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A NOVEL CATIONIC LIPOSOME REAGENT FOR EFFICIENT TRANSFECTION OF MAMMALIAN CELLS

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Summary: A novel cationic derivative of cholesterol, 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), has been synthesized and used to prepare sonicated liposomes with dioleoylphosphatidylethanolamine. This novel cationic liposome reagent facilitates efficient DNA mediated transfection in A431 human epidermoid carcinoma cells, A549 human lung carcinoma cells, L929 mouse fibroblast cells, and YPT minipig primary endothelial cells. The activity was greater than that of a commercial reagent, Lipofectin, and was approximately 4-fold less toxic than Lipofectin when assayed with A431 cells. The reagent is easy to synthesize and stable for at least 6 weeks.

Cationic liposomes are efficient nonviral transfection reagents for animal cells in vitro (for review, see 1). The first such reagent, DOTMA, contains a double-chain quaternary ammonium and ether bonds. This lipid, when mixed with an equimolar amount of DOPE, is effective in transfecting a variety of mammalian cells in vitro (2,3) and in vivo (4). The synthesis of DOTMA is multistep and of relatively low yield (2). The commercial reagent, Lipofectin, which contains DOTMA and DOPE, is also relatively expensive. Although several other cationic liposome reagents have been prepared with commercially available cationic amphiphiles, they are relatively toxic to the treated cells (5-7). We describe here a novel cationic cholesterol derivative which can be

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Abbreviations: CAT, chloramphenicol acetyltransferase; DC-Chol, 3β[N-(N'N'-dimethylaminoethane)-carbarmoyl cholesterol; DOPE, dioleoylphosphatidylethanolamine; DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride.

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synthesized in a single step. Liposomes containing this lipid are more efficient in transfection and less toxic to the treated cells when compared with the Lipofectin reagent.

Materials and Methods

Materials: DOPE was purchased from Avanti Polar Lipids. Cholesteryl chloroformate and N,N-dimethylethylenediamine were obtained from Aldrich. Acetyl CoA, chloramphenicol and CAT were from Sigma. [^{3}H] acetyl CoA (3.18Ci/mmol) was obtained from Amersham. Lipofectin reagent was from GIBCO BRL. Plasmid pUCSV2CAT (constructed by Dr. Mark Magnuson of Vanderbilt University) was amplified in E. Coli and purified by CsCl gradient ultracentrifugation method.(8)

Synthesis of DC-Chol: DC-Chol was synthesized by a simple reaction from cholesteryl chloroformate and N,N-dimethylethylenediamine (Fig.1). A solution of cholesteryl chloroformate (2.25g, 5 mmol in 5ml dry chloroform) was added dropwise to a solution of excess N,N-dimethylethylendiamine (2ml, 18.2 mmol in 3ml dry chloroform) at 0°C. After removal of solvent by evaporation, the residue was purified by recrystallization twice in absolute ethanol at 4°C and dried in vacuo, yielding 0.545g of white powder of DC-Chol (21.8 %). TLC yielded a single spot with Rf=0.57 (chloroform:methanol = 65:35); mp108°C; ^{1}H NMR (250MHz,$CDCl_3$): δ, 2.18 (singlet, 6H, $(\underline{CH_3})_2NCH_2CH_2$); δ,2.36 (triplet,2H,$(CH_3)_2N\underline{CH_2}CH_2NH$); δ,3.21 (quartet,2H,$(CH_3)_2NCH_2\underline{CH_2}NH$); δ,5.18 (triplet, broad, 1H, $(CH_3)_2NCH_2CH_2N\underline{H}CO$).

Preparation of cationic liposomes: A mixture of 1.2 μmol of DC-Chol and 0.8 μmol of DOPE in chloroform was dried, vacuum desiccated, and resuspended in 1ml sterile 20mM HEPES buffer (pH 7.8) in a sterile test tube. After hydration for 24 hours at 4°C, the dispersion was sonicated for 5-10 minutes in a bath sonicator to form liposomes with 150-200nm in average diameter.

