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Review Article

Cytotoxicity issues pertinent to lipoplex-mediated gene therapy in-vivo

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Abstract

Cationic liposomes bind with nucleic acids such as plasmids and oligodeoxynucleotides to form complexes known as lipoplexes. Although these lipoplexes have several advantages over other forms of nucleic acid transfer methods in cell culture and in-vivo, toxicity remains a problem, especially in-vivo. Nevertheless, these carriers have been used in clinical trials against cystic fibrosis and cancer and their usage is attributed mainly to their versatility, especially when it comes to the range of routes available for administration of nucleic-acid-based drugs in-vivo.

Introduction to lipoplexes

Lipoplexes are formed by the interaction of anionic nucleic acids binding to the surface of cationic liposomes eventually forming largely multilamellar lipid–nucleic acid complexes. The nucleic acid molecules persist glued to lipidic molecules with a lipid bilayer surrounding the compacted nucleolipidic particles in one of several different moieties (Choi et al 2001). This heterogeneity may be attributed to the lipidic composition of the vesicles, the manner in which the complexes are formed, the lipid to nucleic acid ratio, the size of the nucleic acid construct, batch-to-batch variation in reagents, and the technique used to treat and visualize these complexes (reviewed in Dass & Su 2000).

In addition to electrostatic attraction, hydrophobic interactions are believed to aid complex formation between lipids and nucleic acids (Wong et al 1996). Hence, depending on the positive (cationic lipid) to negative (phosphate group on nucleic acid) charge ratio, lipoplexes enter cells through electrostatic interaction with such charged residues at the cell surface as sialic acid moieties, or by hydrophobic interaction with the hydrophobic regions of the plasma membrane. A common molecule used in cationic liposome synthesis is the neutral lipid dioleoylphosphatidylethanolamine (DOPE). The role of DOPE is to facilitate membrane fusion or aid in the destabilization of the plasmalemma or endosome (Felgner et al 1994).

Helper lipids such as DOPE are required to stabilize the cationic liposome suspension as cationic lipids repel each other (Zuidam & Barenholz 1998) and to counteract the inhibitory effects of anionic glycosaminoglycans on transfection (Ruponen et al 1999). Liposomes formulated without neutral lipid(s) have inferior rates of transfection (Lasic & Pearlman 1996). That lipoplexes may be stored for as long as a year in sterile water (Cao et al 2000) and may be administered in-vivo via the vascular route (Dass 1998) highlights the versatile usefulness of these vehicles. However, shelf-life is highly dependent on the chemical constituents in the

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formulation as some formulations tend to form macroscopic aggregates over time (Das & Niven 2001).

Factors affecting lipoplex-mediated gene transfer

The success of lipoplex-mediated DNA transfer is dependent on numerous factors and these may explain the inherent variability of lipofection (lipoplex-mediated transfection), particularly in cell culture (Wheeler et al 1996; Dass et al 2000). Factors that influence lipoplex-mediated nucleic acid transfer in-vivo can be categorized as formulation-associated effects and cell culture-associated effects. The former can be further sub-categorized as lipid-related effects, formulation modification-related effects and complex-related effects, while the latter can be divided into cell-related effects and culture condition-related effects.

Lipid-related effects include the cationic lipid component making up the vesicle (Dass 1998), cationic to neutral lipid ratio (Dass et al 2002), and the type of neutral lipid in the vesicle (Boussif et al 2001). Formulation modification-related effects include docking of lipoplexes on to larger solid particles such as polystyrene divinylbenzene microspheres (Dass et al 2000), inclusion of fusogenic viral coat proteins (Namoto et al 1998), or the presence of other non-lipidic biomolecules in the precomplexed liposomes (Chen et al 2000). Complex-related effects include the time lipoplexes are allowed to mature before addition to cells (Yang & Huang 1998), DNA to liposome ratio (Bergan et al 2000), and size of the lipoplexes (Jaaskelainen et al 2000).

Cell-related effects include cell type (Son et al 2000), cell seeding density (Lascombe et al 1996), whether the culture is a primary line or subculture of a primary (Harrison et al 1995), and stage of the cell in the growth cycle (Pickering et al 1994). Culture condition-related effects include the presence of serum in the transfection mixture (Hwang et al 2001), dilution of lipoplex suspension (Staggs et al 1996), the time lipoplexes are incubated with cells (Zabner et al 1995), type and concentration of salts and other biomolecules present in the liposome–nucleic acid mixing medium (Wasan et al 1999).

Although these studies relate primarily to cell culture transfection with lipoplexes, they may, with caution, be extrapolated to the in-vivo setting. Certain issues highlighted by these findings in cell culture are very relevant in-vivo when toxicity is considered. For instance, the cytotoxicity owing to alteration of the liposome to nucleic acid ratio in cell culture can be taken to be a fairly reasonable indication of potential toxicity in-vivo. Such direct observations as the size of lipoplex aggregates in-vitro are very informative when the step towards in-vivo applications with these lipoplexes is to be taken. However, in certain cases, results in cell culture cannot be extrapolated directly to what may occur in-vivo. A good example is the use of immunoliposomes, which function in specific delivery to "target cells" in culture (Watanabe et al 2000), but fail to perform adequately in specific targeting in-vivo.

Clinical trials using lipoplexes

Although these vehicles have been proven to be non-toxic in several studies, including phase I and II clinical trials, slight cases of toxicity still occasionally emerge as discussed below. To date, there are clinical trials with lipoplexes for a variety of ailments, including cystic fibrosis and cancer. Administration of lipoplexes to patients has been performed by various routes, including the intranasal (Hyde et al 2000; Noone et al 2000), intratumoral (Hui et al 1997; Stopeck et al 2001), intraperitoneal (Hortobagyi et al 2001), intrathoracic (Hortobagyi et al 2001), intraarterial (Nabel et al 1994) and intrapleural (Xing et al 1998) routes. Such a variety of administration routes parallel those examined with lipoplexes in the numerous preclinical studies (reviewed in Dass & Burton 1999).

