was monitored by an increase in EtBr fluorescence, as described for DNA complexation.

To study the influence of pH on lipid reduction and DNA release, complexes were formed in 10 mM Hepes, 10 mM Pipes and 10 mM Mes (all from Sigma) at a pH of 5.0, 6.0, 7.0 or 8.0. The effect of the salt concentration on DNA release was also studied, using complexes prepared in 1.2, 5, 20, 50, 100 and 300 mM NaCl.

Cell culture and transfection

HeLa cells were grown at 37 °C in a 5% CO₂/95% air incubator in Eagle's minimum essential medium with the addition of 2 mM L-glutamine, 1% minimum essential medium non-essential amino acid solution (100× stock solution), 50 units/ml penicillin, 50 µg/ml streptomycin and 10% (v/v) fetal bovine serum. All

media and additives were obtained from Gibco-BRL Life Technologies (Gaithersburg, MD, U.S.A.).

Before transfection (36 h), cellular suspensions of 500 µl were seeded into 24-well culture plates to reach a final concentration of 1 × 10⁵ cells/ml. This dilution showed approx. 80% confluency after 36 h. Before transfection, wells were rinsed twice and subsequently incubated with 500 µl of medium without fetal bovine serum. DNA–lipid-complex solutions (50 µl) containing 0.5 µg of plasmid DNA in 75 mM NaCl were added to each well. Cationic, colloiddally stable lipid–DNA complexes were chosen for this experiment. After transfection (2 h), the serum-free medium was supplemented with 10% (v/v) fetal bovine serum. Cells were grown for 36 h at 37 °C in 5% CO₂.

Luciferase assay

Luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI, U.S.A.). Cell layers were rinsed twice with 500 µl of PBS and lysed with 250 µl of Promega cell-culture lysis reagent for 30 min at 20 °C. After 5 min of centrifugation at 12000 *g* and 4 °C, 10 µl aliquots of the supernatant lysate were assayed for luciferase activity using Promega luciferase substrate. Luminometric measurements were performed with a Lumat LB9501 luminometer from EG, Berthold (Evry, France) or a Victor² 1420 Multilabel Counter (Wallac, Turku, Finland). Luciferase activity was quantified by integration over 10 s and expressed as relative light units (RLU) for data from the Lumat LB9501 or quantified over 1 s and expressed as counts/s for the Victor² instrument. To compare RLU and counts/s values, measurements of 500 identical samples of different expression levels were carried out with both luminometers. A coefficient of 10.8 was found to convert counts/s data into equivalent RLU values. To determine the luciferase activity/µg of protein, the protein concentration of the lysate was obtained by using a Pierce BCA assay kit (Rockford, IL, U.S.A.).

Cellular uptake of lipid–DNA complexes

Plasmid DNA was radiolabelled using CpG-methylase (CpG is short for cytidine–guanosine) and the substrate *S*-adenosyl-L-[methyl-³H]methionine (New England Biolabs, Beverly, MA, U.S.A.). The DNA–lipid complexes were formed with [³H]DNA as described above, and HeLa cells were transfected [25]. After 36 h, cell layers were rinsed twice with PBS to remove adsorbed complexes from the cell surface. Luciferase activity and protein concentration were determined as described above. Aliquots (150 µl) from the lysate supernatant were mixed with 12.5 ml of ready-safe scintillation liquid (Beckman, Fullerton, CA, U.S.A.), and radioactivity was counted by liquid scintillation on a 1414 WinSpectral scintillation counter (Wallac). Radioactivity was measured as c.p.m. The amount of internalized DNA was expressed as a percentage of the total radioactivity that was added to cells in the course of transfection.

