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Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes

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Abstract

Cationic liposomes mediate efficient transfection of mammalian cells, but the manner in which cells internalize and process cationic liposome-DNA complexes has not been well characterized. We exposed several cell types, including human and murine erythroleukemia cells, African green monkey kidney cells (CV-1), isolated rat alveolar type II cells and alveolar macrophages to DNA-cationic liposome complexes containing *N*-(1-2,3-dioleyloxypropyl)-*N*,*N*,*N*-triethylammonium (DOTMA) and Dioleylphosphatidylethanolamine (DOPE). The morphology of liposome–cell interactions was assessed by electron microscopy. Liposome preparations were complexed to colloidal gold particles or to both plasmid DNA and gold particles. Cells treated with DOTMA liposome-DNA complexes demonstrated endocytosis of the liposome-DNA complexes in coated pits, which were seen in early endosomes, late endosomes, and lysosomes. In isolated alveolar type II cells, the gold-labelled DOTMA lipid apparently mixed with the contents of lamellar bodies. In most cells, gold particles were dispersed throughout the cytoplasmic matrix. In a small proportion of CV-1 and U937 cells, a membrane system resembling the endoplasmic reticulum developed within the nucleus. This novel structure was also present in nuclei after they were isolated from CV-1 cells and then mixed with DOTMA-containing liposomes. Membranes which form after exposure to DOTMA-containing liposomes were 10 nm in thickness as compared to the approx. 8 nm thickness of endogenous cellular membranes. Based on these morphologic observations, we propose that the main route of entry of cationic liposomes into cells is by endocytosis. In some instances, the endosomal compartment releases its cationic liposome-DNA contents into the cytoplasmic matrix. Occasionally, liposomes may enter the nucleus by fusion with the nuclear envelope, creating vesicular and reticular intranuclear membranes. It is not clear at present which, if any of these morphological observations correlates with transfection mediated by cationic liposomes.

Keywords: Cationic liposome; Gene transfer; Endocytosis; Transfection

1. Introduction

The pathway(s) by which exogenous DNA enters and ultimately is transported to the nucleus of transfected cells are not well understood. Virus infection serves as a model for the delivery of exogenous DNA into cells, with its subsequent transcription in the nucleus. A small number of viruses including HIV-1, appear to fuse with the plasma membrane, thus introducing their nucleic acid core directly into the cytoplasm [1]. Most other viruses and a wide variety of macromolecules appear to enter the cell by endocytosis, with later entry into the cytoplasmic matrix through early or late endosomal or lysosomal membranes [2–8]. The majority of such molecules are degraded in lysosomes without perturbing the homeostasis of the recipient cell. However, virus infection or transfection of cells requires that sufficient amounts of the internalized nucleic acids reach the nucleus in functional form.

With the advent of molecular cloning, an increasingly wide range of cloned genes linked to their own or heterologous promoter-enhancer elements are now available. Numerous techniques, including calcium phosphate precipitation, DEAE-dextran, electroporation, and lipofection all permit expression of transfected genes in a large number of cell types [9–17]. Each method mediates cytoplasmic delivery of a small portion of DNA and its subsequent incorporation into the nucleus. Among the variety of cellu-

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lar transfection techniques commonly used, we have observed that only lipofection (using cationic liposomes) successfully introduced a glucocorticoid receptor derivative, over-expressed and purified from *Escherichia coli*, into mammalian cells in functional form [12]. The liposome-mediated delivery of this glucocorticoid receptor

