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Review

Biochemical and biophysical characteristics of lipoplexes pertinent to solid tumour gene therapy

Crispin R. Dass *

Johnson & Johnson Research, 1 Central Avenue, Australian Technology Park, Eveleigh 1430, Australia

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Abstract

Cationic liposomes have become the reagent of choice for transfer of nucleic acids such as plasmids and oligodeoxynucleotides to cells in culture and in vivo. Whilst these reagents have several advantages over other forms of nucleic acid transfer methods, toxicity remains a significant problem, especially in vivo. Recent studies have also highlighted the immunostimulatory nature of these cationic vesicles when complexed to plasmid DNA, a phenomenon that may be harnessed for efficacious usage against tumours. Current research in this dynamic technological field is aimed at the development of cationic lipids that have negligible toxic effects and enhanced transfection capabilities.

Keywords: Cationic liposome; Lipoplex; Gene therapy; Cancer; Nucleic acid

1. Introduction

Since 1987, when a cationic lipid was reportedly used to formulate the first vesicle used for introduction of plasmids into cells (Felgner et al., 1987), numerous cationic liposomes (CLs, also called cytofectins) have been synthesised and used for delivery of nucleic acids into cells in culture, in animals and in patients enrolled in phase I and II clinical trials. In comparison to other gene delivery modes, such as viral vectors, CLs are simple and quick to formulate, are not as biologically hazardous as viral vectors, are readily available commercially, and may be relatively easily adapted for specific applications. Other chemical reagents such a dendrimers and porphyrin derivatives have only recently entered the field and a great deal of work remains to be done before these are used clinically.

After the initial 'boom' in use of CLs for both cells in culture and in animals, the realisation that they had certain significant limitations, prompted a shift in their design. One major issue was the degree of toxicity that CLs exhibited in cultured cells and that these effects were drastically pronounced in several animal studies. When low doses of CLs are employed in vivo, transfection results are only slightly better than naked gene delivery, thus signalling the need for administration of higher doses, which then tend to be toxic. In the past 5 years, a major re-emphasis on vehi-

* Tel.: +61-2-83965800; fax: +61-2-83965811.
E-mail address: cdass@medau.jnj.com (C.R. Dass).

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cle safety has been implemented, whilst not detracting from the positive attributes of each CL. The present review looks at some such factors and how these have been overcome, or may be overcome, to allow the field of gene therapy to proceed in step with other forms of treatment modalities for diseases such as cancer and cystic fibrosis (CF).

2. Nucleic acid delivery using lipoplexes

Lipoplexes, also called cationic lipid–DNA-complexes (CLDCs), are formed by the interaction of anionic nucleic acids binding to the surface of CLs eventually forming multilamellar lipid–nucleic acid complexes. The negatively-charged nucleic acids are attracted to the surface of the positively-charged vesicles, initially forming complexes with nucleic acid molecules docked on to the surface of the cationic vesicles (Fig. 1). In the case of double-stranded DNA, 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).

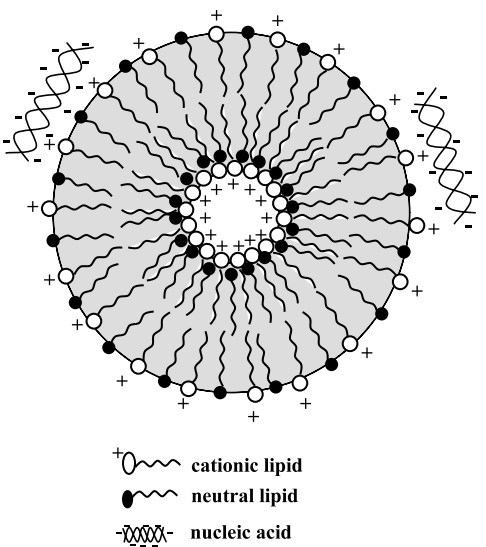

Fig. 1. Formation of lipoplexes. Anionic nucleic acid molecules are attracted to the surface cationic charge of the liposomes (made from a cationic and a neutral lipid mixture) forming complexes that eventually form compact nucleolipidic particles of various morphologies and sizes.

Such a heterogeneity in complex morphology may be attributed to the lipidic composition of the vesicles, the manner in which the complexes were formed, the lipid–nucleic acid ratio, the size of the nucleic acid construct, batch-to-batch variation in reagents, and the technique used to treat and visualise these complexes (reviewed in Dass and Su, 2000; Table 1). 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 (Liu and Huang, 2002), lipoplexes may 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 and aid in the destabilisation of the plasmalemma or endosome (Felgner et al., 1994; Hafez et al., 2001). In addition, helper lipids such as DOPE are required to stabilise the cationic liposome suspension as cationic lipids repel each other (Zuidam and Barenholz, 1998) and to counteract the anti-transfection effects of anionic glycosaminoglycans noted with other carriers such as polyethyleneimine (PEI) and dendrimers (Ruponen et al., 1999). Liposomes formulated without neutral lipid(s) have inferior rates of transfection (Lasic and Pearlman, 1996; Mui et al., 2000), whilst varying rates of transfection may result from varying ratios of cationic–neutral lipid used to formulate the liposomes (Farhood et al., 1995; Dass et al., 2002). As mentioned above, the success of cationic liposome-mediated DNA transfer is dependent on numerous factors (Table 1) which may explain the inherent variability of lipofection (lipoplex-mediated transfection), particularly in vivo (Wheeler et al., 1996; Dass et al., 1997a, 2000). Furthermore, while these vehicles have been proven to be non-toxic in several studies, including phase I and II clinical trials, varying

Table 1
Factors influencing cationic liposome-mediated nucleic acid transfer in cultured cells

Lipid-related effects
- The cationic lipid component making up the vesicles (Dass, 1998; Crook et al., 1998)
- The neutral lipid in the vesicles (Boussif et al., 2001)
- Cationic–neutral lipid ratio in vesicle (Dass et al., 2002)
- Chemical treatment of liposomes prior to addition of DNA (Kariko et al., 1998)
- Toxicity due to formulation (Pleyer et al., 2001)

Formulation–modification-related effects
- Stabilisation of liposomes with non-lipidic agents such as PEG (Saravolac et al., 2000; Semple et al., 2000)
- Presence of fusogenic viral coat proteins (Namoto et al., 1998)
- Presence of replication-defective adenoviral vectors (Dunphy et al., 1999)
- Presence of other non-lipidic biomolecules that enhance transfection (Chen et al., 2000; Yamazaki et al., 2000)
- Docking of liposomes on larger solid particles (Dass, 1998)
- Constitution of formulation (solvent) medium (Boussif et al., 2001)

