CHAPTER 2

NEW LIPID- AND GLYCOLIPID-BASED NANOSYSTEMS FOR TARGETED GENE DELIVERY: CHOLENIMS, GLYCOCLIPS, GLYCOLIPIDS AND CHITOSAN

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Abstract: Cationic lipid vesicles and polypeptides represent common non-viral gene delivery systems for in vitro and in vivo applications. New non-viral vectors for targeted gene delivery, namely, mono-, di- and tricholesterol derivatives of oligoethyleneimine, glycolipids and chitosan derivatives are reported in this chapter. Testing of genotoxicity, cytotoxicity and gene transfer activity against transformed monolayer and suspension cell cultures is carried out for all of these mediators of gene transfer. Experimental results show that GLYCOLIPID VI containing a lactose residue, which was used to form liposomes for gene delivery into tissues (using ^{14}C-adenosine-labeled or plasmid DNA), expressed the affinity of corresponding lipoplexes for kidney, liver, and spleen tissues. GLYCOLIPID VI is a prospective tool for designing new generation of nonviral vectors for targeted gene delivery to tissues. In addition, mCHIT preparation demonstrated high gene transfer activity (β-Gal and CSEAP plasmids) for both monolayer and suspension cell lines

Keywords: cholesteroyl derivatives of oligoethylenpropylenimine; cationic lipid; cationic glycolipid; lactosolipid, modified chitosan; cytotoxicity; genotoxicity; gene transfer; gene delivery

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Abbreviations: CLIP: cationic lipid; GLYCOCLIP: cationic glycolipid; DOPE: dioleyl phosphatidyl ethanolamine; PC: phosphatidyl choline; CHOLENIM: monocholesteroyl ester of ethylen/propylene/imine co-oligomer; TsO: p-toluenesulfonate; RGGN: rat Gasserian ganglion neurinoma; RLU: relative luminescence unit

1. INTRODUCTION

Cationic lipid based vesicles and polypeptides represent common non-viral delivery systems for *in vitro* and *in vivo* functional gene transfer for gene therapy purposes [1–5]. There exist a great variety of types of non-viral vectors [1, 6, 7]. They possess a number of advantages comparing to the viral vectors: they are not immunogenic like adenoviruses, not randomly integrated into genome like retro viruses, not infectious, not patogenic (oncogenic) and cheap. Neutralizing DNA negative charge they facilitate adsorbic endocytosis of self-assembled complexes between plasmid DNA and polycation and/or cationic lipid particle – lipoplexes. Another possiblity for genosomes to be internalized is receptor-mediated endocytosis [8–10]. The most promissing approach to the latter mechanism of targeted gene transfer/delivery is to employ specific oligosaccharide-conjugated vector systems [11, 12]. Systems for targeted delivery and receptor-mediated gene transfer could be also designed on the basis of polycations, but mainly using coupling with carbohydrates [10]. Polycations conjugated with carbohydrate residues were introduced into gene transfer field, and appeared to be one of the most effective group of transfection agents due to the moieties employed responsible for the receptor-mediated gene transfer [12, 13]. A number of chitosan preparations were recently reported as gene transfer and delivery systems [14–16]. Galactose derivative of cholesterol was introduced to provide gene targeting to hepatocytes [17]. In our study we emploied the incapsulation of reporter plasmid DNA into new delivery systems based on glycolipids, which are combining the advantages of both gene transfer mechanisms: non-specific (adsorbic endocytosis) and receptor-mediated ones, along with DNA incapsulation into hydrophobic oligocations.

Here we report new systems for nanotherapy comprising encapsulation of reporter genes into lipoplexes based on the use of cholesterol derivatives of oligoethylenpropylenimine I-III (CHOLENIMs) [18, 19]; cationic glycolipid containing glucose moiety V (GLYCOCLIP) [20], liposomal preparations based on lactosylated lipid (GLYCOLIPID) VI [21]; as the cytofectins and helper phospholipids, for gene transfer and delivery. Evaluation of the cyto- and geno-toxicity and gene delivery activity of these lipoplex and glycolipoplex systems were carried out in cell culture. To this end we also used modified natural polycationic polysaccharide, chitosan–modified chitosan derivative (mCHIT) VII, which can be prepared by deacetylation of chitin – linear poly–(N-acetyl-glucosamine) followed by methylation of deacetylated chitosan.

2. MATERIALS AND METHODS

All reagents used in this study were of reagent grade. Organic solvents were distilled before use. All lipid preparations (Sigma; Avanti) were stored at –80°C.