Cell culture and transfection: A431 cells, A549 cells, and L929 cells (from American Type Culture Collection) were maintained in D-

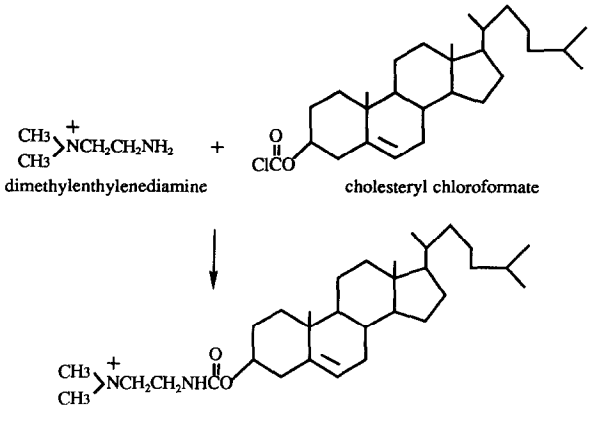

Fig. 1. Reaction scheme for the synthesis of 3β[N-N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol).

MEM (high glucose), F12 nutrient mixture, and McCoy's medium, respectively, supplemented with 10% fetal calf serum. YPT minipig endothelial cells obtained from Drs. Mark Steward and Gary Nable, Howard Hughes Medical Institute, University of Michigan Medical Center, were grown in medium 199 containing 10% fetal calf serum. Cells were plated into 35mm plates the day before transfection. They were about 80% confluent. One to ten minutes before transfection, cationic liposomes were added to the bottom of a 12 X 75mm polystyrene culture tube, followed by the addition of 1ml plasmid DNA diluted in McCoy's medium without serum. The mixture was added to the cells which had been washed once with serum free medium. After 5 hours at 37°C, the incubation medium was replaced with serum-containing medium and cells were harvested 36 hours after the transfection.

CAT assay: CAT activity was determined by the benzene extraction method (9), with two modifications: 0.05μCi [^{3}H] acetyl CoA and 1nmol unlabelled acetyl CoA were used for each assay and the cell extracts were heated at 60°C for 5 minutes to inactivate interfering enzyme activity (10). Protein concentration was determined by Bio-Rad Protein Microassay method, using BSA as standard.

Results and Discussion

Small liposomes (150-200 nm in diameter) could be easily prepared with mixtures of DC-Chol and DOPE (4:6, 5:5, or 6:4, molar ratio) by sonication in a low ionic strength buffer. These liposomes are efficient in mediating the transfection of pUCSV2CAT plasmid DNA into four different types of cultured mammalian cells (Fig. 2). No appreciable cytotoxicity was noted in the transfected cells. Of the cells tested, A431 human epidermoid carcinoma cells showed the highest transfection activity. These cells were chosen for more detailed studies.

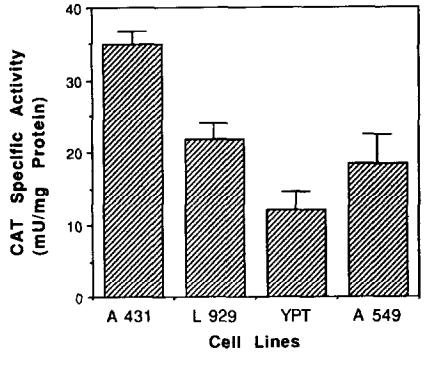

Fig. 2. Transfection activity of cationic liposomes containing DC-Chol and DOPE (6:4). The amount of liposomes used was 24 μg/ml for A431 cells and 21 μg/ml for all others. The amount of DNA used was 4 μg/ml for all types of cells. Data is shown as mean ± s.d.(n=3).

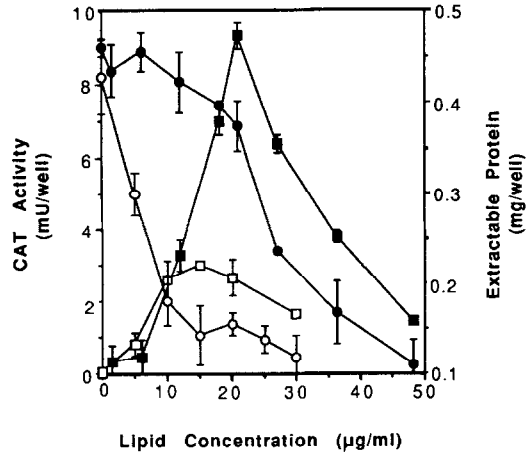

Fig. 3. Transfection activity and toxicity of Lipofectin and liposomes composed of DC-Chol and DOPE (6:4) in A431 cells. Four μg/ml DNA was used for all wells. CAT activity is shown in squares and the amount of extractable protein is shown in circles. Cells were treated with either Lipofectin (open symbols) or liposomes composed of DC-Chol and DOPE (closed symbols). Data is expressed as mean ± s.d.(n=3).