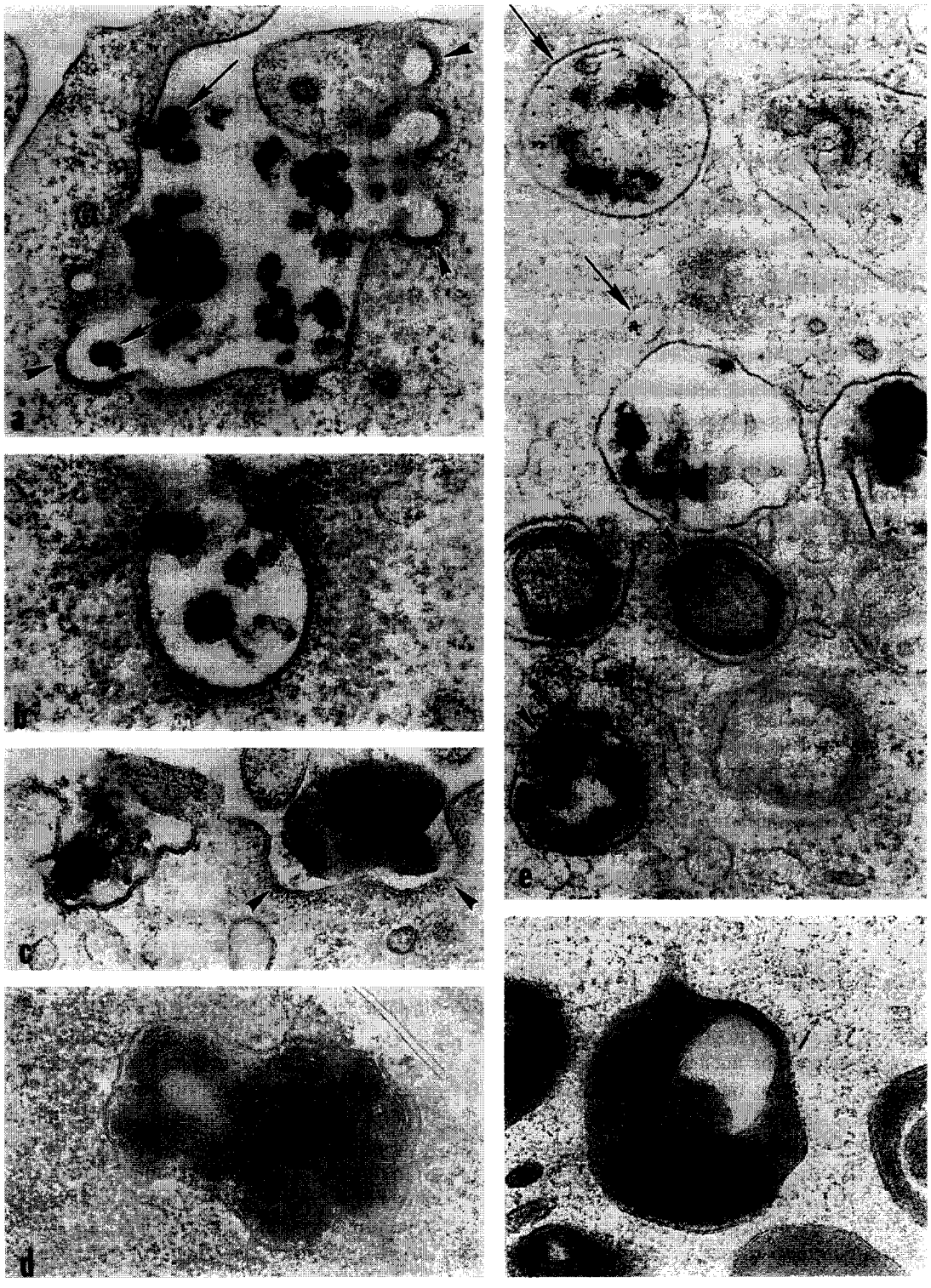

Fig. 1. Transmission electron micrographs of rat alveolar macrophages, following a 1 h interaction with DOTMA/DOPE MLV liposomes, complexed with plasmid alone (a and b) or streptavidin gold (c–e). The cationic lipid complexes are in coated pits and endosomes. Intact liposomes aggregate in early endosomes (d) but the liposomes are not distinguishable in late endosomes (arrows) and apparent lysosomes (arrowheads) (e). The gold label appears in a dense body (arrowhead) (f). This sequence of micrographs represents our interpretation of the general mode of cationic lipid entry into all cell types examined that were transfected by this carrier. Magnifications: (a) 37 000; (b) 65 000; (c) 54 000; (d) 56 000; (e) 48 000; (f) 40 000.

derivative significantly enhanced transcription of a glucocorticoid response element linked to a chloramphenicol acetyltransferase (CAT) reporter gene [12]. This suggests that cationic liposome-DNA complexes containing DOTMA, DOPE and DNA may interact with cultured cells in a unique manner. The present study reports on the interaction of cationic liposomes with a variety of cell lines grown in tissue culture, peripheral blood monocytes (PBM) freshly isolated from human blood, alveolar type II cells, and alveolar macrophages (AM) isolated from rat lung. We studied a variety of different cell types in order to try to establish more general principles of interaction between cationic liposome-DNA complexes and cultured cells. These cells were selected on the basis of their utility for further studies.

Our microscopic observations indicate that the general mode of entry of cationic liposome-DNA complexes into cells resembles that of receptor mediated endocytosis. Infiltration of some of the lipid material into the nucleus, creating novel vesicular and reticular intranuclear membranes, is observed in a small percentage of CV-1 and U937 cells examined. Further studies are still needed to understand the mechanism by which such events are related to transfection itself.

2. Materials and methods

2.1. Cells and cell culture

CV-1 (African green monkey kidney), U937 (human myelocytic leukemia), murine erythroleukemia (MEL) cells, and K562 cells (human erythroleukemia cells were obtained from the American Type Culture Collection (Rockville, MD). CV-1 and MEL cells were maintained in Dulbecco minimum essential medium (DME)-H-21 with 5% fetal bovine serum (FBS) at 37°C and 7% CO_2. Rat alveolar type II cells [18] and rat alveolar macrophages [19] were isolated and purified as previously described. Type II cells were maintained in DME-H-16 with 5% FBS at 37°C and 7% CO_2. Isolation of human peripheral blood monocytes was performed as described previously [20] and liposome-mediated transfections were performed as previously described [12]. Isolation of cell nuclei from CV-1 and U937 was performed as previously described [21].

2.2. Liposomes

Small unilamellar vesicles (SUV) composed of DOTMA/DOPE (1:1) or phosphatidylserine/DOPE (1:1) were prepared, as described in [12]. Multi-lamellar vesicles (MLV) were prepared as described in [22]. DOTMA liposome-streptavidin gold complexes were formed by mixing 600 ng of streptavidin colloidal gold containing particles of 10 nm diameter (E-Y Laboratories, San Mateo, CA) in phosphate buffered saline with 10 nmol of DOTMA/DOPE liposomes in 1 ml of the media appropriate for the cell type to which the complexes were added. After incubating 5 min, the 1 ml mixture was added to $2 \cdot 10^6$ cells growing in 5 ml of medium in 60 mm Falcon plastic dishes, and fixed for EM at 5 min, 30 min, 1 h or 24 h later. DOTMA liposome-plasmid DNA-streptavidin gold complexes were prepared by pre-mixing 600 ng of streptavidin gold with 5 mg of a Rous sarcoma virus (RSV)-chloramphenicol acetyltransferase (CAT) gene plasmid [19], and subsequently adding 10 nmol of DOTMA/DOPE-containing liposomes. CV-1 cells were then transfected with RSV-CAT-DOTMA liposomes, either alone or pre-mixed with streptavidin gold, and subsequently processed as described above. (Cells remained > 90% viable, as assessed by trypan blue exclusion, following exposure to DNA-liposome or DNA-liposome-gold complexes for up to 48 h.)