RESULTS

Cationic lipids

In the present study various spermine-containing cationic lipids were used (see Figure 1). With regard to the position of the disulphide linker, three groups of lipids were distinguished. In the first group, the linker was introduced in a hydrophilic environment. In lipids of the second group, one alkyl chain was linked to the molecule by a disulphide bond, thus placing the disulphide linker in a more hydrophobic environment. The first-group lipid RPR 132621 exhibits the molecular structure of a

Figure 2 DNA complexation and colloidal stability of reducible lipid-DNA particles

These parameters were determined by using dynamic light scattering (continuous line) and residual EtBr fluorescence (dashed line) as described in the Materials and methods section. The data are given for lipids bearing the disulphide bond in different positions within the molecule: group 1 in (a) and (b), group 2 in (c) and (d). Left-hand y-axis, particle size (nm); right-hand y-axis, residual EtBr fluorescence. The size of colloidally unstable, aggregated particles is indicated as 800 nm, although it was presumably larger, but out of the detection limit of the particle analyser. DNA formed complexes with all lipids tested, and the complexes resulting from lipid-DNA compaction showed particle sizes corresponding to the three-zone model of colloidal stability. a.u., arbitrary fluorescence units.

gemini-like lipid. Such lipids contain two identical moieties that are linked by a spacer, which in the case of RPR 132621 is a disulphide bond. Cationic lipids of the third group contain no disulphide bond, and either represent non-reducible analogues of disulphide lipids (the standard lipid RPR 120535 and the non-reducible asymmetric lipid RPR 203769), or correspond to the reduction products of reducible lipids, such as the monoalkyl lipid RPR 228091. This RPR 228091 is composed of a spermine-derived amino acid, coupled to a single C_{18} alkyl chain, and thus constitutes a detergent. These lipids represent three categories of molecules, which are expected to form DNA complexes with different biophysical and biological properties.

Formation of DNA-lipid complexes

In order to monitor DNA complexation by the RCLs and to characterize the resulting lipid complexes, the sizes of lipid-DNA particles and the residual fluorescence of EtBr were determined. Figure 2 shows the mean diameter of lipid-DNA complexes assayed by dynamic light scattering and displays EtBr fluorescence as a function of the lipid-DNA charge ratio (+/-), as defined in the Materials and methods section. In addition, SAXS experiments were performed with lipid-DNA complexes.

The formation of complexes with DNA for the symmetric disulphide lipid RPR 128522 is given in Figure 2(a): mixtures of this cationic lipid and DNA at a charge ratio (+/-) below a value of 1 resulted in small particles with a mean diameter < 200 nm (zone A). Free DNA is detected in this zone, as shown by the relatively high levels of EtBr fluorescence. At a higher

charge ratio (+/-), between 1 and 2.3, colloidally unstable particles (zone B) were observed. These zone-B complexes formed large aggregates and their DNA was highly compacted, as shown by minimal EtBr fluorescence. On the contrary, when DNA was complexed by RPR 128522 at charge ratios > 3 (zone C), small and stable particles were observed. Again, DNA was highly compacted, as shown by EtBr fluorescence. A similar pattern of zones A, B and C was observed for complexes formed from DNA and liposomes obtained from a combination of RPR 128522 and DOPE as co-lipid (results not shown). All cationic lipid-DNA interactions outlined in this paper adhere to this three-zone model of size/colloidal stability (Figures 2a-2d), which depends on the lipid-DNA charge ratio (+/-), as has been proposed previously [22,26].

To characterize lipid-DNA complexes and to define their structure, SAXS experiments were undertaken (Figure 3). The two lipids of group 1, the symmetric disulphide lipid RPR 128522 and the gemini-like lipid RPR 132621, yielded different structures. For RPR 128522 complexes, a scattering peak at 83 Å was measured (Figure 3a). The half-width of the scattering peak corresponded to an effective domain size of 200 Å, which indicated the repetition of 3-4 periodicities. The same structural parameters have been found previously for DNA complexes with the similar standard lipid RPR 120535 [22]. On the contrary, gemini-like lipid RPR 132621 complexes revealed different structural features in that a scattering peak was measured at 60 Å and the second-order reflection at approx. $60/\sqrt{3}$ might indicate a hexagonal structure (Figure 3b). SAXS measurements were also performed for complexes resulting from the interaction of DNA

Figure 3 SAXS measurements

This analysis reveals a defined particle structure of each lipid–DNA complex. Experimental conditions for SAXS experiments are given in the Materials and methods section. The charge ratios (+/–) were 0.7 for RPR 132621–DNA complexes (**b**), and 2.0 for DNA complexes with RPR 128522 (**a**) and RPR 203769 (**c**). The final DNA concentration in complex formulations was 1.5 mg/ml. (**a**) A scattering peak was measured at 83 Å. (**b**) A peak at 60 Å, together with the second-order reflection at 38 Å, suggest a hexagonal structure. (**c**) RPR 203769–DNA complexes showed a first-order scattering peak at 64 Å. See Figure 6 for a comparison of these structures with the respective complexes after DTT reduction. a.u., arbitrary units.