2.1. Cholenims

2.1.1. Synthesis and properties

Cholenims were synthesized as described earlier [18]. Cholenim I is tris- [2- *N*- (3-aminopropyl) aminoethyl] amine monocholesteroyl formiate; cholenim II, tris- [2-*N*- (3- aminopropyl) aminoethyl] amine dicholesteroyl formiate; and cholenim III, tris- [2- *N*- (3- aminopropyl) aminoethyl] amine tricholesteroyl formiate. Salmon sperm genomic DNA (1.7×10^4 kDa) was fragmented by mild sonication to duplexes with an average size of 4 kb. After dialyzing aqueous DNA solution (1.5 mg/ml) against 10 mM NaCl and 1 mM Tris-HCl (pH 7.2), its concentration was determined spectrophotometrically ($\lambda = 260$ nm) using the molar extinction coefficient $\varepsilon = 6600\ M^{-1}cm^{-1}$ [22]. Melting temperature of DNA duplexes in buffer solution was 72°C at a hyperchromic effect of 40%, indicating that the two-helix native structure of the duplexes was retained during sonication of genomic DNA. Melting curves of the complexes between genomic DNA fragments and cholenims were recorded on aVS4-2P spectrophotometer at 260 nm; the accuracy of measurements of temperature was $+/-0.5$ °C. Pyrene fluorescence spectra [23] were recorded on an MPF-44B Perkin-Elmer spectrofluorometer. Circular dichroism spectra of the lipoplexes containing the pCMV-SPORT-β-Gal plasmid (BioLifeTech, catalogue no. 10586-04) and cholenims were recorded on a Jasco J-600 spectropolarimeter. Electron micrographs of lipoplexes were obtained on a JEM 100B electron microscope at accelerating voltage of 80 kV. Briefly, an aliquot of the cholenim/DNA lipoplex was placed on a copper grid covered with a collodion film and dried. Excess complex was removed, and the remainder was negatively stained with 4% aqueous uranyl acetate. After removal of the dye, the film was dried. Micrographs were obtained on Kodak photographic plates.

2.1.2. Cytotoxicity, genotoxicty and gene transfer

The effectiveness of gene transfer using the cholenim lipoplexes was studied with eukaryotic cells RGGN-1 (NGUK-1, rat Gasserian gland neurinoma) and PC-12 (rat adrenal gland pheochromocytoma). Cells were cultured in an RPMI-1640 medium (Flow, United Kingdom) supplemented with 10% fetal bovine serum (PANECO) and 50 μg/ml gentamycin at 37°C in 5% CO_2 in a CO_2 incubator (Flow, United Kingdom) [4]. To form transfection complexes, plasmid DNA and cholenims were mixed, shaken on a Vortex, and incubated at room temperature for 30 min. RGGN-1 and PC-12 cells were transfected with the pCMV-SPORT-β-Gal plasmid 24 h after passage of cells in 96-well plates (5×10^4 cells per well). For this purpose, cultural liquid was removed from wells, and the monolayer was washed with a serum-free medium. Then, the DNA/cholenim lipoplex in a serum-free medium was

added to cells, and plates were incubated at 37°C for 5 h in 5% CO_2.Thereafter, an equal volume of culture medium containing 20% serum was added to cells, and incubation continued for another 48 h. After transfection, the medium was thoroughly removed from wells without disrupting the monolayer, and lysing solution containing 0.1% Triton X-100 and 0.25 M Tris-HCl (pH 8.0) was added to cells. Then, cells were frozen at −70°C and thawed at room temperature for 10 min. The activity of the marker β-galactosidase gene was determined as described [4,24], using chlorophenol-red-β-D-galactopyranoside (*N*-Gal; Sigma, United States) as a standard. Incubation was conducted in a phosphate buffer (pH 8.0) containing 1 mg/ml *N*-Gal, 1 mMgSO_4, 10mM KCl, 50 mM mercaptoethanol and 0.5% bovine serum albumin at 37°C until color development (15 min). The enzyme content in samples was determined using dilutions of the standard β-galactosidase sample (Sigma, United States). Liposomes were obtained by evaporation from reverse phase with subsequent sonication at 4°C for 5 min. Liposomes consisting of phosphatidylcholine and dicholenim (1 : 1, w/w) were used to transfer β-galactosidase gene at the lipid-to-DNA ratio of 1.6:1 (w/w) using intravenous injections, as described [4]. To detect expression of the bacterial β-galactosidase gene, mouse organs (kidneys, liver, heart, lungs, intestine, and spleen) were frozen at −80°C. Pieces of tissue were used to prepare sections (25 μm thick) on a cryostat microtome, which were then mounted on slides.

2.2. Glycoclip

Proton magnetic resonance (^{1}H-n.m.r.) spectra were measured with radiospectrometer "Bruker" MSL-200 (200 MHz) in $CDCl_3$ with $Si(CH_3)_4$ as internal standart. Mass-spectra were recorded with MSBKH time-off-flight mass-spectrometer ("Elektron", Sumy-city, Ukraine) with the ionization by nuclear fragments of californium-252; accelerating voltage was +/− 5 kV or +/− 20 kV. Optic rotation angles were measured with Jasco photoelectric spectropolarimeter, model DIP 360 (Japan).