2.3. Liposome-DNA complexes

DOTMA/DOPE liposome-RSV-CAT complexes used to transfect CV-1 cells had a mean diameter of approx. 100 nm, as determined by laser light scattering, and bore a charge ratio (the ratio of lipid cationic charges to DNA anionic charges) of 1.33. The interaction of cells with DNA-liposome complexes, as assessed either by transfection of RSV-CAT plasmid or by EM, did not appear to differ whether the complexes were made with SUV or with MLV.

2.4. Fixation and processing for electron microscopy

DOTMA/DOPE liposomes and cells in tissue culture or cells freshly isolated from blood or lung were fixed in 1.5% glutaraldehyde in 0.1 mmolar sodium cacodylate buffer containing 1% sucrose, pH 7.4, at room temperature for 1 h. Following tannic acid and uranyl acetate enhancement, tissue was dehydrated in a graded series of alcohols and embedded in Epon 812 resin (Ernest F. Fullam, Latham, NY), sectioned on Sorval MT 2 microtome using

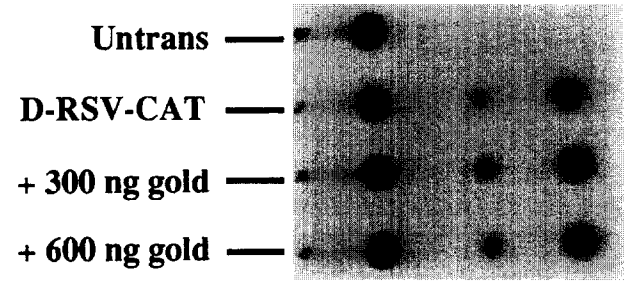

Fig. 2. CV-1 cells were plated and transfected as described previously [12]. Individual plates were either not transfected (Untrans) or transfected with 5 μg of RSV-CAT complexed with 10 nmol of DOTMA/DOPE SUV liposomes (D-RSV-CAT), or the same amounts of the RSV-CAT-DOTMA liposome complex mixed with 300 (+300 ng gold) or 600 (+600 ng gold) ng of streptavidin gold.

diamond knives, and examined with a JEOL 100CX transmission electron microscope operating at 80 kV. Measurement of membrane thickness was performed by magnifying the negatives 200 000 × and examining them with a ruled eye piece.

3. Results

We have used electron microscopy to study the interaction between DOTMA/DOPE liposomes (either as liposomes alone, or as plasmid DNA-liposome complexes, with or without streptavidin gold) and the various cell types (CV-1, U937, K562, MEL cells, alveolar macrophages, and alveolar type II cells). The most frequent observation obtained from this study was that the liposomes were adhering to the cell surface near or in coated pits as shown in rat alveolar macrophages (Fig. 1a–e). This interaction is common to well-defined examples of receptor-mediated endocytosis [2]. All cells which appear to have contacted cationic liposome-DNA complexes internalize the complexes after their binding to the