Table 1 Variation of HPLC retention times of reducible lipids upon treatment of DNA–lipid complexes with DTT

Pure complexes (charge ratio, 4.0) or mixtures of complexes with DTT (final concentrations: DTT, 5 mM; lipid, 1 mg/ml) were analysed by HPLC under experimental conditions described in the Materials and methods section. The runs were performed before, 1 min after and 1 h after DTT addition. Retention times of reduced lipids are given in min. All reducible lipids that had been formulated into complexes were reduced, although their reduction times differed. The percentages indicated in parentheses for RPR 13261 and RPR 132775 indicate the percentages of reduced or unreduced lipids (quantification by integration of the peak areas). RPR 128522 was reduced about 1 min after DTT addition, whereas reduction products of e.g. RPR 202059 could only be detected 1 h after DTT addition.

	Retention time (min)		
	Before DTT addition	1 min after DTT addition	1 h after DTT addition
RPR 128522, symmetric lipid (group 1)	18.6	23.55*	23.62*
RPR 132621, gemini-like lipid (group 1)	13.92	14.15 (59%)	14.5 (17%)
		12.26 (41%)	12.34 (83%)
RPR 132775, asymmetric lipid (group 2)	13.48	13.55 (95%)	13.83 (8%)
		12.54 (5%)	11.92 (92%)
RPR 202059, asymmetric lipid (group 2)	14.04	14.08	18.68*

* Precipitated, solubilized in ethanol and dichloromethane.

with the non-reducible asymmetric lipid RPR 203769 and revealed a first-order scattering peak at 64 Å (Figure 3c).

Together, these measurements of mean lipid–DNA-complex diameter and of residual EtBr fluorescence showed that RCLs complexed DNA in the same way as described for the non-reducible standard RPR 120535. Depending on the charge ratio (+/–), three zones of colloidal stability were monitored and the residual EtBr fluorescence of free DNA decreased with higher lipid concentrations. Moreover the DNA–lipid complexes exhibited a defined particle structure, which was not identical for all DNA–lipid complexes, as shown by SAXS experiments. Particle organization depended rather on the structure of the cationic lipid molecule, and structurally similar lipids showed a similar arrangement in DNA complexes.

Reduction of disulphide lipids formulated into DNA complexes

HPLC analysis of pure lipids and of DNA–lipid complexes was performed to monitor whether RCLs complexed with DNA were still accessible to reduction. These measurements were carried out at three different time points, (i) before, (ii) 1 min and (iii) 1 h after mixture with the reducing agent DTT, and retention times of the compounds from the three measurements were compared. All runs of pure lipids (RPR 128522, RPR 132621, RPR 132775 and RPR 202059) showed completely reduced lipid 1 h after the addition of DTT (results not shown). In contrast, retention times of non-reducible lipids, which were applied as a control, were not altered by the addition of DTT. Furthermore, variations in the time of lipid reduction were observed, presumably corresponding to the chemical context of the disulphide bond. Lipids bearing disulphide bonds in the hydrophilic part of the molecule (group 1) were reduced within 1 min of the addition of the reducing agent. In contrast, lipids of group 2, which bear the reducible linker in the alkyl chain (the asymmetric disulphide lipids RPR 132775 and RPR 202059), were cleaved more slowly. The asymmetric lipid RPR 132775, whose disulphide bridge is introduced in the C₅ alkyl chain, was partially degraded within 1 min. In contrast, the other asymmetric lipid RPR 202059 was cleaved only within 1 h (results not shown). A similar variation in the