Cell-related effects
- Cell type (Son et al., 2000)
- Whether the culture is primary or subcultures of the primary (Harrison et al., 1995)
- Stage of cell in the growth cycle (Pickering et al., 1994)
- Cell seeding density (Lascombe et al., 1996)

Culture-condition-related effects
- The constitution of the cell culture medium (Escriou et al., 1998)
- Presence of serum in transfection mixture (Hwang et al., 2001)
- Time liposome–DNA complexes are incubated with cells (Zabner et al., 1995)
- Dilution of liposome–DNA complex suspension (Staggs et al., 1996)
- The type and concentration of salts and biomolecules present in the liposome–DNA mixing medium (Fasbender et al., 1995; Wasan et al., 1999)

Complex-related effects
- Time lipoplexes are allowed to mature prior to addition to cells (Yang and Huang, 1998)
- Size of the liposome–DNA complexes (Kawaura et al., 1998; Jaaskelainen et al., 2000)
- DNA to liposome ratio (Bergan et al., 2000; Harada et al., 2000)
- The pH of the liposome–DNA mixing medium (Turek et al., 2000)

degrees of toxicity still emerge occasionally as discussed below.

That lipoplexes may be stored for as long as a year in sterile water depending on the constituents (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 formulation as some formulations tend to aggregate with time (Das and Niven, 2001). The common routes of administration of lipoplexes in vivo, particularly for delivery to tumours, are listed in Table 2. 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 due to the space restrictions for doing surgery in these small animals as well as due to 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 intratumoural route, the most direct route possible to the lesion site, has been used in both rodent and human studies. Intratumoural 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 mestastatic process. When carrying out intratumoural administrations, studies commonly fail to report the effect of such dosing on the tumour architecture and definitely never on whether the injections are capable of dislodging tumour cells. The intraarterial 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).

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. While the path from the exterior of the cell to the nucleus with these lipidic carriers is not defined completely

(major current hypothesis depicted in Fig. 2), that the transgene is able to express itself at all is an amazing feat per se.

Some of the earlier generation CLs such as DMRIE and DC-Chol were tested in clinical trials (Table 3), but the resultant biological (therapeutic) effects with these vesicles were at best marginal, and the formulations were hampered by toxicity issues. Recent research has pinpointed certain features of CLs that 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). In any case, these features, whilst not determining better transfection capacity in cell culture, allowed better nucleic acid delivery in vivo. Thus, in vitro and cell culture results have to be treated with caution and cannot necessarily be used to extrapolate the potential of a nucleic acid carrier in vivo. Other factors such as particle diameter become more important when these vesicles are introduced in vivo.

3. Size considerations for lipoplex-mediated nucleic acid transfer to tumours

The vascular bed is well-developed in many types of tumours, occasionally better than the surrounding normal tissues (Jain, 2001). For such tumours, liposomal delivery of genetic material especially with large liposomes (diameter > 1 μm) may prove to be best. For such vesicles, which

Table 2
Potential routes for administration of lipoplexes to tumours

Intravenous injection	
Mouse	Stewart et al., 1992; San et al., 1993; Zhu et al., 1993; Saijo et al., 1994; Lesoon-Wood et al., 1995; Liu et al., 1995; Parker et al., 1995; Thierry et al., 1995; Clarke et al., 1996; Osaka et al., 1996; Stephan et al., 1996; Yokoyama et al., 1996; Hofland et al., 1997; Hong et al., 1997; Liu et al., 1997; McLean et al., 1997; Xu et al., 1997; Bei et al., 1998; Curtis et al., 1998; Griesenbach et al., 1998; Mahato et al., 1998; Smith et al., 1998; Song and Liu, 1998; Song et al., 1998; Barron et al., 1999a,b; Bragonzi et al., 1999; Dow et al., 1999a,b; Liu et al., 1999; McClarrinon et al., 1999; Ochiya et al., 1999; Xu et al., 1999; Anwer et al., 2000a; Bragonzi et al., 2000; Delepine et al., 2000; Floch et al., 2000a; Ishiwata et al., 2000; Meyer et al., 2000; Mizuta et al., 2000; Ren et al., 2000; Tu et al., 2000; Boussif et al., 2001; Hwang et al., 2001; Loisel et al., 2001; Mohr et al., 2001; Sakurai et al., 2001; Uyechi et al., 2001
Rat	Leibiger et al., 1991; Tsan et al., 1995; Lee et al., 1998; Jeschke et al., 1999; Pampinella et al., 2000
Duck	Tagawa et al., 1996
Rabbit	Nabel et al., 1992a; Conary et al., 1994; Canonico et al., 1994
Pig	Nabel et al., 1992a; San et al., 1993
Monkey	Parker et al., 1995
Intraarterial injection	
Rat	Dass et al., 1997a; Dass, 1998; Lee et al., 1998; Schmid et al., 1998a; Rainov et al., 1999; Dass et al., 2000; Marley et al., 2000; Pampinella et al., 2000; Madry et al., 2001; Minchin et al., 2001
Rabbit	Leclerc et al., 1992; Nabel et al., 1992a; Losordo et al., 1994; Takeshita et al., 1994; Wright et al., 1998
Dog	Lim et al., 1991; Chapman et al., 1992
Pig	Nabel et al., 1990, 1992b, 1993a; Muller et al., 1994; Armeanu et al., 2000
Human	Nabel et al., 1994
Direct intratumoural injection	
Mouse	Stewart et al., 1992; Son and Huang, 1994; Yagi et al., 1994; Seung et al., 1995; Egilmez et al., 1996; Son and Huang, 1996; Szala et al., 1996; Zerrouqi et al., 1996; Nomura et al., 1997; Takakuwa et al., 1997; Xu et al., 1997; Bucur et al., 1998; He et al., 1998; Mizuno and Yoshida, 1998; Dunphy et al., 1999; Hottiger et al., 1999; Natsume et al., 1999; Endo et al., 2000; Lee et al., 2000; Yerulshami et al., 2000
Rat	Zhu et al., 1996; Nomura et al., 1997; Yanase et al., 1998
Human	Nabel et al., 1993b, 1996; Pauli et al., 1998; Morse, 2000