In animal studies, lipoplexes have been introduced via other routes, such as intravenous, intracerebral, as an eye droplet, as an enema preparation and direct injection into such tissues as kidney, liver and skeletal muscle (reviewed in Dass & Burton 1999). It is of some interest to note that the intravenous route, the most common route in animal studies (Dass & Su 2000), has not been evaluated in humans. This may be owing to the fact that the intravenous route presents as a convenient (fairly non-invasive) route through which repeat administrations may be given to small animals such as mice or rats. Although repeat administrations via the intravenous route are also possible clinically, it can be proposed that because of the aggregate nature of these complexes, direct administration into the blood circulation should be avoided given the risk of causing embolisms in capillary beds. Furthermore, direct contact with blood would cause a quicker inactivation of lipoplexes owing to the activity of serum factors such as those belonging to the complement pathway (Yang & Huang 1997). Intradermal, subcutaneous or intramuscular injections may also be fraught with risk if the lipoplexes cause an immunological reaction at the site of injection.

At present, the intraperitoneal route may be the most attractive way for "systemic" administration of lipoplexes, but repeat doses may become non-viable after a certain period owing to patient discomfort. In any case, distribution of complexes from the peritoneum into the circulation is not expected to be reproducible from one individual to another. The role of the lymphatics in draining the peritoneal cavity and removing the reservoir of lipoplexes will need to be addressed as will the immune status of the patient, as a greater presence of immune cells such as macrophages in the peritoneal cavity may rapidly deplete the depot at that site.

In cancer genotherapy, for tumours visible at the surface such as the head-and-neck carcinomas and melanomas at other sites, the direct intratumoral route is undoubtedly the best method for treatment. This has been tested in numerous preclinical studies (reviewed in Dass & Burton 1999) and also in a range of clinical studies (Nabel et al 1993; Hui et al 1997; Stopeck et al 2001). All the above clinical studies have delivered plasmid constructs expressing genes for a major histocompatibility complex protein, in the hope of enhancing immune reaction against the invading tumour cells.

The types of cationic liposomes used for cystic fibrosis genotherapy include those containing cationic lipids such as DC-Chol (Caplen et al 1995; Gill et al 1997; Hyde et al 2000), DMRIE (Sorscher et al 1994), DOTAP (Porteous et al 1997), EDMPC (Noone et al 2000) and GL-67 (Ruiz et al 2001). The main hurdle with cystic fibrosis genotherapy using lipoplexes remains the presence of thick and relatively dehydrated mucus secretions characteristic of the disease. The presence of surfactants in lung tissue may also pose some problems owing to their interaction with the lipids constituting the lipoplexes administered via the nasal/tracheal route. In any case, none of the above clinical trials have reported immunological reactions with lipoplexes or signs of general toxicity especially within the pulmonary system.

For cancer genotherapy using the adenovirus subtype-5 E1A gene, the DC-Chol cationic liposome has been used (Xing et al 1998; Hortobagyi et al 2001), and for cancer immunogenotherapy with such genes as the HLA-B7 gene, the DC-Chol cationic liposome has been a common choice (Nabel et al 1993, 1994, 1996; Hui et al 1997; Stopeck et al 2001). The major problems include the inability to express the foreign protein in all cancerous cells, thus the chances of relapses are very high. Different mechanisms for injecting lipoplexes into tumours are needed, incorporating novel ways to homogeneously deliver complexes both spatially and temporally to reach as many cells as possible. The development of vesicles that gain entry into cells more readily, while not causing excessive toxicity (in the case of leakage from injection site), may help alleviate some of the difficulties encountered in this area.

Reflection on the available routes for administration of lipoplexes in-vivo

The intravenous route has been commonly used in murine studies, whereas the intraarterial route has been reserved for larger animals. The intraarterial route is not feasible in mice because of the obvious space restrictions for doing surgery in these small mammals as well as the small size of their arteries. In contrast, various arteries may be cannulated in the rat, including the carotid, renal and the main aortic vessel. The intra-arterial route, one of the most promising routes for homogeneous delivery to tumour tissue, has also been trialled in the clinical setting with lipoplexes (Nabel et al 1994).

The intratumoral route, the most direct route possible to the lesion site, has been used in both rodent and human studies. Intratumoral injection depends largely on the site of the tumour and also on issues such as the possibility of cells becoming dislodged from the primary growth and travelling to establish secondary neoplasms in other distal sites, an unnecessary metastatic process. When carrying out intratumoral administrations, studies commonly fail to report the effects of such dosing on the tumour architecture, the distribution of the nucleic acids within the tumour mass, and whether the injections are capable of dislodging tumour cells.

Lipoplexes have been shown to enter cells via clathrin-involved endocytosis, and to become entrapped in endosomes, being released from these vesicular structures and gaining entry into the perinuclear area before finally being taken up into the nucleus (Cao et al 2000). Friend et al (1996) describe vesicular and reticular intranuclear membranes probably resulting from fusion of lipoplexes with the nuclear envelope. An alternative pathway that has commonly been proposed is that the nucleic acid molecules are released from their lipidic carriers before escaping from the endosome/lysosome. Although the path from the exterior of the cell to the nucleus with these lipidic carriers is not well defined, that the transgene is able to express itself at all in target cells after travelling from the site of administration is in itself an amazing feat. One of the major hurdles would be the very slow rate of diffusion of large nucleic acid molecules such as plasmids greater than 2000 base pairs in length in the cytosol, with diffusion being nearly impossible once in the nucleus (Lukacs et al 2000).