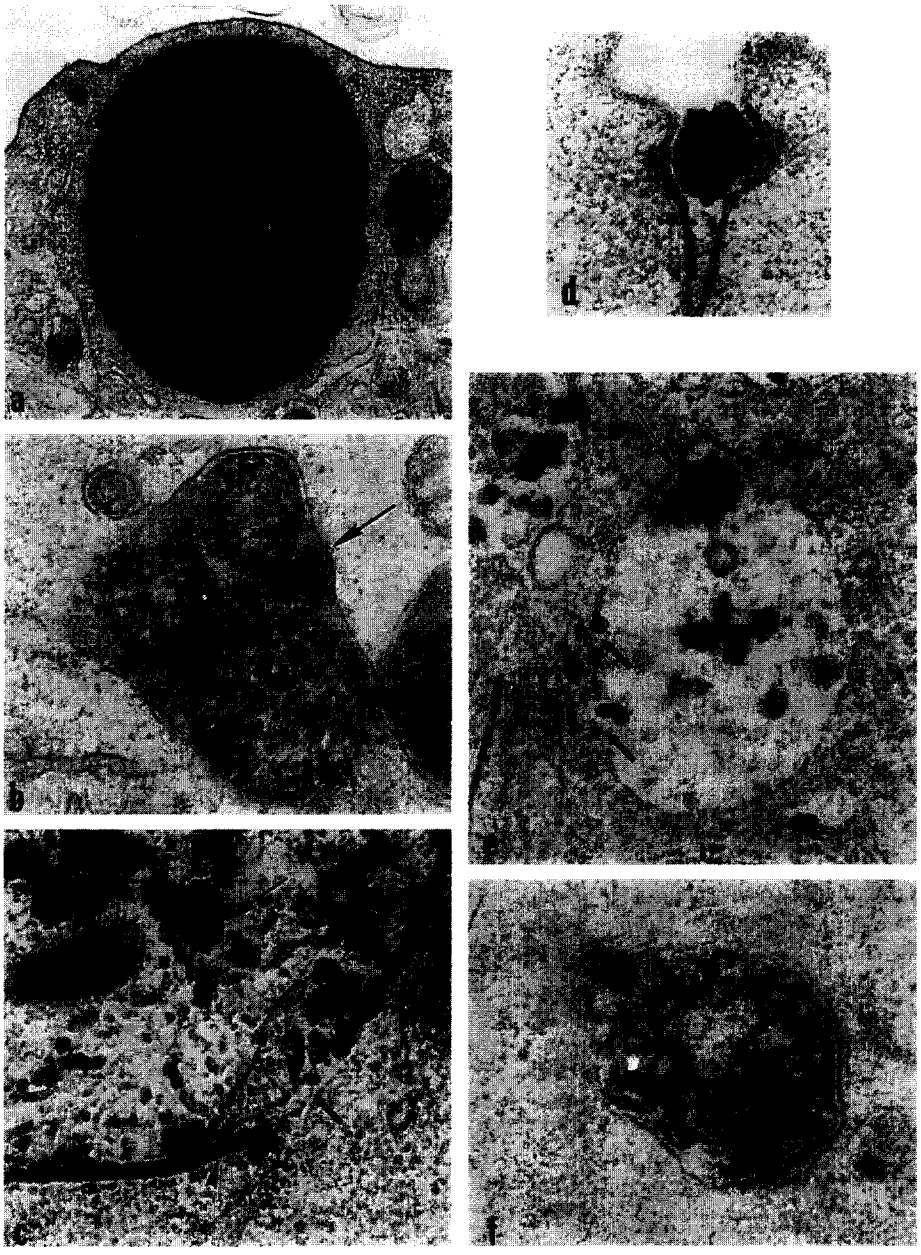

Fig. 3. Transmission electron micrographs of alveolar type II cells, following a 1 h interaction with gold-labeled DOTMA/DOPE MLV liposomes. (a) An intact lamellar secretory granule in an isolated cell. (b) Liposome-gold complexes mixed with the contents of a lamellar secretory granule. (c) DOTMA/DOPE liposomes mixed with the lamellar body contents form extended bilayers and short discs, frequently disrupting the limiting membrane [arrow] and other organelle membranes which they contact. (d) An extracellular gold-labeled liposome. (e and f) The gold label appears together with the liposome [arrow] in a disrupted [bold arrows] endosome membrane (e), and in apparent lysosomes (f). Magnifications: (a) 23 000; (b) 45 000; (c) 54 000; (d) 74 000; (e) 50 000; (f) 48 000.

plasma membrane. Cells maintained at 4°C had liposomes only on the cell surface and in superficial coated pits and vesicles (data not shown). Endocytosis of DNA-cationic liposome complexes was observed at 5 min and proceeded both at room temperature and at 37°C. The liposomes alone and liposome preparations complexed to RSV-CAT plasmid DNA appeared to enter endosomes rapidly, but they became altered morphologically after entering what appears to be the late endosomal compartment (Fig. 1e).

In order to follow the intracellular fate of liposome contents, we labeled DOTMA/DOPE liposomes with gold particles (Fig. 1). The streptavidin gold particles, which bear a net negative charge, bind to the liposomes by electrostatic interaction with the cationic lipid head group. The amount of streptavidin gold used in these studies had no effect on the ability of DOTMA/DOPE liposome-RSV-CAT plasmid complexes to transfect cells (Fig. 2). This indicates that the inclusion of streptavidin gold in the liposome-DNA complex does not alter the characteristics of the complex (size, surface charge, etc) in ways that significantly affect its transfection efficiency (Fig. 2). Thus, based on transfection efficiency, the behavior of the avidin gold-cationic liposome complexes should reflect that of these liposomes complexed to plasmid DNA alone. However, the behavior of gold particles may not be representative of the liposome-associated DNA. The avidin gold-liposome complexes could be seen contacting the cell surface, associated with clathrin coat assembly, plasma membrane invagination, and the formation of vesicles which pinch off from the plasma membrane and rapidly lose their coat (Fig. 1a–c). The labeled liposomes proceeded to early and late endosomal compartments and appeared in dense bodies and other vacuoles generally acknowledged as secondary lysosomes (Fig. 1e and f). The identification of intact liposomes became difficult and the lipid bilayer structure commonly disappeared by the time they enter secondary lysosomes, although the gold remained evident (Fig. 1e and f).