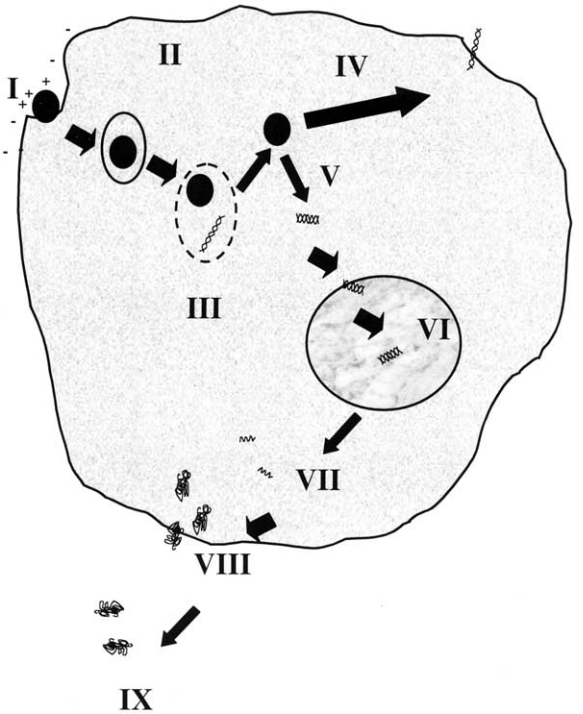

Fig. 2. Intracellular transport of pDNA by cationic liposomes. The steps involved include: (I) binding of lipoplex to anionic surface of cell; (II) transport of lipoplex from plasma membrane into interior of cell in endosome; (III) maturation of endosome into lysosome, which releases lipoplex (release of naked DNA also possible) into cytosol; (IV) efflux of naked DNA to cell exterior; (V) movement of naked DNA towards nucleus; (VI) intranuclear transport of DNA; (VII) transcription of DNA into mRNA which moves out into cytosol; (VIII) translation of mRNA into protein which performs its function within cell or is ferried to the exterior (secretion); (IX) externalised protein is transported to other sites in the circulation (in vivo) or remains in medium (cell culture).

should be retained in the first capillary bed encountered due to their size, such administration must be into an artery upstream of the target site (Dass et al., 1997b, 2000). Tail vein injections result in rapid retention of the majority of liposomal material in the lung vasculature, the first capillary bed encountered via this route, which may prove to be useful for therapy of lung metastases. Small vesicles (diameter ≤ 200 nm) escape the capillary bed and are predominantly taken up by the organs of the reticuloendothelial system (RES), the liver and spleen (Liu et al., 1992; Litzinger et al., 1994). Lipoplexes with diameters ranging from 200 to 400 nm are taken up rapidly by the liver and spleen when administered via the tail vein in mice (Osaka et al., 1996). Thus, proper studies with lipoplexes are needed to determine the true pharmacokinetics of complexes differing in size. Intraarterial delivery of cationic large vesicles (diameter > 1 μm) rather than intermediate vesicles (between 200 nm and 1 μm in diameter) may be more feasible for delivery of nucleic acid molecules to tumours due to the above-mentioned reasons. It has been shown that lipoplexes ranging in diameter from 400 nm to 1.4 μm were most effective for transfection in cell culture (Kawaura et al., 1998). However, in vivo, vesicles greater than 400 nm in diameter may not be able to extravasate through the permeable tumour vasculature pores, and thus pass straight through to be taken up by the RES (Juliano and Stamp, 1975; Kong et al., 2000).

A certain degree of targeting may be achieved by delivering therapeutic genetic constructs as close as possible to the diseased site via a catheter (Dass et al., 1997b; Santiago et al., 1999; Dass et al., 2000). This would ensure a maximum dose of therapeutic nucleic acid molecules to the target site since dilution in the blood, enzymatic degradation, and interaction of the nucleic acid with the biological surroundings such as the vessel wall would be minimised. It has to be kept in mind that certain tumours have a pore cutoff size ranging from 200 nm to 1.2 μm in diameter (Hobbs et al., 1998), which should allow ease of extravasation of particles as large as lipoplexes and most nanoparticles. However, vessel pore size in other tumour types may be restricted to less than 100 nm (Kong et al., 2000) signaling the potential for employing smaller and novel vehicles such as cyclodextrins (reviewed in Dass and Jessup, 2000) or porphyrins (Dass, 2002).

In general, modification of liposome size enables a liposome technologist to alter the pharmacokinetics of a given drug. Liposome diameter influences plasma half-life (Litzinger et al., 1994; Harashima et al., 1995) and site of accumulation (Nagayasu et al., 1996) in vivo. Size may also dictate the quantity of drug delivered, its metabolism and release characteristics in the

study organism (Ferdous et al., 1996). Intricately involved with release characteristics, size of liposomes may also affect the toxicity of the administered drug (Zou et al., 1995). These factors, also relevant to delivery of nucleic acids by liposomes, make particle diameter an important factor in determining the anti-tumour efficacy of a therapeutic agent. However, the formation of heterogeneous complexes commonly noted during and after lipoplex formulation, means that studies should be done with these complexes to monitor their biodistribution under specified conditions. However, the effect of both the route of administration and the site of the target tissue on lipoplex biodistribution comes into play as a factor above that of what the physicochemical characteristics of the vehicle per se are.

4. Biological characteristics of a tumour important for lipoplex delivery

As stated above, the tumour vasculature is more permeable compared to normal tissue vasculature. In addition, neovascularisation of tumours usually leads to newly formed vessels that are leaky due to fragile basement membranes (Liotta et al., 1976). Tumour cells in culture and in vivo secrete vascular endothelial growth factor, VEGF, which apart from increasing the permeability of tumour vessels, aids in the accumulation of excess fluid commonly associated with tumours (Brock et al., 1991). Increased permeability would facilitate movement of lipoplexes from vessels into tumour interstitium. If lipoplexes are targeted to the tumour vascular bed using such devices as microspheres (Dass et al., 1996; Dass and Burton, 1999b) or microplexes (Dass et al., 1999, 2000), then possible side-effects of therapeutic nucleic acids in other tissues should be mitigated. Highly selective delivery is important particularly when dealing with the vascular system, since generation of new tissue, such as that involved in the menstrual cycle, is dependent on vessel regeneration (reviewed in Ferrara, 1999).