Some of the earlier generation cationic liposomes

such as DMRIE and DC-Chol were tested in clinical trials, but the resultant biological (therapeutic) effects with these vesicles were at best marginal, and the formulations were hampered to various extents by toxicity issues. Recent research has pinpointed certain features of cationic liposomes that serve to enhance their capability for nucleic acid delivery in-vivo. These include the cationic head group and its neighbouring aliphatic chain being in a 1,2-relationship on the backbone, ether bond for bridging the aliphatic chains to the backbone, and paired oleyl chains acting as the hydrophobic tether (Ren et al 2000). These features, although not determining better transfection capacity in cell culture, allowed better nucleic acid delivery in-vivo and are biodegradable. Thus, as mentioned before, in-vitro and cell culture results have to be treated with caution and cannot necessarily be used to extrapolate the potential of a carrier in-vivo.

Toxicity owing to cationic liposomes and lipoplexes in cell culture

Lipoplex-mediated toxicity is closely associated with the charge ratio between the cationic lipid species in the formulation and the nucleic acid, as well as the dose of lipoplexes administered (Dass et al 2002). Higher charge ratios are generally more toxic to a variety of cell types, including cancer cell lines. In addition, different reagents have different degrees of toxicity to cells, and toxicity is cell-specific. There are currently in excess of 30 different commercial varieties of cationic liposome formulations available. Because of cytotoxicity, delivery of lipoplexes in-vivo has to be as close to the target site as possible to minimize side-effects. The issue of non-specific efficacious effects (some of which are attributed to toxicity) of lipoplexes as well as other cationic polymeric carriers (Bielinska et al 1996; Lambert et al 1998; Xu et al 1998) has to be taken into consideration.

Several disadvantages of using cationic liposomes and lipoplexes have emerged over the past 25 and 12 years of development and usage, respectively. In cell culture, lipoplexes cause changes to cells, including cell shrinking, reduced number of mitoses, and vacuolization of the cytoplasm (Lappalainen et al 1994). Activity of certain proteins such as protein kinase C may also be affected detrimentally by cationic amphiphiles (Aberle et al 1998). Other toxic effects of cationic lipids in cells in culture include haemolysis (Senior et al 1991), fusion between erythrocytes (Sakurai et al 2001), enhanced superoxide production by neutrophils (Ferencick et al 1990), decreased production of IgG and IgM by human peripheral blood mononuclear cells (Jahnova et al 1994), and down-regulation of nitric oxide and tumour necrosis factor-α (TNF-α) synthesis (Filion & Phillips 1997a).

Recent attempts have been made to formulate cationic liposomes from degradable amphiphilic chemicals that can be metabolized following uptake into cells (Choi et al 2001; Pleyer et al 2001). These contrast with the non-biodegradable nature of lipids used in earlier formulations such as DOTMA. A clear distinction between cationic liposome- and lipoplex-mediated toxicity both in cell culture and in-vivo needs to be made as these are more than occasionally confused. Once nucleic acids are complexed to cationic liposomes, the toxicity is expected to decrease owing to neutralization of the positive charge.

Toxicity of cationic liposomes and lipoplexes in-vivo

One of the earliest in-vivo studies, that of Adams & Hamilton (1977), demonstrated that when cationic liposomes were administered via intraocular instillation, an inflammation of the eyes occurred. Intracerebral injection in mice resulted in epileptic seizures and, in severe cases, fatality (Taniguchi et al 1988). Positively charged liposomes have also been noted to cause complement activation via the alternative pathway (Chon et al 1991). When administered intraarticularly into knee joints, these vesicles incited an inflammatory response (Nita et al 1996). Intratracheal administration has shown that although a significant quantity of plasmid DNA (pDNA) enters the nuclei after release from lipoplexes, these cells undergo cell death and hence express low levels of the transgene (Uyechi et al 2001). Cationic liposomes have also been noted to induce acute systemic inflammatory reactions (Malone 1996) and to evoke macrophage and neutrophil infiltration into the lungs of mice when administered intratracheally (Freimark et al 1998).

Litzinger et al (1996) demonstrated that after intravenous injection of lipoplexes into mice, emboli were noted to form in the circulation. These complexes were found to be highly toxic when administered orally, provoking a dramatic hypothermia resulting in death in some mice (Filion & Phillips 1997b). In carrageenan and in sheep red blood cell challenge inflammatory models, these liposomes were found to have strong anti-inflammatory activity (Filion & Phillips 1997b). Intracoronary administration of lipoplexes has been noted to cause aggregated-lipoplex-mediated microinfarctions leading to false positive gene transfer results (Wright et al 1998). Anti-single-strand-DNA antibodies have also

been induced when using these vesicles to deliver DNA (Filion & Phillips 1998).

Infusion of lipoplexes via the renal artery in rats was accompanied by nephrotoxicity and a resultant lack of transgene expression (Madry et al 2001). Hepatotoxicity was noted when localized delivery of lipoplexes via the portal vein was performed in mice (Mohr et al 2001). Lack of expression owing to toxicity is probably because of the very localized route of delivery since administration of lipoplexes via the aorta to kidneys in rats resulted in expression in both kidney parenchyma and a parasitic tumour in the kidney in other studies (Dass 1998; Dass et al 2000). It may also be owing to the dose and charge ratio of lipoplexes delivered. Large aggregates of lipoplexes may also cause problems because of microinfarction, causing tissue ischaemia and possible myocardial damage when administered intraarterially (Wright et al 1998). Administration of lipoplexes via the intraarterial route in rats decreases the half-life of pDNA from 3.1 (free) to 2.6 days in rats (Minchin et al 2001). This may be owing to a rapid removal by the reticuloendothelial system in-vivo. Thus, the effects of the complexes may prove counterproductive in-vivo even though enhancing the uptake of nucleic acids in cells in culture.