The cationic lipids used are *rac*-N-[2,3-di (octadecyloxy) propyl] pyridinium p-toluenesulfonate (IV, CLIP) that was synthesized by interaction of *rac*-1,2-di-O-octadecyl-3-O-(4-toluenesulfonyl)glycerol with pyridine (90°C, 4 hrs.) with the yield of 85%. Properties: R_f 0.6 (silicagel (Merck), chloroform/methanol, 4:1); mass spectrum: m/z for $[M\text{-}TsO^-]^+$ 658.7; ^{1}H-n.m.r., δ: 0.86 (t, J 7, 6H, $2(CH_2)_{15}CH_3$), 1.24 (br. s, $2(CH_2)_{15}$ CH_3), 1.55 (m, 4H, $2OCH_2$ CH_2), 2.33 (s, 3H, $C_6H_4CH_3$), 3.25 (t, 4H, J 7.1, $2OCH_2CH_2$), 3.3–3.5 (m, 2H, CH_2 $OC_{18}H_{37}$), 3.85 (m, 1H, $CHOC_{18}H_{37}$), 4.61 (d. d, J 8.5, 13; 1H, CH_2N^+), 7.16 (m, 2H) and 7.71 (m, 2H, $C_6H_4CH_3$), 8.04 (m, 2H), 8.52 (m, 1H) and 8.89 (m, 2H, $C_5H_5N^+$).

rac- 1,2-Dioctadecyl-3-O- (2,3,4-tri-O-acetyl-6-deoxy-6-pyridinium-β-D-glucopyranosyl) glycerol p-toluenesulfonate, GLYCOCLIP, V was synthesized by the glycosylation of *rac*-1,2-dioctadecylglycerol [25] with 6-O-(4-toluenesulfonyl)-2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide in the presence of $Hg(CN)_2$ and $HgBr_2$ as previously described [26], followed by interaction of the resulting compound (R_f 0.54, silicagel, petrol.ether/ether, 1:1.5) with pyridine. Properties: $[\alpha]^{20}D$ –4.3° (Cl.5, chloroform/methanol, 3:2); R_f 0.45 (silicagel, chloroform/methanol, 4:1); mass

spectrum: m/z for $(M\text{-}TsO^-)^+$ 946.2; ^{1}H-n.m.r., δ: 0.85 (t, J 7, 6H, $2(CH_2)_{15}CH_3$), 1.27 (br. s, $2(CH_2)_{15}CH_3$), 1.52 (m, 4H, $2OCH_2CH_2$), 2.00, 2.15 and 2.35 (s, 9H, $3(COCH_3)$, 2.61 (s, 3H, $C_6H_4CH_3$), 3.27–3.67 (m, 9H, $2OCH_2CH_2$), $CHOCH_3$, OCH_2CHCH_2O protons of Gro), 4.02–4.22 (m, 1H at C-5 Glc); 3.97–5.37 (m, 6H at C-2, C-3, C-4, C-5 and C-6 Glc); 7.12 (m, 2H) and 7.72 (m, 2H, $C_6H_4CH_3$); 8.10 (m, 2H), 8.57 (m, 1H) and 8.97 (m, 2H, $C_5H_5N^+$).

2.2.1. Liposome preparation

GLYCOCLIP/DOPE (1:1), GLYCOCLIP/DOPE/CHOLENIM (1:1:2), and CLIP/PC (1:1, w/w) liposomes were prepared by the reverse phase evaporation technique normally providing small monobilayer particles [27]. GLYCOCLIP-based liposomes were prepared by slow addition of ether lipid solution to water at 50°C, followed by complete evaporation of organic solvents under reduced pressure and oil pump as described [28]. The value of +/− charge ratio was 1.0 for CLIP/PC vesicles, 1.6 for GLYCOCLIP/DOPE ones, and 3.2 in the case of mixed GLYCOCLIP/DOPE/CHOLENIM liposomes. The size of lipoplex particles formed of liposomes used and plasmid DNA is ca. 100-200 nm (the data are not shown), which is normal for *in vitro* experiments. Oxidation index of liposomal lipids (PC, DOPE), OD^{233}/OD^{215} ratio, was measured after extraction from liposomal preparations, and it didn't exeed 0.1–0.2.

2.2.2. Lipofection procedure

CHO cells were maintained in the RPMI 1640 medium with L-glutamine, and 10% fetal calf serum. The cells were washed, and incubated at 37°C in serum-free OPTIMEM medium (Boeringer-Manheim) before transfection. Genosomes (3 μg of pCMV-Luc/3 μg of liposomes in 100 μL of medium) were added to the CHO cell monolayer (2×10^5 cells) up to 1 ml of total volume, and were incubated for 4 hours (37°C, 4.5% CO_2) (including 15 min period on microshaker 326M) [29]. Medium was then removed, cells were washed twice with HEPES buffer, and incubated with full medium for 24 hrs (postincubation). Then the lysis buffer was added. DNA-liposomes complexes (2 μg of DNA/2 μg of lipid) were prepared by mixing in OPTIMEM medium, added to cells, and incubated in the same way [3]. Luciferase activity was measured after 30 min incubation in the lysis buffer using Promega kit with LUMAT luminometer. The transfection efficiency values were represented as relative lumenescense units (RLU). The data in all cases represent the means of 4 series of independent experiments (four experiments each) with standart deviation ($M+/-\sigma$). The statistical significance was evaluated by Student t-test ($p < 0.05$).