Theoretically at least, once a tumour's blood supply is curbed or completely inhibited, the tumour itself should be eradicated. Interestingly, Thurston et al. (1998) has shown that angiogenic VECs in murine tumours internalised lipoplexes at a greater rate than anionic, neutral or sterically-stabilised liposomes. Such selectivity in up-

Table 3
Published clinical trials using lipoplexes

Phase	Gene	Route	Cationic lipid	Reference
I	Adenovirus 5 E1A	Intrapleural	DC-Chol	Xing et al., 1998
I	Ad5 E1A	Intraperitoneal	DC-Chol	Xing et al., 1998
I	Ad5 E1A	Intrathoracic	DC-Chol	Hortobagyi et al., 2001
I	Ad5 E1A	Intraperitoneal	DC-Chol	Hortobagyi et al., 2001
I	CFTR	Intranasal	GL-67	Ruiz et al., 2001
I	HLA-B7	Intratumoural	DC-Chol	Nabel et al., 1993b
I	HLA-B7	Intraarterial	DC-Chol	Nabel et al., 1994
I	CFTR	Intranasal	DMRIE	Sorscher et al., 1994
I/II	CFTR	Intranasal	DC-Chol	Caplen et al., 1995
I/II	CFTR	Intranasal	DC-Chol	Gill et al., 1997
I/II	CFTR	Intranasal	EDMPC	Noone et al., 2000
I/II	CFTR	Intranasal	DC-Chol	Hyde et al., 2000
I/II	HLA-A2	Intratumoural	DC-Chol	Hui et al., 1997
I/II	HLA-B13	Intratumoural	DC-Chol	Hui et al., 1997
I/II	H-2K	Intratumoural	DC-Chol	Hui et al., 1997
II	HLA-B7	Intratumoural	DMRIE	Nabel et al., 1996
II	α1-Antitrypsin	Intranasal	DOTMA	Brigham et al., 2000
II	HLA-B7 + β2-microglobulin	Intratumoural	DMRIE	Stopeck et al., 2001
II	CFTR	Intranasal	DOTAP	Porteous et al., 1997

take should render it possible to target tumour VECs with genetic medicine using lipoplexes. Similar findings have been documented elsewhere when lipoplexes were administered via the tail vein of mice (Liu et al., 1997; Chen et al., 1999; Anwer et al., 2000a) and intraarterially (Rainov et al., 1999). It has been noted that cationic lipid-encapsulated albumin undergoes increased extravasation across the blood-brain barrier (Fenart et al., 1999). This may be quite important for systemic gene delivery to the brain and its resident tumours.

It has also been noted that uptake of antisense strands is much faster in leukaemic human cell lines than in normal cells from the same patient (Calabretta et al., 1991; Zhao et al., 1996). Normal brain cells of rats do not permit entry of plasmids as much as brain tumour (glioma) cells (Nishi et al., 1996). A transplanted tumour line in the kidneys of rats shows greater expression of a foreign gene delivered free than the normal kidney parenchyma (Dass et al., 1997b; Dass, 1998; Dass et al., 2000). Such a mechanism may exist because of the greater division rate of mutated cells. Dividing cells undergo breakages in their cellular membranes, thereby allowing genes to get into the nucleus. Cells treated with aphidocolin, a cell cycle-arresting agent, exhibited 20-fold lower reporter gene activity delivered by lipoplexes than non-arrested cells (Mortimer et al., 1999). In addition, when growth arrest was lifted, transgene expression was found to coincide with the transition of cells from the G2/M phase into the G1 phase of the cell cycle.

Alternatively, greater expression of the delivered transgene in tumour cells may be explained by a more demanding blood supply to the tumour. Accumulation of the macromolecule dextran, which is unable to extravasate out of the microvasculature, in murine subcutaneous tumours increases in a linear fashion with increasing tumour volume (Anwer et al., 2000b), largely due to an increased blood supply in growing tumours. In addition, this study showed that larger tumours had a greater expression of a reporter gene than smaller tumours. Regardless of the mechanism, these findings have great implications for vascular-based gene delivery to solid tumours.

On the downside, cancer cells often occupy less than 50% of a tumour mass. One to 10% of the volume is made up by the vasculature (Jain, 2001), and the rest of the tumour volume consists predominantly of a collagen-rich matrix, the interstitium. To reach a tumour cell, the active agent must traverse the endothelial barrier and through the often more viscous interstitial matrix. Additionally, in tumours, pressure in the interstitium is higher than that within blood vessels (Boucher et al., 1996). Hence, movement of large molecular and multimolecular entities, such as nucleic acids and liposomes, respectively, through vessels occurs mainly by diffusion (Jain, 2001). However, in regions of the tumour where interstitial pressure is low, movement of large molecules occurs via convective transport caused by 'solvent drag'.

Ample consideration should be given to the fact that tumours seen clinically contain well-supplied rapidly growing regions interspersed with poorly perfused, often necrotic areas (reviewed in Murray and Carmichael, 1995). In solid tumour tissue, blood vessels become tortuous, with variable intercapillary distances and compression and occlusion of lumens. Insufficient perfusion results eventually in necrosis in certain pockets within the tumour and also hypoxic areas containing otherwise viable tumour cells. This heterogeneity poses a problem to drug delivery be it from a distance or via direct intratumoural injection. Ideally, the drug has to reach the periphery of the tumour, a region that is characterised by vigorous cellular turnover, but even this is not a consistent feature of all tumours (Jain, 2001).

In addition, studies characterising the permeability of tumour vasculature normally rely on small (1–2 mm) tumours, cells of which are closer to an afferent vessel than those cells in larger tumours with hypoxic pockets (Kong et al., 2000). Thus, there are various limiting factors in the delivery of nucleic acid agents to solid tumours. While the efficacy of the nucleic acid construct is somewhat dependent on matters such as the expression level in cells or resistance against endonucleases, it is largely reliant on the route of delivery to the tumour. At present, the vascular route is the best mode of administration since it provides the therapeutic construct direct access to the rapidly growing regions of the tumour.

Further enhancement in delivery of genetic medicine to tumours 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 stabilised antisense lipid particles, SALPs (Leonetti et al., 2001; Mui et al., 2001). Of all these vesicles, SALPs have shown the greatest promise due to the following reasons. SALPs are essentially formulated from an ionisable aminolipid and polyethylene glycol (PEG) for stabilisation during the formulation process and more importantly, during travel through the vasculature. These 80–140 nm multilamellar vesicles exhibit significantly prolonged plasma circulation time when compared to free nucleic acids and lipoplexes (Semple et al., 2000). They also mask the cationic lipid as it travels through the vasculature and the cationic lipid is unmasked when the target site is reached by successive loss of the PEG entities. tumour uptake studies reveal that enhanced uptake in tumour cells is possible with these novel carriers, due to the larger plasma area-under-the-(concentration/time) curve and increased resistance to degradation (Leonetti et al., 2001). In any case, stabilised formulations of lipoplexes retain their transfection capability longer in vivo than their non-stabilised counterparts (Hong et al., 1997). However, regardless of the choice of CL, the port of delivery will have a huge bearing on how efficacious the nucleic acid is at the diseased site.