Loss of gene expression owing to lipoplex-mediated enhanced immunogenicity

Following systemic delivery of either adenoviral vectors or lipoplexes, transgene expression is relatively transient. Lung expression of intravenously delivered lipoplexes decreases by approximately 1 log per week from the maximal expression observed one and two days after administration (Li et al 1999; Tan et al 1999). Several mechanisms may be responsible for this phenomenon: (i) generation of neutralizing antibody against the foreign gene product; (ii) cytokine-mediated promoter shutdown; and (iii) eradication of the expressing cells through apoptosis, innate or adaptive immune reactions. Such mechanisms have major implications since repeat administrations are impossible because of cumulative immune responses and net transgene expression decreases over time. This phenomenon has been the real stumbling block for various forms of gene therapy, including those utilizing viral vectors.

In-vitro, it has been demonstrated that TNF-α and interferon-γ (IFN-γ) down-regulate gene expression from such common promoters as cytomegalovirus, independent of the genetic composition of the gene transfer vector (Qin et al 1997). To confirm this, administration of neutralizing antibody against IFN-γ significantly enhanced in-vivo expression several weeks after commencement of gene delivery (Qin et al 1997). There have been numerous reports of intravenous administration of synthetic vectors containing bacterially derived DNA inciting the production of cytokines such as TNF-α, IFN-γ and interleukin-12 (Dow et al 1999; Li et al 1999; Tan et al 1999; Whitmore et al 1999; Bramson et al 2000).

As mentioned above, cytokines are important negative regulators of gene expression since administration of anti-cytokine antibodies results in enhanced levels of gene expression (Li et al 1999). Importantly, cytokine production seems to be directly associated with the presence of stimulatory cytosine-guanine (CpG) motifs in vector DNA, since the methylation or elimination of these motifs reduces plasma cytokine levels (Li et al 1999; Yew et al 2000). Furthermore, the use of dexamethasone, a commonly used immunosuppressant, reduces cytokine generation in-vivo and improves the level and duration of gene expression (Tan et al 1999).

Although gene expression levels in the lung have been improved by neutralizing or eliminating cytokine production, the overall rate of decrease in gene expression over time is unaffected, suggesting that additional mechanisms are at work (Li et al 1999; Tan et al 1999). Apoptosis of transgene-expressing cells is one hypothesis put forward to explain this loss in expression (Li et al 1999). Intravenous administration-mediated toxicity may be owing to the increase of transaminase levels in mice, which display histopathological lesions in the liver, but no adverse effects in the lung at the same dose (Loisel et al 2001). This release of transaminases is attributed to the presence of unmethylated bases in the pDNA sequence.

The future for cationic liposomes and lipoplexes in nucleic acid transfer

Further enhancement in delivery of genetic medicine in-vivo may be attained with other vesicles such as immunoliposomes (Watanabe et al 2000), thermosensitive liposomes (Gaber et al 1996; Needham et al 2000), coated lipoplexes (Stuart et al 2000) or stabilized antisense lipid particles (SALPs) (Leonetti et al 2001; Mui et al 2001). Immunoliposomes and thermosensitive liposomes, although showing some promise in cell culture, have not readily been adapted for in-vivo usage.

Of the above-mentioned vesicles, SALPs have shown the greatest promise for several reasons. SALPs are formulated from an ionizable aminolipid and polyethylene glycol (PEG) for stabilization during the formulation process and, more importantly, while in transit

through the vasculature. SALPs are 80–140 nm in diameter and are multilamellar vesicles that exhibit significantly prolonged plasma circulation time when compared with free nucleic acids and lipoplexes (Semple et al 2000). They also mask the cationic lipid as it travels through the vasculature, unmasking the cationic liposome as the target site is reached by a successive loss of the PEGylated lipids.

Tumour uptake studies reveal that enhanced uptake in tumour cells is possible with SALPs, because of the larger area under the plasma concentration–time curve and increased resistance against nuclease-mediated degradation (Leonetti et al 2001). Coated lipoplexes may have some potential since these are cationic liposomes coated with PEG-conjugated lipids to increase circulation time in-vivo. More work needs to be done in-vivo to better characterize these vesicles and develop mechanisms for increasing nucleic acid delivery. In any case, stabilized formulations of lipoplexes retain their transfection capability longer in-vivo in comparison with their non-stabilized counterparts and this in itself is a major improvement (Hong et al 1997).

Summary

Cationic lipid–DNA complexes (lipoplexes) are one of the most efficient ways of delivering nucleic acids into cultured cells and have been used in-vivo with varying degrees of success. However, nucleic acid delivery has been traditionally hindered by the toxicity associated with these formulations. Novel formulations that are more biocompatible especially in-vivo are being tested, but further research needs to be done before these carriers are introduced into clinical trials. A recent finding has been the enhancement of general immunogenic recognition of foreign nucleic acids complexed to cationic lipids. Such immunostimulation may prove to be further disadvantageous for nucleic acid delivery with lipoplexes. Provided that the toxic effects attributed to cationic lipids can be alleviated (important because of lessons learnt from the other liposome formulations available), the next few years should prove to be quite decisive on whether lipoplexes will be more readily introduced into the clinical setting against such ailments as cystic fibrosis and cancer.