Figure 4 DNA release after DTT treatment of lipid-DNA complexes

(a) Kinetics of DNA release from RPR 128522-DNA complexes with charge ratios of 0.4 (zone A), 1.7 (zone B) and 4.0 (zone C) was assessed by residual EtBr-fluorescence increase. Plasmid DNA is released from complexes of zones A and C 20 min after DTT addition. To support DNA release from complexes of zone B, the incubation temperature was shifted to 37 °C after 30 min. (b and c) The residual EtBr fluorescences of RPR 202059- and RPR 132775-DNA complexes before (continuous line) and after DTT addition (dashed line) are compared. After DTT-induced reduction, (b) RPR 202059-DNA complexes release DNA, whereas in (c) DNA compaction by RPR 132775 is maintained. (d) DNA complexation by reduced lipids or the analogues of reduced lipids. The monoalkyl lipid RPR 228091, which is an analogue of reduced RPR 132775 and RPR 132621, exhibits DNA complexation (dashed line) and a colloidal stability (continuous line) comparable with cationic reducible lipids (see Figure 2). Spermine exhibits no DNA-complexing ability (dotted line), and no particles could be measured by dynamic light scattering. a.u., arbitrary fluorescence units.

lipid-reduction time was also observed for lipids complexed to DNA (Table 1).

In summary, the HPLC measurements confirmed that disulphide-bearing lipids are sensitive to reduction. Even when formulated into DNA complexes, lipids are still sensitive to reduction. These various lipids also exhibit similar differences in their reduction kinetics, either as pure/uncomplexed lipids or DNA-associated in complexes.

DNA release

To challenge the concept of enhanced DNA release from RCLCs upon reduction, DNA-release experiments were performed. Complex dissociation and the resulting DNA release were measured by EtBr fluorescence. In the presence of DTT, glutathione or cysteine, DNA release was only observed from DNA complexes formed with the symmetric and asymmetric disulphide lipids RPR 128522 and RPR 202059 (Figures 4a and 4b; results not shown for cysteine and glutathione). Figure 4(a) displays the kinetics of DNA release from RPR 128522-DNA complexes of zones A, B and C, respectively. DNA was completely released from complexes of zones A and C 20 min after addition of DTT. In contrast, a delayed release was observed for DNA complexes from zone B. Plasmid-DNA complexes formed with the asymmetric reducible lipid RPR 202059 released DNA upon addition of DTT to a lesser extent (Figure 4b): complexes

of zones A, B and C had to be incubated with DTT for 12 h at 37 °C to completely release DNA. Glutathione-induced release did not reach 100%. Cysteine completely released DNA from complexes of zone A, but released only traces of DNA from complexes of zones B and C (results not shown). In contrast there was no DNA release from the complexes after the reduction of RPR 132621, the gemini-like lipid (results not shown), or of RPR 132775, the second asymmetric disulphide lipid (Figure 4c), during the experiments. The control DNA-lipid complexes formulated with corresponding lipid analogues of group 3, which bear no disulphide bond, did not release DNA upon the addition of DTT (results not shown).

In order to explain why RPR 128522- and RPR 202059-DNA complexes released DNA upon lipid reduction whereas the other analysed lipid complexes did not, we studied the DNA-complexing abilities of reduced lipids. We tested whether (i) the reduced disulphide lipids, (ii) their analogues (e.g. the monoalkyl lipid RPR 228091) and (iii) spermine maintained a DNA-complexing ability. The product of RPR 128522 reduction represents spermine; the product of RPR 202059 reduction represents a spermine molecule with a short hydrophobic chain (C_5). The results show that spermine itself did not lead to DNA compaction, as shown by the absence of a decrease in EtBr fluorescence with increasing spermine concentration (Figure 4d). Additionally, DTT was added to micellar solutions of RPR 128522, RPR 202059 and RPR 132621, and the reduced

Figure 5 Influence of pH value and NaCl concentration on DNA release from lipid–DNA complexes

These parameters were determined according to the procedures described in the Materials and methods section. Measurements were performed with RPR 202059–DNA complexes at a charge ratio of 6.0. (a) The residual EtBr fluorescence of complexes in the presence (dashed line) and absence (continuous line) of DTT at different pH values. For DNA release, a pH value higher than or equal to 6.0 is essential. (b) The residual EtBr fluorescence of complexes at different salt concentrations was determined before (continuous line) and after (dashed line) the addition of the reducing agent. Higher salt concentrations support efficient DNA release from lipid–DNA complexes. a.u., arbitrary fluorescence units.