5. Route of administration as an important determinant for selective delivery of lipoplexes to primary tumours and metastases

After an intratumoural injection, Nomura et al. (1997) found out that approximately 50% of naked plasmid DNA (pDNA) is eliminated from the tumour within 2 h, while more than 90% of pDNA is retained in the tumour when delivered as lipoplexes. In both cases however, a great variation in gene transfer was observed between tumours, and cancer cells in the needle-track zone were transfected the most. Szala et al. (1996) demonstrated that when the *Escherichia coli* cytosine deaminase gene under the control of a tumour-specific promoter was administered intratumourally, and 5-fluorocytosine was given intraperitoneally, tumour growth was inhibited dramatically. This effect was attributed to liposome toxicity, increased transfection of surviving tumour cells, and a bystander effect whereby the easily diffusible 5-fluorouracil (biologically active derivative of 5-fluorocytosine) caused destruction of nearby cells. Intratumoural injection of lipoplexes into a human basal cell carcinoma model resulted in transgene expression detected predominantly in the epidermis, and to a lesser extent, in the dermis of mice (Hottiger et al., 1999). In contrast to both tail- and intraportal-vein injection of lipoplexes, administration by direct intratumoural injection in hepatic tumours results in high levels of expression and an accompanying low level of expression in the surrounding liver parenchymal cells (Mohr et al., 2001). One of the drawbacks of intratumoural administration, regardless of the carrier, is that the delivered nucleic acids are present predominantly in the needle-track region as mentioned above and noted elsewhere (Lee et al., 2000).

Lipoplexes are confined to the capillary network and homogenously distributed throughout the lung lobe when administered via the tail vein (Uyechi et al., 2001). In contrast, intratracheal administration results in regional distribution, concentrating around bronchioles and distal airways. Not all the bronchioles were stained with lipoplexes, suggesting that the airway-administered solution became channeled through certain bronchiolar pathways. Endothelial cells (ECs) were stained after intravenous administration, and epithelial cells were stained after intratumoural administration. This study went to confirm that ECs are quite amenable to gene delivery via lipoplexes injected systemically. Lung, heart, spleen, muscle and liver were the major sites of transgene expression when lipoplexes were administered intravenously in mice, while some activity in kidney and nil reporter gene expression was noted in blood, bone marrow or brain (Hofland et al., 1997). Maximal expression was observed 24 h after administration and activity decreased 10-fold by day 5 for all tissues. In the lung, both intersti-

tial cells and ECs were transfected, while other tissues displayed a predominant expression in ECs. Other studies have shown that the lungs are a major site of lipoplex deposition when complexes are administered via the tail vein (Hong et al., 1997; Mohr et al., 2001).

It has been noted that empty CLs may be needed to enable the lipoplexes from gaining better entry into cells and hence rendering more transgene expression when administered intravenously (Smith et al., 1998). However, intratumoural administration did not result in an added advantage of using empty liposomes to complement subsequent delivery of lipoplexes. In a separate study (Griesenbach et al., 1998), intratracheal administration of lipoplexes resulted in a predominant DNA deposition in epithelial cells lining the bronchioles whereas intravenous delivery led to deposition in the alveolar region of the lung, including type II alveolar epithelial cells. Transgene expression in lungs may prevail for more than 50 days after injection of lipoplexes, but expression is predominantly located to the ECs in the lungs (McClarrinon et al., 1999).

Delivery to the different cell populations in the lungs may be selective (Bragonzi et al., 2000) depending on the route of administration (intratracheal versus intravenous) and the type of cationic transfection reagent used (lipoplexes versus PEI). Such delivery has been attributed to the interaction of these carriers with the plasma membrane of ECs and an overcoming of the surfactant barrier present on lung surfaces. Apart from the surfactant barrier, airway inflammation frequently found in congenital and acquired lung disorders may interfere with nucleic acid delivery by direct administration through instillation or aerosolised lipoplexes (Bragonzi et al., 1999). Lung accumulation may be avoided using lipoplexes with a net negative charge on their surface, due to a higher pDNA–cationic lipid charge ratio (Ishiwata et al., 2000). This may have particular bearing on whether the primary tumours and metastatic growths are in the lungs or livers of diseased animals.

As implied above, the route of lipoplex administration is an important determinant of the quantity of nucleic acids reaching the target tissue and hence the desired pharmacodynamic effect. For instance, Curtis et al. (1998) demonstrated that compared to intravenous, intramuscular, oral, subcutaneous and intraperitoneal routes of administration, the intratumoural route was best for targeting lipoplexes to the mouse gallbladder columnar epithelium, where biliary fibrosis due to CF occurs. For delivery to regenerating skeletal muscle tissue, intraarterial administration is significantly better than both the intravenous and intracardiac routes (Pampinella et al., 2000). In mice given intravenous injection of lipoplexes (Osaka et al., 1996), at 2 min, the distribution of lipoplexes were as follows: lung > liver > spleen (red pulp) > kidney (cortex). At 24 h, the ranking shifted to liver, spleen (red pulp) > kidney (cortex) > lung, blood. Significantly greater expression of the transgene was noted with complexes than with free pDNA at both early time-points and at 24 h post-injection. The 24-h samples revealed that there was an accumulation of complexes in Kupffer cells, RES cells in the marginal zone of the spleen, and diffusely along alveolar septae with scattered accumulations in alveolar macrophages. Tail vein injection localises lipoplexes to the lung and spleen of mice whereas portal vein injection results in significant expression in liver (Mohr et al., 2001). In addition, portal vein injection allows a low but detectable level of transgene expression in intrahepatic tumours in contrast to negligible delivery via tail vein injection.

For delivery to tumours, the intraarterial route may present as the best route since it allows an upstream administration of lipoplexes in relation to the lesion. Selective delivery of free pDNA (Dass, 1998; Dass et al., 1997b, 2000), lipoplexes (Dass et al., 2000), microspheres (Dass et al., 1997b) and microplexes (microsphere-lipoplex vehicles; Dass et al., 2000) was achievable to intrarenal tumours when complexes were infused via the aorta and channeled to proceed into the renal arteries by a temporary clamping of the aorta just below the renal artery branch sites. Intracarotid delivery of lipoplexes into rats permitted the transfection of 30% of tumour cells in a brain tumour model (Rainov et al., 1999). Transient vascular 'permeabilisation' of the brain tu-

mour barrier with bradykinin increased this efficiency to 50%. An added advantage of intraarterial delivery is that the 'target' tissue is homogenously transfected.