References

Aberle, A. M., Tablin, F., Walker, N. J., Gruenert, D. C., Nantz, M. H. (1998) A novel tetraester construct that reduces cationic lipid-associated cytotoxicity. Implications for the onset of cytotoxicity. *Biochemistry* **37**: 6533–6540

Adams, D. O., Hamilton, T. A. (1977) The cell biology of macrophage activation. *Ann. Rev. Immunol.* **2**: 283–318

Bergan, D., Galbraith, T., Sloane, D. L. (2000) Gene transfer in vitro and in vivo by cationic lipids is not significantly affected by levels of supercoiling of a reporter plasmid. *Pharm. Res.* **17**: 967–973

Bielinska, A., Kukowska-Latallo, J. F., Johnson, J., Tomalia, D. A., Baker, J. R., Jr (1996) Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers. *Nucleic Acids Res.* **24**: 2176–2182

Boussif, O., Gaucheron, J., Boulanger, C., Santaella, C., Kolbe, H. V., Vierling, P. (2001) Enhanced in vitro and in vivo cationic lipid-mediated gene delivery with a fluorinated glycerophosphoethanolamine helper lipid. *J. Gene Med.* **3**: 109–114

Bramson, J. L., Bodner, C. A., Graham, R. W. (2000) Activation of host tumoral responses by cationic lipid/DNA complexes. *Cancer Gene Ther.* **7**: 353–359

Cao, A., Briane, D., Coudert, R., Vassy, J., Lievre, J., Olsman, E., Tamboise, E., Salzmann, J. L., Rigaut, J. P., Taillandier, E. (2000) Delivery and pathway in MCF7 cells of DNA vectorized by cationic liposomes derived from cholesterol. *Antisense Nucleic Acid Drug Dev.* **10**: 369–380

Caplen, N. J., Alton, E. W. F. W., Middleton, P. G., Dorin, J. R., Stevenson, B. J., Gao, X., Durham, S. R., Jeffery, P. K., Hodson, M. E., Coutelle, C., Huang, L., Porteous, D. J., Williamson, R., Geddes, D. M. (1995) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat. Med.* **1**: 39–46

Chen, Q.-R., Zhang, L., Stass, S. A., Mixson, A. J. (2000) Co-polymer of histidine and lysine markedly enhances transfection efficiency of liposomes. *Gene Ther.* **7**: 1698–1705

Choi, J. S., Lee, E. J., Jang, H. S., Park, J. S. (2001) New cationic liposomes for gene transfer into mammalian cells with high efficiency and low toxicity. *Bioconjug. Chem.* **12**: 108–113

Chon, A., Cullis, P. R., Devine, D. V. (1991) The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol.* **146**: 4234–4241

Das, A., Niven, R. (2001) Use of perfluorocarbon (Fluorinert) to enhance reporter gene expression following intratracheal instillation into the lungs of Balb/c mice: implications for nebulized delivery of plasmids. *J. Pharm. Sci.* **90**: 1336–1344

Dass, C. R. (1998) Targeted delivery of DNA for cancer therapy. PhD thesis, Charles Sturt University, Wagga Wagga, Australia

Dass, C. R., Burton, M. A. (1999) Lipoplexes and tumours. *J. Pharm. Pharmacol.* **51**: 755–770

Dass, C. R., Su, T. (2000) Delivery of lipoplexes for genotherapy of solid tumours: role of vascular endothelial cells. *J. Pharm. Pharmacol.* **52**: 1301–1317

Dass, C. R., Walker, T. L., Kalle, W. H. J., Burton, M. A. (2000) A microsphere–lipoplex (microplex) vector for targeted gene therapy of cancer. II. In vivo biodistribution study in a solid tumour model. *Drug Deliv.* **7**: 15–20

Dass, C. R., Walker, T. L., Burton, M. A. (2002) Liposomes containing cationic dimethyl dioctadecyl ammonium bromide (DDAB): formulation, quality control and lipofection efficiency. *Drug Deliv.* **9**: 11–18

Dow, S. W., Fradkin, L. G., Liggitt, D. H., Wilson, A. P., Heath, T. D., Potter, T. A. (1999) Lipid–DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. *J. Immunol.* **163**: 1552–1561

Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J.,

Border, R., Ramsey, P., Martin, M., Felgner, P. L. (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* **269**: 2550–2561

Ferencick, M., Lacko, I., Devinsky, F. (1990) Immunomodulatory activity of some amphiphilic compounds. *Pharmazie* **45**: 695–696

Filion, M. C., Phillips, N. C. (1997a) Anti-inflammatory activity of cationic lipids. *Br. J. Pharmacol.* **122**: 551–557

Filion, M. C., Phillips, N. C. (1997b) Toxicity and immunomodulatory activity of some liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim. Biophys. Acta* **1329**: 345–356

Filion, M. C., Phillips, N. C. (1998) Major limitations in the use of cationic liposomes for DNA delivery. *Int. J. Pharm.* **162**: 159–170

Freimark, B. D., Blezinger, H. P., Florack, V. J., Nordstrom, J. L., Long, S. D., Deshpande, D. S., Nochumson, S., Petrak, K. L. (1998) Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid: cationic lipid complexes. *J. Immunol.* **160**: 4580–4586

Friend, D. S., Papahadjopoulos, D., Debs, R. (1996) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochim. Biophys. Acta* **1278**: 41–50

Gaber, M. H., Wu, N. Z., Hong, K., Huang, S. K., Dewhirst, M. W., Papahadjopoulos, D. (1996) Thermosensitive liposomes: extravasation and release of contents in tumor microvascular networks. *Int. J. Radiat. Oncol. Biol. Phys.* **36**: 1177–1187