2.3. Lactosylated Lipid, GLYCOLIPID, VI

In this study, we used DMSO, $CaCl_2$ (chemical purity and tissue-culture grades), egg phosphatidylcholine and cholesterol from Fluka, X-Gal (5-bromo-4-chloro-3-indolyl-1,3-*D*-galactopyranoside) from Aldrich, and N-Gal (chlorophenol-red-β-*D*-galactopyranoside). All solutions were sterilized using 0.22-μm nitrocellulose membranes (Millipore). Reagents and media were prepared in autoclaved deionized

water. The modified glycolipid, lactosolipid, was synthesized from lactose thioderivative by the method described [30, 31]. This method allows obtaining neutral and positively charged glycolipids with symmetrical and asymmetrical aglycone structure. The last stage of this synthesis and the removal of protective groups are shown in the scheme. Thiogalactose **1** at the double bond of dihexadecyl ester of maleic acid **2** (scheme) was attached using triethylamine as an activator of reaction. The structure of synthesized compound **3** was confirmed by the results of 1H NMR and IR spectroscopy and mass spectrometry.

2.3.1. DNA, liposomes and lipoplexes

^{14}C-adenosine-labeled DNA was isolated from *E. coli* cells grown on a Luria-Bertani medium with adding ^{14}C-adenine (56 mCi/mmol, Izotop, Russia) by the standard procedure [32]. ^{14}C-DNA was sonicated at 22 kHz with an UZDN-2T disintegrator (Russia) for 15 min, with 30-s intervals after each minute of sonication, at 0°C. In total, ten sonication cycles were performed. As a result of this procedure, 4.6kb fragments were obtained (electrophoretic data). To obtain preparative amounts of the pCMV-SPORT-β-Gal plasmid (BioLifeTech, catalogue no. 10586-04), *E. coli* XL-1 cells transformed with this construct were cultured in a fermenter (shaker) at 37 ± 0.5°C for 14–16 h (night culture) in a Luria-Bertani liquid microbiological medium (ratio, 800 ml of medium per 4 l of air) supplemented with 50 mg/ml ampicillin as a selective component of cells carrying the plasmid with the corresponding marker gene.

To form nucleoliposome complexes (lipoplexes), ^{14}C-adenosine-labeled or plasmid DNA was mixed with liposomes and incubated for 30 min. Experiments were performed with four- to six-month-old inbred ICR mice weighing 36–40 g. Lipoplexes containing 80 μg of ^{14}C- adenosine-labeled DNA (65000 cpm per mouse) and 160 μg of phosphatidylcholine/lactosolipid liposomes were injected to anesthetized mice through a glass capillary into the portal vein of the liver. One day after injection, operated animals were euthanized; their internals were extracted, weighed, and lyzed in 0.6 N KOH at 37°C. Lysates were neutralized with 0.6 N $HClO_4$ and loaded on filters. Then, filters were dried and placed into flasks with scintillation liquid. The radioactivity trapped on the filters was measured in a Rakbeta counter. Polybilayer liposomes used to transfect mice *in vivo* were formed from a mixture containing phosphatidylcholine (70 mol %), lactosolipid (20 mol %), and dicholenim (10 mol %) by evaporation from reverse phase, as described [27]. Solutions of original lipids were stored at –80°C and liposomes were stored at 4°C under nitrogen. Liposomes were used within two weeks.

2.3.2. Cells, cell survival and genotoxicity determination

Rat Gasserian ganglion neurinoma (RGGN) cells were cultured in the RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum, and 50 μg/ml gentamycin. RGGN cells were seeded after the treatment with 0.02% EDTA (24-well plates) for their growing and DNA synthesis measurements. The initial cell density was

5×10^4 per well. RGGN cells were incubated unsealed in CO_2-incubator (5% CO_2, 37°C), liposomes were added 24h after cell passing [18, 33].

^{14}C-Thymidine (56 mCi/mmol, "IZOTOP", Russia) (5 mcCi) was added to 1 ml of the culture medium 24 hrs after liposomes. Cells were washed with cold Hanks medium 2 hrs after labeling, and fixed overnight with a cold mixture ethanol/"ice" acetic acid (9:1) to remove the unbound ^{14}C-thymidine. The cell monolayer was stained with 0.2 % crystal violet in 2 % aq. ethanol solution, the stained cells were washed with water, and the dye was eluted with 10% aq. acetic acid. Cell number was measured as the optical density value at 595nm with O.D.^{595} value equaled to 0.1 corresponding to 32,500 cells [34]. Then the cells were lysed with 0.3 N KOH overnight at 37°C, the pH value of the mixture was adjusted to 7, and the radioactivity value was counted using Bray's solution.