Current clinical evaluations of lipoplexes as pDNA carriers are focussing on delivery to phagocytes, tumour cells, endothelium and some parenchyma cell types (Morse, 2000). This does not come as a surprise as these sites are usually passively targeted when lipoplexes are administered systemically. Nevertheless, it is heartening that tumour cells are included on this list, but as this review describes, more targeting of therapeutic nucleic acids to neoplastic tissues requires additional research into developing better carriers. The effects of cellular characteristics and tissue architecture on lipoplex uptake need to be taken into account as certain cells may carry out endocytosis at one pole and not the other (basal versus apical membranes). Finally, it is envisaged that when the negative toxic aspects (below) of both CLs and the resultant lipoplexes are culled, a better class of carriers will definitely be developed.

6. General toxicity due to CLs and lipoplexes

Toxicity encountered when using lipoplexes is normally 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 CL formulations available. Due to toxicity, in vivo delivery of lipoplexes has to be as close to the target site as possible to minimise side effects. The issue of non-specific efficacious effects, some of which are due to toxicity, of lipoplexes as well as other cationic polymeric carriers (Bielinska et al., 1996; Lambert et al., 1998; Xu et al., 1998) is to be taken into consideration in studies using these carriers.

Several disadvantages of using CLs in general and lipoplexes specifically have surfaced over the past 25 years. In cell culture, lipoplexes cause several changes to cells, which include cell shrinking, reduced number of mitoses, and vacuolisation 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 induction of haemolysis (Senior et al., 1991), induction of 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 synthesis (Filion and Phillips, 1997b). Recent attempts have been made to formulate CLs from degradable amphiphilic chemicals that can be metabolised following uptake into cells (Choi et al., 2001; Pleyer et al., 2001). These contrast the non-biodegradable nature of lipids used in earlier formulations such as DOTMA in Lipofectin™. A clear distinction between CL- and lipoplex-mediated toxicity both in cell culture and in vivo needs to be drawn, as these are more than occasionally confused.

One of the earliest in vivo studies, that of Adams and Hamilton (1977), demonstrated that when CLs 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 while a significant quantity of pDNA enters nuclei after release from lipoplexes, these cells undergo cell death and hence express low amounts of the transgene (Uyechi et al., 2001). CLs have also been noted to induce acute systemic inflammatory reactions (Malone, 1996) and macrophage and neutrophil infiltration into the lungs of mice when administered intratracheally (Freimark et al., 1998).

Litzinger et al. (1996) reported that following intravenous injection of lipoplexes into mice, emboli formed 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 and Phillips, 1997a). In carrageena and in sheep red blood cell challenge inflammatory models, these liposomes were found to have a strong anti-inflammatory activity (Filion and Phillips, 1997a). 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 and 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). Hepatoxicity was noted when localised delivery of lipoplexes via the portal vein was performed in mice (Mohr et al., 2001). Lack of expression due to toxicity is probably due to the very localised 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 (Dass, 1998; Dass et al., 2000). It may also be due to the dose and charge ratio of lipoplexes delivered. Large aggregates of lipoplexes may also cause problems due to 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 due to a rapid removal by the RES in vivo. Thus, the toxic effects of the complexes may prove counterproductive in vivo whilst enhancing the uptake of nucleic acids in cells in culture. Current research is focussed on finding alternative chemistries for lipids that would not be as toxic but still maintain or even increase transfection levels in cultured cells and especially in vivo.

7. Dependency of transfection and toxicity on chemistry of transfection lipids

While the transfection capability of lipoplexes have been documented extensively in most papers, a concomitant examination of the toxic effects of such drug delivery is lacking, especially in vivo applications. It is thus a relatively tricky task in trying to weigh one CL against another for delivery of a particular type of nucleic acid into a particular region in a certain species of test animal to achieve levels of efficacious effect that are beyond what is capable with administration of naked nucleic acid strands. Transfection in cell culture is in some ways more prone to variability due to differences in cell culture reagents (example serum batch), cell line, cell passage number, amongst other things (for more comprehensive list, refer to Table 1). Toxic effects experienced in a particular set of conditions do not necessarily mean that similar effects would be noted if the study were to be done again under the same conditions. The major difference between conventional drugs and genotherapeutic constructs is that the latter need to latch on to the cell's inherent genetic machinery and either express a therapeutic transgene or downregulate a specific deleterious gene.

Cationic lipids used for CL formulation are composed of three basic domains (Fig. 3): a positive charged head group, a hydrophobic region, and a linker that tethers the polar and non-polar regions. The polar and hydrophobic domains of cationic lipids may have dramatic effects on both transfection and toxicity levels. Two major types of hydrophobic moities are utilised, namely aliphatic chains and cholesterol-based derivatives. Linker bonds may be of these major types: ester, ether, carbamate and amide. The headgroup often consists of primary, secondary, or tertiary amines, but quaternary ammonium salts, guanidino, and imidazole groups have also been trialed (Heyes et al., 2002).

It has been shown that for cholesterol-containing cationic lipids, those with the cholesterol anchor linked in a 'T-shape' configuration are most efficient when instilled into murine lungs (Lee et al., 1996). Thus, the cationic lipid 'GL67' is best

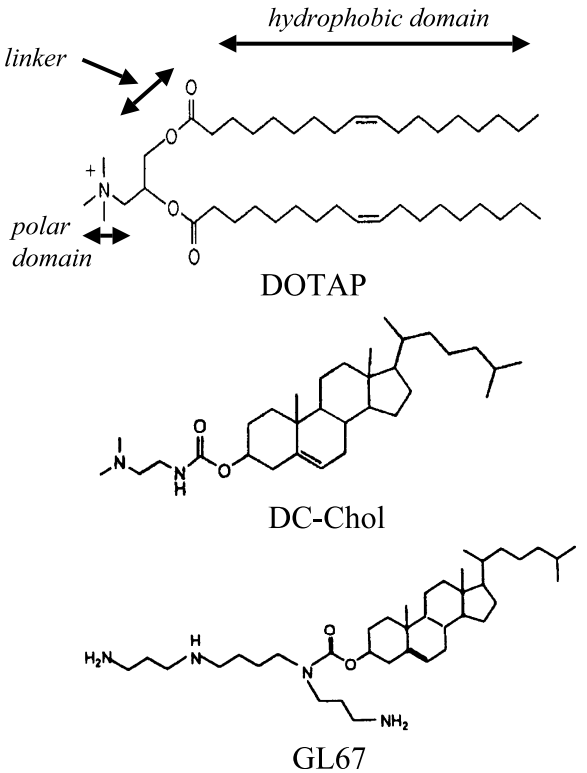

Fig. 3. Cationic lipids commonly used for nucleic acid delivery. Each cationic lipid is made of three domains: polar headgroup, an intervening linker region, and a hydrophobic zone.

amongst its cholesterol-based 'peer' molecules, though toxicity was not assessed in this study. Bennett et al. (1997) showed that the more effective cationic lipids for delivery of plasmids to the lungs of mice were those with the greatest difference between the cross-sectional areas of the polar and hydophobic domains. In addition, headgroup structures that promote close polar domain association in combination with fatty acyl *cis*-unsaturation were most active in the lung. Additional polarity in the headgroup region, such as the use of hydroxylethyl group, has been noted to aid in in vivo pulmonary transfection (Balasubramaniam et al., 1996). Floch et al. (2000a) demonstrated that substitution of the nitrogen atom in the polar headgroup with either phosphorus or arsenic results in a substantial improvement in transfection efficiency in cultured cells and in murine lungs when administered intravenously.