Gill, D. R., Southern, K. W., Mofford, K. A., Seddon, T., Huang, L., Sorgi, F., Thomson, A., MacVinish, L. J., Ratcliff, R., Bilton, D., Lane, D. J., Littlewood, J. M., Webb, A. K., Middleton, P. G., Colledge, W. H., Cuthbert, A. W., Evans, M. J., Higgins, C. F., Hyde, S. C. (1997) A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **4**: 199–209

Harrison, G. S., Wang, Y., Tomczak, J., Hogan, C., Shpall, E. J., Curiel, T. J., Felgner, P. L. (1995) Optimization of gene transfer using cationic lipids in cell lines and primary human $CD4^+$ and $CD34^+$ hematopoietic cells. *Biotechniques* **19**: 816–823

Hong, K. L., Zheng, W.-W., Baker, A., Papahadjopoulos, D. (1997) Stabilization of cationic liposome–plasmid DNA complexes by polyamines and poly(ethylene glycol)–phospholipid conjugates for efficient in vivo gene delivery. *FEBS Lett.* **400**: 233–237

Hortobagyi, G. N., Ueno, N. T., Xia, W., Zhang, S., Putnam, J. B., Weiden, P. L., Willey, J. S., Carey, M., Branham, D. L., Payne, J. Y., Tucker, S. D., Barthalomeusz, C., Kilbourn, R. G., De Jager, R. L., Sneige, N., Katz, R. L., Anklesaria, P., Irahim, N. K., Murray, J. L., Theriault, R. L., Valero, V., Gershenson, D. M., Bevers, M. W., Huang, L., Lopez-Berestein, G., Hung, M. C. (2001) Cationic liposome-mediated E1A gene transfer to human breast and ovarian cancer cells and its biologic effects: a Phase I clinical trial. *J. Clin. Oncol.* **19**: 3422–3433

Hui, K. M., Ang, P. T., Huang, L., Tay, S. K. (1997) Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA–liposome complexes. *Gene Ther.* **4**: 783–790

Hwang, S. H., Hayashi, K., Takayama, K., Maitani, Y. (2001) Liver-targeted gene transfer into a human hepatoblastoma cell line and in vivo by sterylglucoside-containing cationic liposomes. *Gene Ther.* **8**: 1276–1280

Hyde, S. C., Southern, K. W., Gileadi, U., Fitzjohn, E. M., Mofford, K. A., Waddell, B. E., Gooi, H. C., Goddard, C. A., Hannavy, K., Smyth, S. E., Egan, J. J., Sorgi, F. L., Huang, L., Cuthbert, A. W., Evans, M. J., Colledge, W. H., Higgins, C. F., Webb, A. K., Gill, D. R. (2000) Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **7**: 1156–1165

Jaaskelainen, I., Peltola, S., Honkakoski, P., Monkkonen, J., Urtti, A. (2000) A lipid carrier with a membrane active component and a small complex size are required for efficient cellular delivery of antisense phosphorothioate oligonucleotides. *Eur. J. Pharm. Sci.* **10**: 187–193

Jahnova, E., Ferencick, M., Nyulassy, S., Devinsky, F., Lasko, I. (1994) Amphiphilic detergents inhibit production of IgG and IgM by human peripheral blood mononuclear cells. *Immunol. Lett.* **39**: 71–75

Lambert, G., Fattal, E., Brehier, A., Feger, J., Couvreur, P. (1998) Effect of polyisobutylcyanoacrylate nanoparticles and lipofectin loaded with oligonucleotides on cell viability and PKC alpha neosynthesis in HepG2 cells. *Biochimie* **80**: 969–976

Lappalainen, K., Jääskeläinen, I., Syrjänen, K., Urtti, A., Syrjänen, S. (1994) Comparison of cell proliferation and toxicity assays using two cationic liposomes. *Pharm. Res.* **11**: 1127–1131

Lascombe, I., Mougin, P., Vuillermoz, C., Adessi, G. L., Jouvenot, M. (1996) Gene transfer into subcultured endometrial cells using lipofection. *Biotechniques* **20**: 88–91

Lasic, D. D., Pearlman, R. (1996) Liposomes and lipidic particles in gene therapy. In: Rosoff, M. (ed.) *Vesicles*. New York, Marcel Dekker, pp 477–489

Leonetti, C., Biroccio, A., Benassi, B., Stringaro, A., Stoppacciaro, A., Semple, S. C., Zupi, G. (2001) Encapsulation of c-*myc* antisense oligodeoxynucleotides in lipid particles improves antitumoral efficacy in vivo in a human melanoma line. *Cancer Gene Ther.* **8**: 459–468

Li, S., Wu, S.-P., Whitmore, M., Loeffert, E. J., Wanh, L., Watkins, S. C., Pitt, B. R., Huang, L. (1999) Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am. J. Physiol.* (*Lung Cell Mol. Physiol.*) **276**: L796–L804

Litzinger, D. C., Brown, J. M., Wala, I., Kaufmann, S. A., Var, G. Y., Farrell, C. L., Collins, D. (1996) Fate of cationic liposomes and their complex with oligonucleotide in vivo. *Biochim. Biophys. Acta* **1281**: 139–149

Loisel, S., Le Gall, C., Doucet, L., Ferec, C., Floch, V. (2001) Contribution of plasmid DNA to hepatotoxicity after systemic administration of lipoplexes. *Hum. Gene Ther.* **12**: 685–696

Lukacs, G. L., Haggie, P., Seksek, O., Lechardeur, D., Freedman, N., Verkman, A. S. (2000) Size-dependent DNA mobility in cytoplasm and nucleus. *J. Biol. Chem.* **275**: 1625–1629

Madry, H., Reszka, R., Bohlender, J., Wagner, J. (2001) Efficacy of cationic liposome-mediated gene transfer to mesangial cells in vitro and in vivo. *J. Mol. Med.* **79**: 184–189