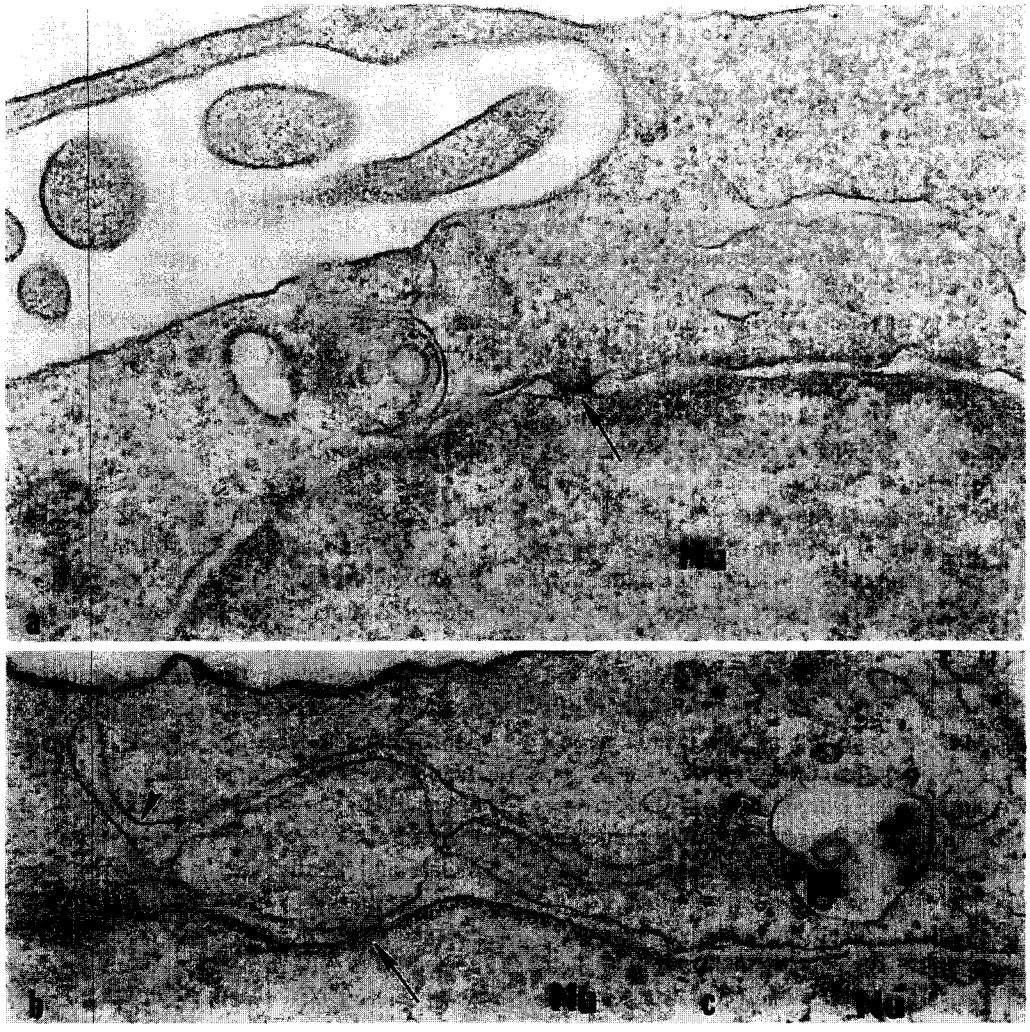

Fig. 4. Transmission electron micrograph of U937 cells following a 1 h incubation with gold-labeled MLV liposomes. (a) Free gold label is seen in the cytoplasm near nuclear pores (arrow), as well as (b) in portions of endoplasmic reticulum continuous with the nuclear envelope (arrowhead), and in (c) endosomes containing liposome-gold complexes. Nucleus (Nu). Magnifications: (a) 42 000; (b) 50 000; (c) 42 000.

In isolated rat alveolar type II cells maintained in tissue culture medium and exposed to liposomes, some intact DOTMA/DOPE liposomes-streptavidin gold complexes appeared in lamellar bodies, the cells secretory product (Fig. 3b), as well as in tubular and vesicular endosomes. Alveolar type II cells did not endocytose streptavidin gold particles in the absence of liposomes (data not shown). Gold appearing free in the secretory granules must therefore be a consequence of liposome endocytosis and incorporation into the secretory pathway (Fig. 3).

In all type II cells to which DOTMA/DOPE liposomes were added, with or without associated gold, lamellar body lipids assumed truncated bilayer configurations (Fig. 3c) and fragmented membranes with which they contacted. The lamellar bilayers fragmented into discs (Fig. 3c and e), interacted with the membrane confining the lamellar bodies (Fig. 3b) and fused with all organelles including endosomes (Fig. 3e), the rough surfaced endoplasmic reticulum, and the nuclear envelope (Figs. 4 and 6). Continuity with or association with the nuclear envelope was seen most dramatically in CV-1 cells, which are highly susceptible to DOTMA liposome mediated transfection [12].

One of the most striking additional observations is that in all populations of cells examined which demonstrated endocytosis of liposomes, approx. 20% of the cells revealed colloidal gold outside the endosomal and lysosomal compartments. Fifty to sixty separate cell preparations were examined. In these instances, the label was observed in the cytoplasmic ground substance, endoplasmic reticulum, and rough surfaced cisternae continuous with the nuclear envelope, as well as in the cytoplasm near nuclear pores (Fig. 4a and b). Commonly, the cells that had gold label free in the cytoplasm and in cisternae also had gold label within late endosomes near the nuclear envelope (Fig. 4c). Also, in one to two percent of viable-appearing CV-1 and occasional U937 cells exposed to the plasmid DNA-DOTMA/DOPE liposome complexes, vesicular and reticular intranuclear membranes developed. A portion of a CV-1 cell nucleus in a control non-transfected CV-1 cell is shown in Fig. 5. Fig. 6 depicts the nucleoplasmic reticulum of a CV-1 cell with DOTMA/DOPE liposomes labeled with streptavidin gold and mixed with cells. The membranes also formed reticulate lamellar structures with the liposomes (Fig. 7b and c). Fig. 7a is a CV-1 cell fortuitously leached of its cytoplasmic matrix, which shows reticular intranuclear structures comparable to that of the endoplasmic reticulum in the cytoplasm. Both have some ribosomes on the bilayers, but the reticulum in the nucleus is composed of 10 nm thick membranes compared with the 8 nm cytoplasmic membranes in these preparations.

To test the possibility that the interaction between nuclei and liposomes is a direct phenomenon, we incubated DOTMA/DOPE liposomes with isolated CV-1 nuclei (Fig. 8a) for 1 h at 37°C. Sixteen percent of the nuclei developed reticular structures (Fig. 8b,c and d), often starting in nucleoli adjacent to the nuclear envelope (8c) and always contiguous with the nuclear envelope itself. Negatively charged phosphatidylserine-DOPE (in a 1 to 1 molar ratio) liposomes did not interact with isolated nuclei, and no lipid bilayer structures were present within them (data not shown). This specific manifestation of nuclear envelope and nucleic acid–liposome interaction appeared confined to cationic lipid-containing liposomes.