The effect of hydrophobic chain length on transfection is controversial (Heyes et al., 2002) and its effect on toxicity has not been adequately addressed to date (van der Woude et al., 1997). In any case, the influence of hydrophobic chain length on both these parameters may well depend on the physicochemical features of the other two regions (Floch et al., 2000a). Assymetry in the hydrophobic regions in regards to the aliphatic chains has been noted to increase transfection in cell culture (Balasubramaniam et al., 1996) as does chain unsaturation (van der Woude et al., 1997). Based on lung transfection in mice, Ren et al. (2000) have come up with the following hypotheses for improving lipofection: polar and hydrophobic domains being in a 1,2-relationship on the backbone, ether linker bond, and paired oleoyl chains as the hydrophobic region of the cationic lipid.

The linker region may also have an affect on both transfection capability and toxicity. For instance, the efficiency of DC-Chol for transfection of cells in culture, with its carbamate linker, was less and toxicity more than that of its close cousin based on a dithiodiglycolic acid [(cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine; CHDTAEA)] linkage (Tang and Hughes, 1999a). Cationic lipids with ester bonds such as DOTAP in the linker zone are more biodegradable and associated with less cytotoxicity in cultured cells (Leventis and Silvius, 1990; Farhood et al., 1992; Aberle et al., 1998; Choi et al., 2001), but ether bonds render cationic lipids better transfecting agents (Ghosh et al., 2000). In cell culture, Tang and Hughes (1999b) have demonstrated that single-tailed cationic lipids with ester linkages can function as nucleic acid vehicles with low cytotoxicity and transfection capacities comparable to the commercial reagents. Aberle et al. (1998) proposed that cytotoxicity due to CLs may occur at a stage before the lipoplexes are encapsulated into endosomes. An increase in the length of the linker segment leads to decreased cytotoxicity in cell culture (Floch et al., 2000a).

Thus, as CLs differ significantly from each other, and minor differences may alter their transfection capability and toxicity, much more re-

search needs to be done to further address these issues. Transfection remains a fairly ill-understood process, and the development of new CL reagents has outpaced research aimed at examining the individual steps involved in the transfection mechanism(s) at play. Extrapolation may not be valid from in vitro testing to cell culture transfection (Mui et al., 2000; Ferrari et al., 2001), and certainly is not recommended between cell culture performance and in vivo nucleic acid delivery (Floch et al., 2000b; Ren et al., 2000). Whilst the task remains a large one, much as it was at the onset of this technology in the late 1980s, basic research has to be done to address such concerns, especially before these CLs become routinely used in patients.

8. Enhanced immunostimulatory activity of nucleic acids complexed as lipoplexes

Apart from general cytotoxicity, specific cases of 'immunotoxicity' have emerged over the past few years. Several studies have demonstrated that administering lipoplexes into the lung lumen provokes the production of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-12 that are accompanied by a pulmonary influx of cells such as polymorphonuclear cells (Schuele et al., 1997; Freimark et al., 1998). However, this induction of cytokine generation is not limited to pulmonary administration, since both the intraperitoneal and intravenous routes have shown similar profiles. Although synthetic vectors have been regarded as 'safe' and 'non-toxic' in comparison to adenoviral vectors which incite strong adaptive immune reactions (Dass and Burton, 1999a), the pDNA component of these vectors and the stimulatory cytosine–phosphate-guanine (CpG) motifs contained within are capable of stimulating dose-dependent innate immune reactions that can, in extreme cases, be lethal. This has been demonstrated by treating mice with a low dose of DNA containing CpG sequences followed by a challenge with a sub-lethal dose of lipopolysaccharide (Ozmen et al., 1994; Sparwasser et al., 1997) in which death occurs due to a sepsis-like syndrome.

Intravenous administration of lipoplexes in mice may cause an immune response that prevents a subsequent administration of lipoplexes into mice after a relatively short interval (Meyer et al., 2000). Repeated systemic gene expression could be achieved upon readministration with a minimal time interval of 14 days between two injections. When administered 6 days apart, subsequent gene expression was inhibited by an initial lipoplex administration containing the pDNA but uninhibited when free pDNA was first injected.

It is interesting that intramuscular administration of vectors fails to incite a robust toxic response, which is not the case with both the intrapulmonary or intravenous routes. As mentioned above, several in vivo studies suggest that intrapulmonary delivery of pDNA vectors is responsible for the generation of proinflammatory Th1-like cytokines and a subsequent influx of infiltrating cells into the lung region (Schuele et al., 1997; Freimark et al., 1998; Yew et al., 1999). In any case, the cationic lipids per se do not elicit an immune response and the effect is not due to the presence of endotoxins in the plasmid preparation (Freimark et al., 1998). A few of these effects are mediated by the nucleic acid component while the involvement of the cationic lipid component has not been ruled out. Alton et al. (1998) proposed that acute, mild flu-like symptoms encountered in a CF clinical trial were due to DNA-dependent effects not observed in the control (lipid alone) group.

Schuele (2000) lists several ways by which the immunostimulatory effects of vector CpG motifs may be reduced. These include methylation of cytosine bases in these motifs, addition of neutralising sequences, elimination of CpG motifs, immunosuppression using chemical or biological approaches, targeting of vectors away from cells of the RES, and inhibition of endosomal acidification. It has been shown that systematic elimination of approximately 50% of the CpG motifs in a plasmid vector resulted in reduction in cytokine secretion by mouse spleen cells in vitro and in mouse lung following intravenous injection or intranasal instillation (Yew et al., 2000). This protocol also increased the level of transgene expression. Passive targeting away from the RES

using such routes as intramuscular injection can also increase the level of transgene expression. Use of immunosuppressants such as dexamethasone results in elevated gene expression following systemic delivery, although the expression is transient (Tan et al., 1999). Agents that inhibit endosomal acidification such as chloroquine and quinacrine, that are already used clinically against malaria, may also have beneficial effects. A low concentration of quinacrine administered together with lipoplexes resulted in decreased cytokine levels in the lung following intranasal instillation, without a significant reduction in transgene expression (Yew et al., 2000).