2.3.3. Animal experiments

Animals that were injected with the complex through the portal vein were euthanized two days after injection. For histochemical analysis, organs were frozen at 80°C immediately after their extracting from mice. Sections of these organs (25 μm thick), obtained using a cryostat microtome, were mounted on slides. Then, 200 μl of PBS (pH 7.5) containing X-Gal (6 mg/ml), 1 mM $MgSO_4$, 4 mM $K_4[Fe(CN)_6]$, and 4 mM $K_3[Fe(CN)_6]$ were poured over slides. X-Gal (6 mg) was preliminarily dissolved in 200 μl of DMSO. Slides were placed in a thermostate (37°C) in a moist chamber. The time required for the development of blue staining as a result of X-Gal degradation (30-50 min) was determined [4]. Thereafter, slides with sections were incubated in 2.5% glutaric aldehyde at 4°C for 2 h. To visualize cell structures (predominantly nuclei), sections were additionally stained with hematoxylin. Then, after successive dehydration in 70, 96, and 100% ethanol, a mixture of ethanol and xylol (1:1), and o-xylol, sections were embedded into Canada balsam drops under cover slips. For spectrophotometric detection of β-galactosidase activity in organs *in vivo*, they were homogenized on ice in PBS (pH 8.0) containing 1 mM Mg^{2+} and 10 mM K^+. Then, 1 ml of the homogenate was mixed with 100 μl of a substrate (chlorophenol-red-β-*D*-galactopyranoside) and 100 μl of mercaptoethanol. The mixture was stirred on a Vortex and divided into two parts (the experimental and the control). The experimental part was incubated in a thermostate at 37°C for 30 min (the optimal time for color development for 0.2 g aliquots), and the control part was incubated on ice. Then, both tubes were centrifuged at 11000 rpm for 7.5 min. The supernatant was collected and stored in the cold. Then, 200 μl of the reaction mixture were added to cuvettes with PBS. The specific activity of β-galactosidase was determined using the standard enzyme (Sigma, catalogue no. 9031-11-2) at different dilutions, by the optical density at 280 nm (D_{580}), which corresponded to the absorption maximum of the reaction product in the visible part of the spectrum. The values of optical density of the standard samples were used to plot a calibration curve that was then used to determine the activity of β-galactosidase in homogenates of organs. The coefficient used for calculation was determined by approximation to linear direct proportionality by the least squares

method using the MS Excel software. The values of D_{580} for homogenates of organs incubated at 37°C were measured relative to the matching samples that were incubated on ice (the control). The activity of transgenic β-galactosidase in organs was determined by the difference in the activity of the enzyme in the experimental and control samples. Using the calibration curve, the activity of β-galactosidase was recalculated to the international units of enzymatic activity (IU) and expressed in IU per gram of organ.

2.4. Modified Chitosan, VII

All chemical reagents used (L-α-phosphatidylcholine, α-tocopherol ester of succinic acid, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and p-nitrophenyl phosphate (Sigma); polyethylenimine (PEI) were of analytical grade.

2.4.1. Plasmids

All recombinant DNA manipulations (transfection, purification of plasmid DNA) were performed according to the protocols described earlier [35]. The following plasmids driven by the IE CMV promoter were used: pEQ176 with bacterial β-Gal gene (a gift from Dr. J. Overbaugh, University of Washington, Seattle, U.S.A.) and pCSEAP plasmid with secreted alkaline phosphatase gene (a gift from Dr. K. Doronin, University of Sant Luis, U.S.A.).

2.4.2. Synthesis

Chitosan preparations containing secondary and tertiary amino groups were prepared by G.G. Krivtsov using two-stage synthesis, intermediate product not being isolated. Initial chitosan preparation (reagent grade, m.m. 312 kDa, polydispercity 6.7, deacetylation degree 85%, 15% of N-acetylglucoseamine residues) was a gift from Dr. D.B. Freiman ("Sonat" Company, Moscow, Russia). We used reductive amination reaction [36] on the first stage to get chitosan preparation containing 20–25% of N-ethylated primary amino-groups. Chitosan (1% solution in 1% aq. acetic acid) was treated by 2% aq. solution of acetaldehyde in the presence of excess of sodium cyanoborhydride for 12 hours at 20°C. Resulted N-ethylated chitosan preparation was precipitated by 4% aq. sodium hydroxide solution, and was washed by water. On the second stage, Eshveiler-Clark reaction [36] was carried out: ethanol (85%), formic acid and formaldehyde (37%) were added to the final N-acetylated chitosan residue the CH_2O and HCOOH to primary NH_2 groups molar ratio being 2:2:1, and reaction mixture was heated 3 hours at 75°C as pointed earlier [37]. After that reaction mixture was left to reach room temperature, and was dialyzed exhaustively against 0.5% aq. acetic acid solution. Resulting N-ethylated (secondary) and N-dimethylated (tertiary) chitosan preparation was lyophilized and analyzed. Primary (40%), secondary (25%) and tertiary (20%) amino groups contents were measured by potentiometric titration. Characteristic viscosity was decreased from 492 $cm^3.g^{-1}$ (for initial chitosan) to 256 $cm^3.g^{-1}$ (for resulting mCHIT). Molecular mass of mCHIT is 60 kDa (gel filtration data).