Malone, R. W. (1996) Toxicology of non-viral gene transfer. In: Walsh, B. (ed.) *Non-viral genetic therapeutics: advances, challenges and applications for self-assembling systems*. Boston, IBC's Biomedical Library Series, pp 4.1.1–4.1.26

Minchin, R. F., Carpenter, D., Orr, R. J. (2001) Polyinosinic acid and polycationic liposomes attenuate the hepatic clearance of circulating plasmid DNA. *J. Pharmacol. Exp. Ther.* **296**: 1006–1012

Mohr, L., Yoon, S. K., Eastman, S. J., Chu, Q., Schuele, R. K., Scaglioni, P. P., Geissler, M., Heintges, T., Blum, H. E., Wands, J. R. (2001) Cationic liposome-mediated gene delivery to the liver and to hepatocellular carcinomas in mice. *Hum. Gene Ther.* **12**: 799–809

Mui, B., Raney, S. G., Semple, S. C., Hope, M. J. (2001) Immune

stimulation by a CpG-containing oligodeoxynucleotide is enhanced when encapsulated and delivered in lipid particles. *J. Pharmacol. Exp. Ther.* **298**: 1185–1192

Nabel, E. G., Yang, Z., Muller, D., Chang, A. E., Gao, X., Huang, L., Cho, K. J., Nabel, G. J. (1994) Safety and toxicity of catheter gene delivery to the pulmonary vasculature in a patient with metastatic melanoma. *Hum. Gene Ther.* **5**: 1089–1094

Nabel, G. J., Gordon, D., Bishop, D. K., Nickoloff, B. J., Yang, Z.-Y., Aruga, A., Cameron, M. J., Nabel, E. G., Chang, A. E. (1996) Immune response in human melanoma after transfer of an allogeneic class I major histocompatibility complex gene with DNA-liposome complexes. *Proc. Natl Acad. Sci. USA* **93**: 15388–15393

Nabel, G. J., Nabel, E. G., Yang, Z. Y., Fox, B. A., Plautz, G. E., Gao, X., Huang, L., Shu, S., Gordon, D., Chang, A. E. (1993) Direct gene transfer with DNA–liposome complexes in melanoma: expression, biological activity, and lack of toxicity in humans. *Proc. Natl Acad. Sci. USA* **90**: 11307–11311

Namoto, M., Yonemitsu, Y., Nakagawa, K., Hasimoto, S., Kaneda, Y., Nawata, H., Sueishi, K. (1998) Heterogeneous induction of apoptosis in colon cancer cells by wild-type p53 gene transfection. *Int. J. Oncol.* **12**: 777–784

Needham, D., Anyarambhatla, G., Kong, G., Dewhirst, M. W. (2000) A new temperature sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. *Cancer Res.* **60**: 1197–1201

Nita, I., Ghivizzani, S. C., Galea-Lauri, J., Bandara, G., Georescu, H. I., Robbins, P. D., Evans, C. H. (1996) Direct gene delivery to synovium. An evaluation of potential vectors in vitro and in vivo. *Arthritis Rheum.* **39**: 820–828

Noone, P. G., Hohneker, K. W., Zhou, Z., Johnson, L. G., Foy, C., Gipson, C., Jones, K., Noah, T. L., Leigh, M. W., Schwartzbach, C., Efthimiou, J., Pearlman, R., Boucher, R. C., Knowles, M. R. (2000) Safety and biological efficacy of a lipid–CFTR complex for gene transfer in the nasal epithelium of adult patients with cystic fibrosis. *Mol. Ther.* **1**: 105–114

Pickering, J. G., Jekanowski, J., Weir, L., Takeshita, S., Losordo, D. W., Isner, J. M. (1994) Liposome-mediated gene transfer into human vascular smooth muscle cells. *Circulation* **89**: 13–21

Pleyer, U., Groth, D., Hinz, B., Keil, O., Bertelmann, E., Rieck, P., Reszka, R. (2001) Efficiency and toxicity of liposome-mediated gene transfer to corneal epithelial cells. *Exp. Eye Res.* **73**: 1–7

Porteous, D. J., Dorin, J. R., McLachlan, G., Davidson-Smith, H., Davidson, H., Stevenson, B. J., Michaelis, U., Naujoks, K., Ho, L. P., Samways, J. M., Imrie, M., Greening, A. P., Innes, J. A. (1997) Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **4**: 210–218

Qin, L., Ding, Y., Pahud, D. R., Chang, E., Imperiale, M. J., Bromberg, J. S. (1997) Promoter attenuation in gene therapy: interferon-γ and tumor necrosis factor-α inhibit transgene expression. *Hum. Gene Ther.* **8**: 2019–2029

Ren, T., Song, Y. K., Zhang, G., Liu, D. (2000) Structural basis of DOTMA for its high intravenous transfection activity in mouse. *Gene Ther.* **7**: 764–768

Ruiz, F. E., Clancy, J. P., Perricone, M. A., Bebok, Z., Hong, J. S., Cheng, S. H., Meeker, D. P., Young, K. R., Schoumacher, R. A., Weatherly, M. R., Wing, L., Morris, J. E., Sindel, L., Rosenberg, M., van Ginkel, F. W., McGhee, J. R., Kelly, D., Lyrene, R. K., Sorscher, E. J. (2001) A clinical inflammatory syndrome attributable to aerosolized lipid–DNA administration in cystic fibrosis. *Hum. Gene Ther.* **12**: 751–761

Ruponen, M., Yla-Herttuala, S., Urtti, A. (1999) Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochim. Biophys. Acta* **1415**: 331–341