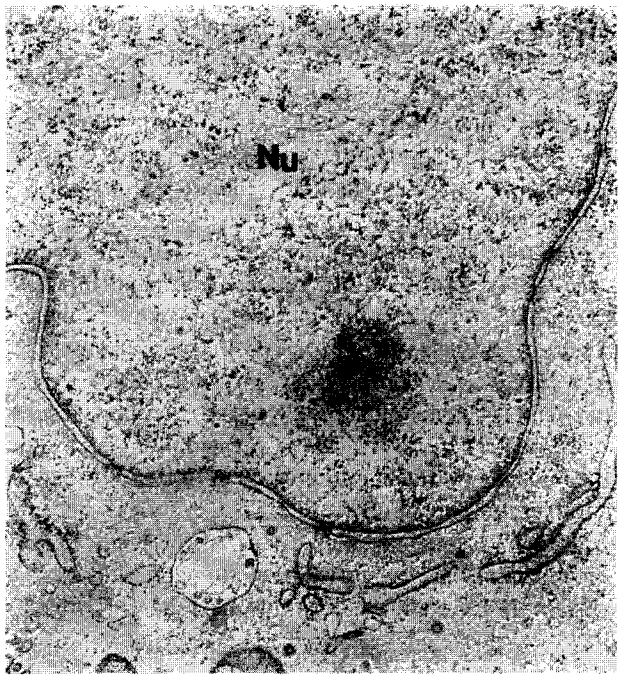

Fig. 5. Electron micrograph of a portion of the nucleus (Nu) and adjacent cytoplasm in an untreated CV-1 tissue culture cell. The nuclear envelope and nuclear pores (arrows) and the dispersed chromatin are clearly defined. Nucleolus (arrow). Magnification: 20000.

4. Discussion

In order to be transcribed, transfected genes must be delivered intracellularly, and ultimately into the nucleus in functional form. Cells do not ordinarily internalize large and/or charged macromolecules, such as plasmid DNA. Therefore, a variety of different techniques have been developed to deliver DNA into cultured cells. The mechanisms of successful carrier-mediated gene delivery are poorly characterized, and various possibilities include: how the carrier-DNA complex interacts with the plasma membrane (endocytosis versus fusion), how it is processed in the cytoplasm (within or outside the endosomal compartment), and finally how it is finally transported into the nucleus. Earlier studies had established that negatively charged liposomes are endocytosed by CV-1 cells via the coated-pit mechanism [24]. Although such liposomes can encapsulate DNA, the transfection efficiency is not better than that obtained by calcium phosphate [25]. Therefore, we have focused our attention to the cationic liposomes because of their higher transfection efficiency [9,12]. We

have examined by electron microscopy the interactions of cationic liposomes with a variety of cultured cell types, as well as freshly isolated human peripheral blood monocytes, rat alveolar type II cells, and rat alveolar macrophages.

Our observations suggest but do not prove that the intracellular pathway by which DNA complexed to cationic liposomes reaches the nucleus in a number of different cell types is through endocytosis, followed by its release from an early endosomal compartment (possibly mediated by cationic lipid fusogens). In some instances, the cationic lipid may react with the nuclear envelope. It is not known whether such interactions may be important for the transfer of DNA into the nucleus. Furthermore, we cannot exclude that cytoplasmic entry could occur following fusion at the plasma membrane level.

The morphologic detection of membrane fusion is difficult to obtain even in synchronized high volume fusion phenomena, such as synaptic vesicle release or during the acrosome reaction in spermatozoa. Therefore, it is not surprising that without rapid freezing or optimizing the number of fusion events, we have found only few continuities between DOTMA-containing liposomes and membranous organelles. However, morphologically definitive

Fig. 6. Transmission electron micrograph of a CV-1 cell, following incubation for 1 h with DOTMA/DOPE MLV liposomes. Some CV-1 cells develop extensive vesicular and reticular membranes (NR) in the nucleus (Nu). The membranes in the nucleus are thicker than the organelle membranes in the cytoplasm (Cyt). Magnification: 21 000.

structures possibly deriving from such fusion events between liposomes and intracellular membranes were obvious in all preparations of cells transfectable by cationic liposomes with small variations between cell types. Direct liposome-organelle membrane fusions were most clearly seen in rat alveolar type II cells. There was frequent introduction of the liposome associated gold particles to all cell compartments including the secretory granules. The blending of the endocytic and exocytic pathways in this cell is striking confirmation that this phenomenon is a frequent occurrence in surfactant secretion. Lipid material from the DOTMA:DOPE liposomes seems to be particularly interactive with the lamellar body lipids, mainly composed of dipalmitoylphosphatidylcholine (DPPC), and also interactive with the lamellar body membrane, endoplasmic, nuclear envelope and even the stable outer mitochondrial membrane [12]. Interaction or mixing with these membranes is evidenced morphologically as thickening of these membranes from 8 to 10 nm. By comparison, the alveolar macrophages fill their endocytic compartment with DOTMA/DOPE liposomes or liposome-gold complexes, without much overt membrane association. Yet, even in this efficient phagocytic system, some gold particles reach the lumen of the endoplasmic reticulum. Moreover, these cells can be transfected using cationic liposomes [19].