Figure 6 Comparison of the structural characteristics of lipid–DNA complexes before and after DTT-induced reduction

SAXS experiments were performed with lipid–DNA complexes (a and b, curves I), reduced lipid–DNA complexes (a, curve II) and complexes formulated from already reduced lipids (b, curve II). Experimental conditions are given in the Materials and methods section. (a) The SAXS spectra of RPR 128522–DNA complexes (curve I) show a loss of structural characteristics after the addition of DTT (curve II). (b) Structural features of complexes formed from untreated RPR 132621 (curve I) show that complexes can also be formed from the RPR 132621 derivative after DTT-induced reduction (curve II), although with different structural characteristics. a.u., arbitrary units.

lipids were used to form complexes with DNA. It was found that the reduction product of the gemini-like lipid RPR 132621 still complexed DNA in contrast to the reduction products of the symmetric and asymmetric disulphide lipids RPR 128522 and RPR 202059, which lost their complexing ability (results not shown). The cationic monoalkyl lipid RPR 228091 (analogue of the reduction products of RPR 132621 and RPR 132775) also displayed DNA-complexing properties, and the diagram size/charge ratio (Figure 4d) showed three zones (A, B and C) according to the three-zone model of cationic lipids described above.

Since media and intracellular compartments exhibit different pH and ionic compositions, we investigated the influence of pH and ionic strength (NaCl concentration) on DNA release from RPR 202059 complexes upon DTT-induced lipid reduction. At pH values below 6.0, DNA release was not observed, whereas NaCl concentrations higher than 50 mM facilitated DNA release (Figure 5). The same results were obtained for DNA–RPR 128522 complexes (not shown).

SAXS experiments were undertaken to study the potential structural changes in DNA–lipid complexes due to lipid reduction. Complexes of DNA with the symmetric disulphide lipid RPR 128522 did not show any organized structure after incubation with DTT, when analysed by SAXS (Figure 6a), which indicates complex dissociation after DTT treatment. In contrast, results showed that the structure of the gemini-like lipid RPR 132621–DNA complexes after DTT addition did not differ from the structure of untreated complexes (results not shown). We also studied by SAXS experiments the complexes formed from the addition of DNA to the RPR 132621 derivative that had undergone preliminary reduction by DTT, which led to dissociated monoalkyl components (Figure 6b). They revealed a scattering peak at 80 Å and a repetition of the periodicity between 5 and 6. These geometric features of DNA complexes obtained with the reduced derivative of RPR 132621 differed significantly from geometrical parameters found for complexes formed with the unreduced lipid, whether or not they had been incubated with DTT after formation of complexes. On the

Figure 7 Transfection activities and complex internalization

Studies of transfection (RLU/μg of protein, indicated by white bars) and internalization of lipid–DNA complexes (DNA internalization %, indicated by black squares) were performed according to the Materials and methods section. Left-hand side, results are given for the reducible asymmetric lipids RPR 202059 and RPR 132775, and for their non-reducible analogue RPR 203769 as a control, at charge ratios of 4.0 and 6.0. The transfection activity of reducible lipids is up to 1000-fold higher when compared with the non-reducible analogue RPR 203769. Internalization results do not correlate with results from transfection-activity studies. Right-hand side, the transfection activities and complex internalization of different formulations were compared. This is shown here for the symmetric reducible lipid RPR 128522, whose DNA complexes were formulated from micelles or liposomes, at charge ratios of 8.0 and 10.0. In the case of RPR 128522–DNA complexes, the particles formed from liposomes exhibited up to 1000-fold higher activity than those formed from micelles. The latter were less internalized during transfection. Error bars represent the S.D. based on the entire population.

contrary, the structural features of the reduced monoalkyl derivative of RPR 132621 resembled the structure of the monoalkyl lipid RPR 228091 (scattering peak at 80 Å, results not shown).