Sakurai, F., Nishioka, T., Saito, H., Baba, T., Okuda, A., Matsumoto, O., Taga, T., Yamashita, F., Takakura, Y., Hashida, M. (2001) Interaction between DNA–cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. *Gene Ther.* **8**: 677–686

Semple, S. C., Klimuk, S. K., Harasym, T. O., Santos, N. D., Ansell, S. M., Wong, K. F., Maurer, N., Stark, H., Cullis, P. R., Hope, M. J., Scherrer, P. (2000) Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. *Biochim. Biophys. Acta* **1508**: 1–15

Senior, J. H., Trimble, K. R., Maskiewicz, R. (1991) Interaction of positively-charged liposomes with blood: implications for their application in vivo. *Biochim. Biophys. Acta* **1070**: 173–179

Son, K. K., Tkach, D., Hall, K. J. (2000) Efficient in vivo gene delivery by the negatively charged complexes of cationic liposomes and plasmid DNA. *Biochim. Biophys. Acta* **1468**: 6–10

Sorscher, E. J., Logan, J. J., Frizzell, R. A., Lyrene, R. K., Bebok, Z., Dong, J. Y., Duvall, M. D., Felgner, P. L., Matalon, S., Walker, L., Wiatrak, B. J. (1994) Gene therapy for cystic fibrosis using cationic liposome mediated gene transfer: a phase I trial of safety and efficacy in the nasal airway. *Hum. Gene Ther.* **5**: 1259–1277

Staggs, D. R., Burton, D. W., Deftos, L. J. (1996) Importance of liposome complexing volume in transfection optimization. *Biotechniques* **21**: 792–797

Stopeck, A. T., Jones, A., Hersh, E. M., Thompson, J. A., Finucane, D. M., Gutheil, J. C., Gonzalez, R. (2001) Phase II study of direct intralesional gene transfer of Allovectin-7, an HLA-B7/β2-microglobulin DNA–liposome complex, in patients with metastatic melanoma. *Clin. Cancer Res.* **7**: 2285–2291

Stuart, D. D., Kao, G. Y., Allen, T. M. (2000) A novel, long-circulating, and functional liposomal formulation of antisense oligodeoxynucleotides targeted against MDR1. *Cancer Gene Ther.* **7**: 466–475

Tan, Y., Li, S., Pitt, B. R., Huang, L. (1999) The inhibitory role of CpG immunostimulatory motifs in cationic lipid vector-mediated transgene expression in vivo. *Hum. Gene Ther.* **10**: 2153–2161

Taniguchi, K., Yamamoto, Y., Itakura, K., Miitchi, H., Hayashi, S. J. (1988) Assessment of ocular irritability of liposome preparations. *J. Pharmacobiodyn.* **11**: 607–611

Uyechi, L. S., Gagne, L., Thurston, G., Szoka, F. C. Jr (2001) Mechanism of lipoplex gene delivery in mouse lung: binding and internalization of fluorescent lipid and DNA components. *Gene Ther.* **8**: 828–836

Wasan, E. K., Harvie, P., Edwards, K., Karlsson, G., Bally, M. B. (1999) A multi-step lipid mixing assay to model structural changes in cationic lipoplexes used for in vitro transfection. *Biochim. Biophys. Acta* **1461**: 27–46

Watanabe, Y., Sawaishi, Y., Tada, H., Sato, E., Suzuki, T., Takada, G. (2000) Immunoliposome-mediated gene transfer into cultured myotubes. *Tohoku J. Exp. Med.* **192**: 173–180

Wheeler, C. J., Felgner, P. L., Tsai, Y. J., Marshall, J., Sukhu, L., Doh, S. G., Hartikka, J., Nietupski, J., Manthorpe, M., Nichols, M., Plewe, M., Liang, X., Norman, J., Smith, A., Cheng, S. H. (1996) A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. *Proc. Natl Acad. Sci. USA* **93**: 11454–11459

Whitmore, M., Li, S., Huang, L. (1999) LPD lipopolyplex initiates a

potent cytokine response and inhibits tumor growth. *Gene Ther.* **6**: 1867–1875

Wong, F. M. P., Reimer, D. L., Bally, M. B. (1996) Cationic lipid binding to DNA: characterization of complex formation. *Biochemistry* **35**: 5756–5763

Wright, M. J., Rosenthal, E., Stewart, L., Wightman, L. M., Miller, A. D., Latchman, D. S., Marber, M. S. (1998) β-Galactosidase staining following intracoronary infusion of cationic liposomes in the in vivo rabbit heart is produced by microinfarction rather than effective gene transfer: a cautionary tale. *Gene Ther.* **5**: 301–308

Xing, X., Chang, J. Y., Hung, M. (1998) Preclinical and clinical study of HER-2/*neu*-targeting cancer gene therapy. *Adv. Drug. Deliv. Rev.* **30**: 219–227

Xu, M., Chen, Q. R., Kumar, D., Stass, S. A., Mixson, A. J. (1998) In vivo gene therapy with a cationic polymer markedly enhances the antitumor activity of antiangiogenic genes. *Mol. Genet. Metab.* **64**: 193–197

Yang, J.-P., Huang, L. (1997) Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. *Gene Ther*: **4**: 950–960

Yang, J.-P., Huang, L. (1998) Time-dependent maturation of cationic liposome–DNA complex for serum resistance. *Gene Ther.* **5**: 380–387

Yew, N. S., Zhao, H., Wu, I.-H., Song, A., Tousignant, J. D., Przybylska, M., Cheng, S. H. (2000) Reduced inflammatory response to plasmid vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mol. Ther.* **1**: 255–262

Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., Welsh, M. J. (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **270**: 18997–19007

Zuidam, N. J., Barenholz, Y. (1998) Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery. *Biochim. Biophys. Acta* **1368**: 115–128