While liposome–membrane interactions involving fusion or lipid mixing take place within milliseconds and are difficult to capture by EM, the presence of free gold in the

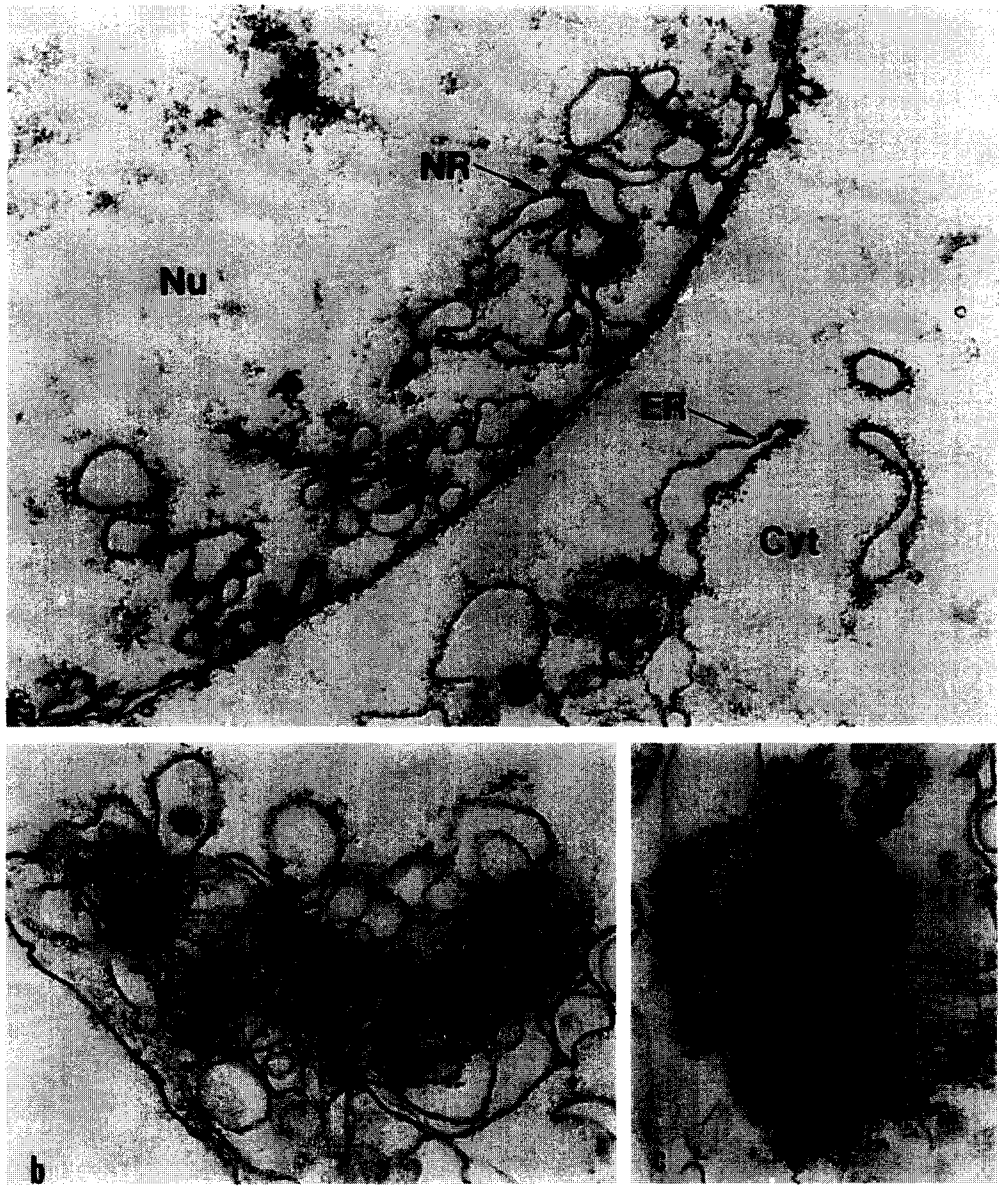

Fig. 7. Transmission electron micrograph of a CV-1 cell, fortuitously leached of its matrix. The intranuclear membranes can be seen to have ribosome-studded portions (arrows), similar in appearance to the endoplasmic reticulum (arrow) (a). Nu, Nucleus. (Cyt), cytoplasm. (b) and (c) reveal 10 nm thick membranes possibly formed from the mixing of DOTMA and native cellular membranous debris. The persistence of the gold label indicates continued electrostatic attraction between the gold and cationic lipid. Magnifications: (a) 25 000; (b) 42 000; (c) 45 000.

cytoplasm constitutes reasonable evidence that such interactions may be involved in the transfer of particulate material through the membranes. Although neither gold particles nor DNA is strictly encapsulated within the lumen of the liposomes, their strong interaction with the liposomal lipid may facilitate their transmembrane passage following lipid mixing at the membrane surface, even if fusion is difficult to define in this case as mixing of contents. At present, however, it is not proven that liposome-associated colloidal gold is representative of the behavior of liposome-associated DNA.

The most striking liposome-induced morphologic change took place in CV-1 cells and in U937 cells, which also showed avid endocytosis of liposome-plasmid DNA complexes. In these cell types, there is a clear interaction between cationic lipids and nuclear envelope. This interaction is most often initiated in the region of the nucleolus, with the development of a novel structure, composed of vesicular and reticular intranuclear membranes. We presume that this reticular structure is composed partly of DOTMA/DOPE lipid, which is diluted among the naturally occurring lipids in the cell. The thickness of these membranes distinguishes it from the 75 Å native membranes of the ER and the plasma membrane. While this is a consequence of DOTMA/DOPE liposome-nuclear envelope interaction, it occurs in this exaggerated manner in only a minority of cells. It is possible that a larger number of transfected cells display this phenomenon of liposome-nuclear envelope interaction, but to a lesser degree which does not produce a morphologically apparent structure.