Together, these results show that not all lipid–DNA complexes studied here release DNA after reduction. (i) Lipids whose DNA complexes do not release DNA maintain their DNA-complexing ability even after reduction, and geometrical parameters of such DNA–lipid complexes were maintained upon DTT treatment. (ii) Lipids whose DNA complexes release DNA upon DTT-induced reduction lose their complexing ability when reduced. Furthermore, the structures of their complexes were destroyed during the course of reduction.

Gene-transfer efficiency

To examine a potential correlation between the biophysical data and reporter-gene activity, transfection experiments were performed on adherent eukaryotic cells. HeLa cells were transfected with cationic lipid–DNA complexes, and reporter-gene activity of complexes with the asymmetric disulphide lipids RPR 202059 and RPR 132775 from group 2 were compared with results with their analogue without any disulphide bonds (RPR 203769). DNA complexes formulated from reducible lipids showed up to 1000-fold higher transfection activity than that of complexes of the unreducible analogue (Figure 7). The gene-transfer efficiency of these reducible lipid complexes represents the same range of activity as DNA complexes of RPR 120535, one of our reference lipids (RPR 120535–DNA complexes give a similar activity to *N*-[1-(2,3-dimethylstyloxy)propyl]-*N,N*-dimethyl-*N*-(2-hydroxyethyl)ammonium bromide–cholesterol–DNA complexes [22]).

Additionally, we observed differences in transfection efficiencies of DNA complexes formed with distinct RPR 128522

formulations. DNA complexes of this symmetric disulphide lipid formed from micelles showed no activity, whereas reporter-gene expression significantly increased when complexes were formed from corresponding RPR 128522–DOPE liposomes (Figure 7). To exclude RPR 128522–DNA-complex disruption and subsequent DNA release before contact with the cell surface, it was ensured that no DNA was released from these complexes in the presence of culture medium (results not shown).

In summary, these results suggest an increased gene-transfer efficiency into eukaryotic cells by DNA complexes formed from selected RCLs. Additionally, an enhancing effect of DOPE for DNA complexes with RPR 128522 was observed.

Cellular uptake of lipid–DNA complexes

Experiments were undertaken to determine the amount of DNA that had been internalized during transfection with lipid–DNA complexes. Tritiated plasmids were used to assess the amount of internalized DNA. Figure 7 illustrates the quantity of ³H-labelled DNA that was found in cells after lysis. Only a very small amount of DNA was internalized, and similar cellular uptake was found for DNA from complexes of different charge ratios (+/-) of zone C. These results were compared and correlated with the transfection activity of the same lipid–DNA particles. With complexes of the asymmetric lipids of the second group, we observed an internalization of approx. 4% for RPR 202059. The more active RPR 132775–DNA complexes were internalized with an efficiency of up to 15% (Figure 7). About 8% of DNA from complexes of the non-reducible asymmetric lipid RPR 203769 was detected within the cells (Figure 7).

In analogy with transfection experiments we also compared the cellular uptake of RPR 128522–DNA complexes formulated from either RPR 128522 micelles or RPR 128522–DOPE liposomes (Figure 7). Results indicate that only a very limited quantity of DNA–RPR 128522 complex from micelles was internalized, and that complexes formed from liposomes were taken up more extensively.

Together, these internalization experiments show that RCLs affect the cellular uptake of its DNA complexes when compared with the non-reducible analogue, but that higher DNA internalization is not always correlated with elevated gene-expression efficiency.

DISCUSSION

We have studied three groups of cationic lipids that were formulated into lipid–DNA complexes, which are expected to be useful in gene therapy (Figure 1). These lipopolyamines consist of a polycationic spermine headgroup and a hydrophobic moiety. Lipids of the first two groups bear a disulphide bond, but at different positions within the lipid. The reducible linker has been introduced either into a hydrophilic part of the molecule (group 1) or into the alkyl chains, which provides a more hydrophobic environment for the disulphide bonding (group 2). Lipids of group 3 bear no disulphide bond and were used as a control.