2.4.3. pH-sensitive amphiphilic liposomes and lypoplex preparation

Liposomes were formed from a mixture containing egg yolk L-α-phosphatidyl choline (Sigma), (Fluka) (or α-tocopherol ester of succinic acid, Sigma) (9:1, mol. %) using reverse phase evaporation technique [27]. by the addition of lipid fraction ether solution to water (55°C, 1 ml/min), followed by exhaustive removal of organic solvent by evaporation under reduced pressure and *in vaccuo*. Nitrogen gas was passed through liposome suspension (conc. 2 mg/ml), liposomes prepared were stored at 4°C under nitrogen and used during three-week period. Plasmid DNA was mixed with liposome suspension (1:10, w/w) to form lipoplex, and magnesium chloride was added to lipoplex complex to reach final volume of 50 μL and Mg (II) ion concentration – of 50 mM.

2.4.4. Cell lines, transfection, and plasmid DNA

Human melanoma cell line (MeWo) and human tumor T-lymphocyte line (Jurcat) were obtained from ATCC bank. HeLa, human osteosarcoma (HOS-1) and human immortalzied premonocyte (U937) cell lines were kindly provided by Dr. T.I. Ponomareva (Institute of Agricultural Biotechnology, Moscow, Russia). HeLa, HOS-1 and human melanoma MeWo cells were grown at 37°C and 5% CO_2 in MEM (HyClone) medium suplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine, and 50 μg/ml of gentamycin. Immortalized premonocyte U937 and transformed lymphocyte Jurcat cells were grown at 37°C and 5% CO_2 in RPMI 1640 (HyClone) medium suplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 mcg/ml of gentamycin. Plasmid DNAs were finally purified by two cycles of centrigfugation in a CsCl gradient. The cells were transfected with plasmid DNA using a number of techniques: Ca $(PO_4)_x$ method [38], amphiphylic liposome- [28, 39, 40] and PEI- [41] mediated transfection. DNA concentration was estimated by measuring the absorbance at 260 nm, and horizontal agarose electrophoresis as well.

2.4.5. pEQ176 plasmid expression testing

Cells were washed with phosphate buffer solution and then fixed with 0.25% glutaraldehyde and 2% formaline solution for 5 mins at 4°C. Cells were covered *in situ* with coloured solution (5 mM yellow blood salt, 5 mM red blood salt, 2 mM $MgCl_2$ and 1% X-Gal indigogenic substrate), prepared in phosphate-salt buffer, after two-fold washing, and cells were incubated in the solution for 2 hrs. The expression level was observed by microscopic study counting blue-coloured cells, and calculated as a percentage of coloured cells from total amount of cells.

2.4.6. Testing of pCSEAP expression level in culture medium

Aliquotes of cultural medium (80 μL) harvested from cell monolayer 4 days after transfection were centrifugated 14,000 rpm, 2 min and heated at 65°C for inhibition of endogenic alkaline phosphatase activity. Equal volume of reactive buffer solution (0.5 M Na_2HCO_3, 0.5 mM $MgCl_2$; pH 9.8) was added to every aliquote, and the

mixture was incubated 10 min at 37°C. 50 μL of 60 mM p-nitrophenylphosphate solution (Sigma) (37°C) was added to every probe, and mixture was incubated for 20–30 min. Optical density at 405 nm was measured with rider "Titertek" (Flow).

3. RESULTS AND DISCUSSION

3.1. Cholenims

3.1.1. DNA encapsulation

To determine the relationship between the structure of cholenims and cholenim-based lipoplexes and their effectiveness in gene transfer, it was necessary to study the interaction between these compounds and nucleic acids, as well as their effect on DNA structure. For this purpose, we used the following physicochemical methods: fluorescence probes, spectrophotometry, circular dichroism spectroscopy, and electron microscopy. The hydrophilic moiety of cholenims includes the groups which are characteristic of the structure of natural polyamines spermine and spermidine, which exhibit affinity to and stabilize DNA helix [42], as well as polyethyleneimine, which display activity in gene transfer [41]. Due to complexity of the melting curves of plasmid DNA, we studied the effect of cholenims on the melting curves of genomic DNA.

Figure 1 (upper field) shows the melting curves of fragments of genomic DNA and its complexes with cholenims. Analysis of these curves showed that the complexes formed by DNA and compounds I, II, or III have a higher melting temperature (by 8, 5, and 4°C, respectively) compared to pure DNA fragments. Thus, these compounds stabilize the DNA helix, with their stabilizing effect decreasing in the following order: compound I > compound II > compound III. The affinity of colenims for the double helix of DNA is different due to different positive charges of their hydrophilic groups and different hydrophobicity/hydrophilicity ratios. Apparently, electrostatic interactions between the amino groups of compounds I and II and the negatively charged phosphate groups of the polynucleotide chain are important of stabilizing complexes. There is a good correlation between the ΔT_{melt} value and the charge of cholenim: the greater the charge, the greater the stabilizing effect (Table 1). Analysis of circular dichroism spectra of the pCMV-SPORT-β-Gal plasmid and its complexes with compounds I–III led us to conclude that they are practically identical and that these compounds do not affect the structure of double helix of DNA, which retains B-conformation (spectra not shown). As a fluorescent probe we used pyrene, whose oscillatory structure of emission spectra is highly sensitive to polarity of its microenvironment. Due to this property, pyrene is widely used in studies of membranes, micelles, and hydrophobic clusters [22, 23].