An experiment was done by using a fixed amount of DNA (4 μg/ml) with various amounts of liposome containing DC-Chol and DOPE (6:4). Lipofectin was also used for comparison (Fig. 3). It is apparent that the transfection activity of the new liposome reagent was greater than that of Lipofectin, although both showed a bell-shape dependence on the lipid concentration. The amount of extractable protein from the treated cells was also determined as an indication of the cytotoxicity of the treated cells. The Lipofectin/DNA complex was quite toxic to the A431 cells, showing a IC_{50} value of approximately 6 μg/ml lipid. Liposomes composed of DC-Chol and DOPE (6:4) were less toxic, with the IC_{50} value being at approximately 25 μg/ml. It is important to note that the maximal CAT activity of the cells transfected with liposomes containing DC-Chol and DOPE (6:4) occurred at 20 μg/ml lipid. At this concentration, the total cellular protein content of the treated cells was about 80% of the untreated control. This is compared to only 12% of the control protein content for cells treated with Lipofectin for the maximal transfection activity (at 15 μg/ml lipid).

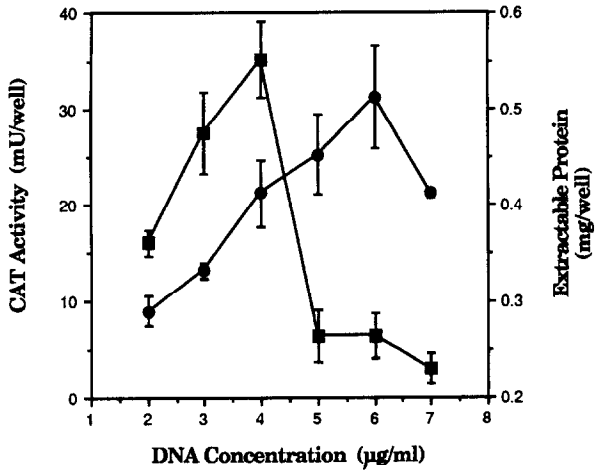

Fig. 4. Transfection activity and toxicity of liposomes composed of DC-Chol and DOPE (6:4) in A431 cells. Twenty four μg/ml liposomes were used for all wells. Both CAT activity (■) and amount of extractable protein (●) are shown. Data is expressed as mean ± s.d.(n=3).

Liposomes containing DC-Chol and DOPE (6:4) were mixed with various amounts of plasmid DNA and added to A431 cells for transfection (Fig. 4). For 24 μg/ml total lipid, the transfection activity increased with DNA concentration to about 10 mU CAT activity per well at 4 μg/ml DNA. Lower transfection activities were observed at higher DNA concentrations. The bell-shaped curve has previously been observed for other cationic lipid dispersions (2,6), and is related to the oversaturation of the cationic charges by the excess DNA. Also shown in the Fig. 4 is the total amount of extractable protein from the treated cells. At low DNA concentrations, some cytotoxicity was observed in the treated cells. This is probably due to the excess cationic liposomes in the medium. At higher DNA concentrations, close to normal amounts of cell protein were observed in the treated cells indicating that excess DNA can mask the toxicity of the cationic liposomes. Using the optimal amounts of liposomes (21-24 μg/ml) and DNA (4 μg/ml), the total amount of cell protein was only reduced by about 20% (Fig. 3 and 4).

DC-Chol, containing a carbamoyl bond, is much more stable than similar amphiphiles with an ester bond (Farhood et al., unpublished

data). Indeed, liposomes containing DC-Chol and DOPE retained full transfection activity after storage at 4°C for 6 weeks (data not shown). Other metabolizable cationic amphiphiles containing a labile ester bond (11) are not expected to be stable due to the ammonium group catalyzed hydrolysis reaction. On the other hand, carbamoyl bond can be hydrolysed by esterases and should be biodegradable once having entered the cells. This would not be the case for those amphiphiles containing ether bonds, such as DOTMA.

In summary, we have prepared a stable transfection reagent which can be synthesized by a simple, inexpensive method. The new reagent is more effective in transfection and less toxic than Lipofectin. It should be valuable for transfection experiments and in gene therapy (12).

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