The ability of the standard lipid RPR 120535 to form complexes when mixed with DNA has been described previously and discussed [22]. We demonstrated that cationic lipids which bear an additional disulphide bond can complex DNA, and also adhere to the three-zone model of colloidal stability (Figure 2). In accordance with this model, cationic lipid–DNA complexes of zone A and C carry an excess of either negative (zone A) or positive (zone C) charges, and consequent repulsion between complexes and colloidal stability is ensured. As expected, near

charge neutrality (zone B), complexes were observed to aggregate and precipitate.

Structural studies were performed to define the supramolecular organization of these complexes, to detect an eventual influence of disulphide bonds on the complex structure, and to obtain information on structural changes of complexes due to lipid reduction. It has been shown previously that DNA–RPR 120535 complexes form multilamellar structures with a periodicity of 80 Å, consisting of four lamellae per particle [22]. Complexes of the symmetric disulphide lipid RPR 128522 showed the same lamellar organization as those containing structurally similar standard lipid RPR 120535, that is three or four lamellae per particle (Figure 3a). In contrast, different structural characteristics were observed for DNA complexes with the gemini-like lipid RPR 132621 (Figure 3b). These particles most likely exhibit a hexagonal structure. Furthermore, DNA complexes formed with the non-reducible asymmetric lipid RPR 203769 show similarity to the structure of DNA complexes with the gemini-like lipid RPR 132621 but differ from RPR 128522– and RPR 120535–DNA complexes (Figure 3c). These differences could originate either from the balance between hydrophobicity and positive charges or from steric considerations. In the gemini-like lipid RPR 132621 and in the asymmetric lipids (e.g. RPR 203769, Figure 1), the number of cationic amines versus the number of alkyl groups in the hydrophobic chains is higher than for RPR 120535 or RPR 128522. This could lead to smaller structures in DNA–lipid particles.

All disulphide-bearing lipids were sensitive to reducing agents, but the kinetics of the reduction reactions probably depended on the chemical environment: the disulphide linker is more readily reduced in hydrophilic environments. This was observed for uncomplexed lipids alone as well as for DNA–lipid complexes. We suggest that disulphide linkers in a hydrophilic environment are more easily accessible to a reducing agent, even when compacted into DNA complexes (Table 1).

After lipid reduction, DNA was only released from DNA complexes that contained the symmetric and asymmetric disulphide lipids RPR 128522 and RPR 202059 (Figures 4a and 4b). DNA complexes with the gemini-like lipid RPR 132621 and the second asymmetric disulphide lipid RPR 132775 remained stable in the presence of a reducing agent. These data confirm that DNA release from lipid–DNA complexes depends on the complexing ability of the reduced lipids. We found that some reduced lipids or analogues of reduced lipids can maintain their complexing ability. For example, the monoalkyl lipid RPR 228091, which is the analogue of the reduction product from RPR 132621 and RPR 132775, containing three positive charges and one hydrophobic chain (C_{18}), still forms complexes with DNA (Figure 4d) and consequently RPR 132621– and RPR 132775–DNA complexes remain stable after reduction. In contrast, upon reduction of RPR 128522 or RPR 202059, the majority of hydrophobic domains are dissociated from the polycationic spermine moiety. As spermine itself did not complex DNA (Figure 4d) under the conditions described (see the Materials and methods section), the latter lipids liberate DNA upon reduction. Comparable results have been observed by F. Tang and colleagues [15–17] using cationic lipids bearing a disulphide linker between the hydrophobic and hydrophilic parts of the molecule. However, their data on DNA release showed a delayed liberation of DNA.

The fact that RPR 202059–DNA complexes released DNA less readily than RPR 128522–DNA complexes upon DTT-induced reduction can be explained by (i) slowed-down reduction kinetics due to the hydrophobic environment of the disulphide bond within RPR 202059, and (ii) the short C_5 carbon chain,

which remains bound to the spermine entity after RPR 202059 reduction. Although RPR 128522 is reduced immediately after the addition of the reducing agent, kinetic differences for DNA release have been observed for DNA 128522 complexes of zones A, B and C. This suggests that aggregated complexes of zone B were less accessible to the reducing agent.