As seen from the results, the value of this ratio almost did not depend on the concentration of cholenim up to the threshold value; further increase in cholenim concentration results in a sharp increase in the I_3/I_1 ratio (in the absence of DNA, this parameter did not depend on the concentration of cholenims within the concentration range analyzed). These values, different for compounds I (6.0×10^{-5} M), II (8.6×10^{-5} M), and III

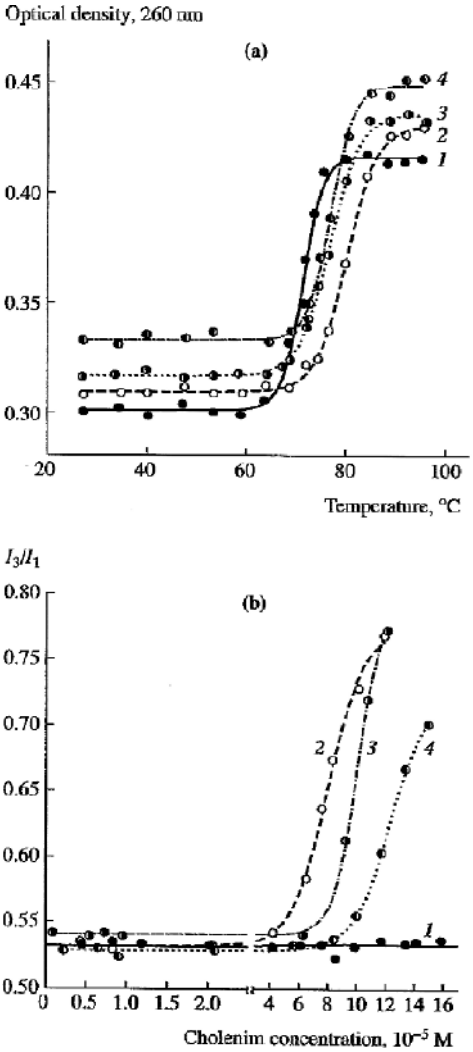

Figure 1. Physicochemical characteristics of the complexes (lipoplexes) DNA-CHOLENIMS. (a) UV melting curves of salmon roe DNA in the buffer containing 10 mM NaCl and 1 mM Tris-HCl (pH 7.2) *(1)* in the control and in the presence of *(2)* monocholenim, *(3)* dicholenim, and *(4)* tricholenim (1.0±0.2). 10^{-4} M. (b) Dependence of pyrene emission spectrum (the I_3/I_1 index) on the concentration of *(2)* monocholenim, *(3)* dicholenim, and *(4)* tricholenim. Curve *1* shows DNA spectrum in the absence of cholenims.

Designations: I_1 and I_3, amplitudes of oscillatory lines of emission spectra of monomeric pyrene at 383 and 372 nm, respectively, in the presence of salmon sperm DNA (45μM by phosphate)

Table 1. Properties of hydrophobic oligocation CHOLENIMS and their lipoplexes

Cholesterol derivatives	T melt., (°C)	ΔT	CMC, M	Charge*	EM, diameter (nm)	Transfection efficacy against PC-12 cells	
						DNA/ cholenim ratio	Picog protein per 10^5 cells
Monocholenim	80	+8	$6.0*10^{-5}$	+2	100–130	3:1	105
						2:1	187
						0,7:1	36
Dicholenim	77	+5	$8.6*10^{-5}$	+1	200–250	3:1	100
						2:1	113
						0,7:1	14
Tricholenim	76	+4	$1.0*10^{-4}$	0	300–340	3:1	56,5
						2:1	31,3
						0,7:1	7,5

Note: ΔT designates an increase in melting temperature of DNA samples in the complex with an oligocation; CMC, critical micelle concentration; EM, diameter of particles of the corresponding complexes with plasmid DNA or DNA fragments (electron microscopy data).

* Calculated for the amino groups at pH 7.0.

(1.0×10^{-4} M), correspond to formation of complexes between these compounds and DNA, which contain hydrophobic clusters where pyrene molecules are inserted, and may be regarded as critical micelle concentrations (CMC). There is a good correlation between the CMC value and the decrease in the total positive charge of polar groups of cholenims. Thus, it can be postulates that cholenims bind with DNA to form a hydrophobic coat around the helix, and that the disadvantageous (in terms of energy) contact between hydrophobic cholesterol residues with aqueous environment at certain concentration results in a decrease in solubility of complexes.