The development of these reticular intranuclear membranes does not seem to require metabolic energy. Isolated

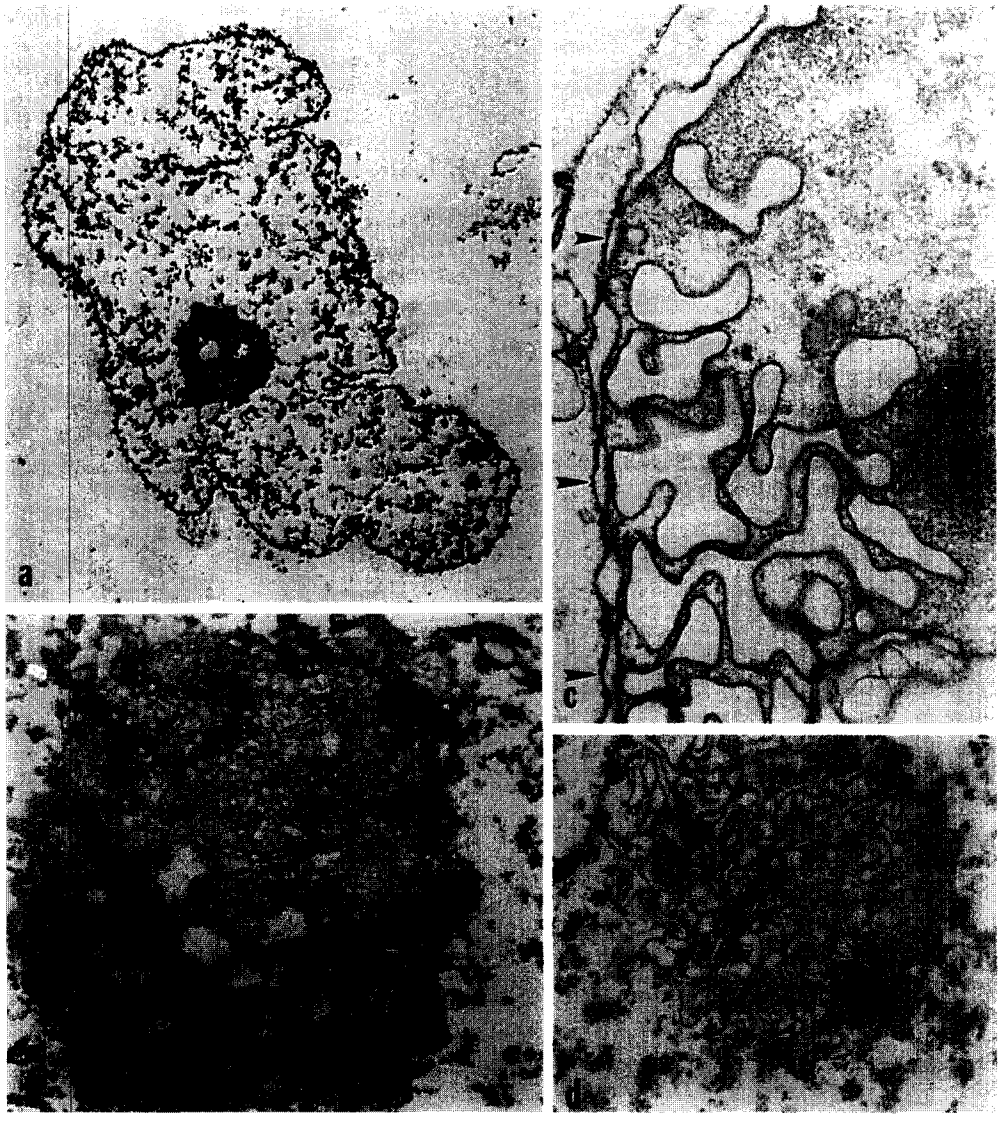

Fig. 8. Transmission electron micrograph of CV-1 cell nuclei. (a) An untreated isolated CV-1 cell nucleus. (b–d) An isolated nucleus incubated for 1 h with unlabeled MLV liposomes. The intranucleus membranes seem to initiate in the remaining nuclear envelope (c), and in nucleoli (b and d). These figures demonstrate a direct interaction between cationic lipids, and the nuclear envelope, nuclear contents. Magnifications: (a) 5000; (b) 17000; (c) 20000; (d) 24000.

nuclei, incubated with cationic liposomes form bilayers within the nuclei quite readily. After mixing with the lipids and proteins of disrupted type II alveolar cells, gold-labeled DOTMA/DOPE liposomes also form bilayers of the same thickness as those seen in the reticular intranuclear membranes.

Because of the rapidity and transience of fusion events, we cannot exclude the possibility that the DOTMA/DOPE liposome-plasmid DNA complexes fuse directly with the plasma membrane [23]. However, our morphological observations indicate disruptive interactions with endosomal/lysosomal membranes rather than with the plasma membrane, suggesting that this may be the most likely route of entry resulting in transfection. Thus it appears that somewhere between the coated pits and the late endosomes, the cationic liposomes facilitate an escape of complexed DNA into the cytoplasm and eventually into the nucleus. However, more definitive studies are needed for a more direct correlation between morphological observations and transfection activity.

Acknowledgements

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