We have observed less-efficient DNA release when using reducing reagents other than DTT, namely cysteine and glutathione. This is probably due to differences in reduction power of the latter two reducing agents. A similar observation has been described previously by F. Tang and colleagues [16].

The effects of pH and ionic strength on DNA release from lipid complexes have been studied with DNA-releasing lipid complexes (RPR 128522 and RPR 202059). Low pH generally impedes reduction, and accordingly no DNA was released at pH values below 6.0 (Figure 5a). Furthermore, facilitated DNA release from complexes at NaCl concentrations higher than 50 mM was detected (Figure 5b). This observation suggests the destabilization of complexes due to an electrostatic interaction/competition with salt ions either before or after reduction, which is in agreement with reports on complex dissociation caused by high salt concentrations [27].

Results from the X-ray scattering experiments which involved complexes in the presence of DTT were consistent with the data from the DNA-release studies: RCLCs that release DNA after DTT incubation lose their geometrical structure in the presence of DTT (e.g. RPR 128522, Figure 6a). In addition, the SAXS results indicate the intrinsic stability of non-DNA-releasing complexes. For example, gemini-like lipid RPR 132621–DNA complexes did not release DNA and showed no change of scattering pattern in the presence of DTT (Figure 6b). Thus, reducible lipids that remain bound to DNA in complexes in the presence of DTT not only keep their property to compact DNA, but their complexes also display high stability, which is demonstrated by a lack of intracomplex structural change and reorganization.

Complexes formed with the symmetric disulphide lipid RPR 128522 showed an increased transfection efficiency with DNA complexes formed from RPR 128522–DOPE liposomes as compared with complexes formed from RPR 128522 micelles (Figure 7). We suggest that DNA from the latter complexes is released too easily and/or too fast in endosomal compartments, or into the cytoplasm where it is possibly digested by nucleases [28]. Countering this effect, DOPE might perform a stabilizing effect on these complexes, which ensures reporter-gene expression. In addition, internalization studies show that complexes from RPR 128522–DOPE liposomes are more easily internalized than from RPR 128522 micelles (Figure 7). Therefore, DOPE might also contribute to an increased transfection activity by positively affecting the cellular uptake of these complexes.

The transfection activity of DNA particles formulated with the asymmetric disulphide lipids of group 2 was up to 1000 times higher than the transfection rate of DNA complexes containing the non-reducible analogue RPR 203769 (Figure 7). Interestingly, RPR 132775 complexes were more active than those of RPR 202059, although only RPR 202059–DNA complexes released DNA in the presence of a reducing agent. One interpretation of these results could indicate that (i) complexes formed from the asymmetric disulphide lipid RPR 202059 and the symmetric disulphide lipid RPR 128522 are too unstable and release DNA too fast and/or easily upon disulphide reduction. (ii) Furthermore, the higher transfection activity of RPR 132775–DNA complexes might be due to a raised membranolytic activity of the C_{18} detergent resulting from RPR 132775 reduction, whereas reduced RPR 202059 bears only a C_5 carbonic chain. However,

uncharacterized processes, different from facilitated DNA release, can also be made responsible for the increased transfection activity.

No general mechanism of reduction in cells has yet been reported. However, several reduction-dependent biological mechanisms such as cytotoxicity concerning diptherietoxin and ricin, and sinbis virus membrane fusion have been described [29–31]. These mechanisms have allowed the importance of freely accessible thiol groups on the cell surface for disulphide reduction to be recognized. Moreover, parts of the Golgi apparatus seem to participate in cellular reduction reactions. The high cytosolic glutathione concentration, which can reach up to 10 mM in the liver, is also reported to affect intracellular reduction [32].

In the present work we have demonstrated the destabilization of several disulphide-lipid–DNA complexes by reduction, which is followed by DNA release. Disulphide lipids that minimize their DNA-complexing capacity due to reduction release DNA easily. In comparison with their non-disulphide analogues, such lipids can provide an improved reporter-gene activity in transfected cells. Additionally, our internalization studies show individual differences in cellular uptake of the cationic lipid–DNA complexes. Future experiments will be designed to directly address the occurrence of intracellular lipid reduction and its influence on intracellular trafficking of transfected DNA.

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