9. Loss of gene expression due to lipoplex-mediated 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 at days 1 and 2 post-administration (Li et al., 1999; Tan et al., 1999). Several mechanisms may be responsible for this phenomenon: (i) generation of neutralising 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 great implications since repeat administrations are impossible and net transgene expression decreases over time. This phenomenon, in fact, has been the real stumbling block for gene therapy.

In vitro, it has been demonstrated that TNF-α and IFN-γ downregulate gene expression from such common promoters as cytomegalovirus (CMV), independent of the genetic composition of the gene transfer vector (Qin et al., 1997). To confirm this, administration of neutralising 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 IL-12 (Dow et al., 1999a; Li et al., 1999; Tan et al., 1999; Whitmore et al., 1999; Bramson et al., 2000). As mentioned above, cytokines proved to be important negative regulators of gene expression since administration of anti-cytokine antibodies resulted in enhanced levels of gene expression (Li et al., 1999). Importantly, cytokine production seems to be directly associated with the presence of stimulatory CpG motifs in vector DNA, since the methylation or elimination of these motifs reduced cytokine levels in the circulation (Li et al., 1999; Yew et al., 2000). Furthermore, the use of dexamethasone, a commonly used immunosuppressant, reduces cytokine generation in vivo and improves both the level and duration of gene expression (Tan et al., 1999).

However, while gene expression levels in the lung have been improved by neutralising or eliminating cytokine production, the overall rate of decrease in gene expression over time was unaffected, suggesting that additional mechanisms were at work (Li et al., 1999; Tan et al., 1999). The loss of transgene-expressing cells by apoptosis is one likely candidate for this loss in expression (Li et al., 1999). Intravenous administration-mediated toxicity may be due 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). Such an increase may be controlled by using less cytotoxic CLs (Floch et al., 2000b). This release of transaminases is attributed to the presence of unmethylated bases such as cytosine and guanine in the pDNA sequence.

10. Use of CpG immunostimulation in gene therapy

Plasmids replicated in *E. coli* cells typically contain CpG motifs at a frequency of 1:16 dinucleotides, which is similar to that found in bacterial DNA (Freimark et al., 1998). Two exploitations of CpG immunostimulation are vaccination and cancer immunotherapy. Numerous publications have shown that immunostimulatory

CpG motifs can be utilised in vaccination stategies that include administration of a plasmid vector encoding the antigen or the antigen per se. Inhibition of tumour growth has been achieved by administration of CpG-containing lipoplexes through various routes of delivery (Parker et al., 1996; Blezinger et al., 1999; Dow et al., 1999a,b; Lanuti et al., 2000; Whitmore et al., 1999; Bramson et al., 2000). These results have been derived from studies using transgenes expressing proinflammatory cytokines such as IL-12 or even vectors containing no foreign transgene (null vectors) (Parker et al., 1996; Whitmore et al., 1999; Lanuti et al., 2000). Growth inhibition of tumours appears to be caused by CpG motifs since methylation of these sequences negates the effect (Whitmore et al., 1999). While there is a therapeutic effect, the exact nature of the inhibition by the CpG-containing motifs on tumour growth is not that clear. Cytokines produced have multiple effects both on the tumour cells and on the tumour vasculature. A case in point is the ability of IL-12, generated in response to CpG-containing motifs, to elicit anti-angiogenic pathways (Duda et al., 2000). Other cytokines such as those elicited in response to IFN-γ (itself a CpG-elicited cytokine), namely IP10 and Mig, are also capable of having anti-angiogenic properties (Strieter et al., 1995).

It has been demonstrated that lipoplexes enhance the immunogenic effect of pDNA (Perrie et al., 2001; Reyes et al., 2001). Not only are the primary tumours eliminated, but also total protection is proffered from subsequent challenge with tumour cells administrated months after the first tumour cell challenge (Lanuti et al., 2000). This rejection is mediated by cytotoxic T-lymphocytes (CTLs) specific for the tumour cell antigen(s). Likewise, oligodeoxynucleotides containing phosphorothioated-CpG sequence motifs inhibit tumour growth via a non-specific mechanism in animal studies (Neckers et al., 1998; Smith and Wickstrom, 1998). Immunostimulation is enhanced with cationic lipid-containing stabilised antisense lipid particles (SALPs; Bramson et al., 2000; Mui et al., 2000). Efficacious value is independent of the Watson–Crick base-pairing down-regulation of the target gene, and this activity is enhanced in the presence of cationic lipids.

Thus, the finding that null vectors can generate inhibition of tumour growth by inciting a state of anti-tumour immunity has driven this emphasis of getting such constructs into cancer clinical trials for 'immunogenotherapy'. For instance, treating intraperitoneal tumours with multiple doses of CpG-containing null vector resulted in both the inhibition of tumour growth and also a protection against subsequent tumour cell challenge given months after the first treatment in mice (Lanuti et al., 2000). CTLs specific for the tumour cell in question were deemed responsible for tumour rejection in the secondary tumour challenge. The authors proposed a 'cross-priming' phenomenon whereby the immune system was 'educated' to react to the tumour-specific antigens by the stimulation of the innate immune responses by CpG motifs in the vector DNA.

Hence, in the next decade when novel CLs are formulated with enhanced transfection capability and negligible toxicity, various therapeutic modalities may be harnessed into the one nucleic acid construct. For instance, a pDNA expressing the wild-type *p53* tumour suppressor transgene may have sufficient motifs to have not only a genotherapeutic effect, but an accompanying immunotherapeutic effect which is elicited by complexing the pDNA with the safe but efficient CL. Indeed, much more remains to be done in developing and testing this technology, but the beneficial effects are anticipated to outweigh the quantity of time, effort and funds dedicated to this task.

11. Summary

CLDCs (lipoplexes) are to date one of the most efficient ways of delivering nucleic acids into cultured cells and are increasingly being used in vivo. However, in vivo 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. An interesting recent finding has been the enhancement of general immunogenic recognition by foreign nucleic acids, and that such immunos-

timulation is enhanced when nucleic acids are administered complexed to cationic lipids. While such immunostimulation may prove to be disadvantageous for nucleic acid delivery with lipoplexes, several studies have shown that, rather paradoxically, such immunorecognition may be exploited against cancer cells in vivo. Provided that the toxic effects attributed to cationic lipids can be alleviated; the next few years should prove to be quite decisive on whether this immunoenhancement aspect of lipoplexes is in fact of adequate worth against cancer.

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