Electron-microscopic study showed that the complexes between genomic DNA and the plasmid with cholenims represent spherical particles with a diameter of 100 to 300 nm. Condensation of 4–6 kb DNA fragments and compound II showed that the size of particles significantly varies. The fact that the size of DNA/cholenim particles is large and almost does not depend on the molecular weight of DNA is unusual for a simple micellar structure. Figure 1 (lower field) shows the dependence of the spectral parameter I_3/I_1, which is the most sensitive to hydrophobicity of microenvironment, on the concentration of cholenims at a constant DNA concentration (I_1 and I_3 are the amplitudes of oscillatory lines of emission spectra of the monomeric form of pyrene at 383 and 372 nm).

3.1.2. Gene transfer and delivery

The results of transfection of PC-12 cells with the complexes containing the pCMV-SPORT-β-Gal plasmid and cholenims are summarized in Table 1. The greatest effectiveness of transfection of PC-12 cells was reached when DNA/cholenim

genosomes were used at a ratio of 2:1. However, significant effectiveness of transfection was also observed at DNA/cholenim ratio of 3:1. Similar results were obtained for the dicholenim-based complex. The effectiveness of transfection in the case of DNA/dicholenim genosomes at ratios of 2:1 and 3:1 was considerably higher than at the ratio 0.7:1 and comparable with the effectiveness of transfection for the DNA/cholenim ratio at the ratio 3:1. Tricholenim was much less effective in gene delivery compared to the other two compounds. In this case, the effectiveness of transfection markedly decreased as the proportion of tricholenim in genosomes increased. The effectiveness of transfection of RGGN-1 cells was 30 and 32 pg protein per 10^5 cells for the DNA/monocholenim complex and 14 and 23 pg protein per 10^5 cells for the DNA/dicholenim complex (ratio, 2:1 and 1:1, respectively). Although this index for RGGN-1 cells in general was considerably lower compared to the effectiveness of transfection of PC-12 cells, this finding also supports the fact that monocholenim and dicholenim may be used as gene carriers *in vitro*. However, it should be noted that, in the case of *in vivo* transfection, there might be another relationship between the effectiveness of gene transfer and qualitative and quantitative composition of cholenim-based complexes. Amphiphilic liposomes consisting of phosphatidylcholine and dicholenim at the ratio 1:1 (w/w) were used to transfer the β-galactosidase gene using intravenous injection at the lipid/DNA ratio 1.6:1 (w/w). Sections of organs were incubated with the substrate X-Gal, which in the presence of β-galactosidase is degraded, yielding the bright blue dye indigo. In preparations analyzed, the reporter DNA was expressed predominantly in endothelial cells of pulmonary vessels and in neighboring cells, which provides evidence that vascular endothelial cells are permeable for our complexes (Figure 2).

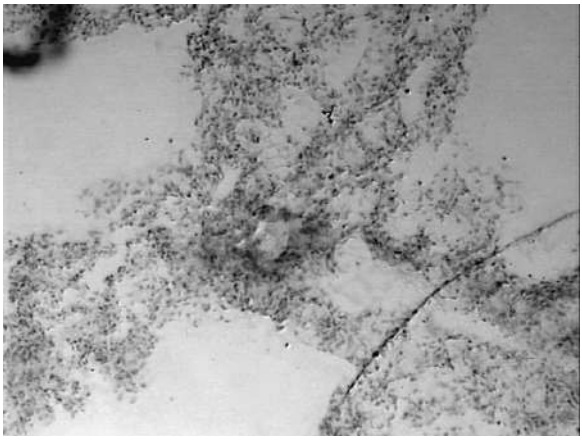

Figure 2. Histochemical preparation of ICR mouse lung after injection into the portal vein of the liver of lipoplexes formed by the pCMV-SPORT-β-Gal plasmid and liposomes PC/DICHOLENIM (1:1). Staining around the blood vessel is the result of degradation of the substrate X-Gal by bacterial β-galactosidase. Magnification, 200; computer processing; AXIOSKOP 20 Carl Zeiss

This distribution pattern is characteristic of cationic liposomes injected intravenously. Thus, the introduction of the cholesterol fragment into the structure of oligoethylene imines improves the characteristics of the corresponding complexes: increases the hydrophobicity/hydrophilicity ratio, stabilizes the lipoplex, and ensures optimal CMC values. Our data confirm the existence of stable DNA/cholenim complexes and electrostatic interaction in them of positively charged groups with negatively charged phosphate groups of DNA, with the deoxyribose phosphate backbone being apparently involved in the stabilization of genosomes. Compounds I–III interact with DNA to form a hydrophobic coat around its double helix. The high effectiveness of DNA/cholenim lipoplexes in gene transfer *in vitro* is probably determined by their complete dissociation in the cytosol before the nuclear membrane, because this ability of lipoplexes is a key characteristic required for transfection [43].

3.2. Glycoclip

3.2.1. Cyto- and genotoxicity

Potential cyto- and genotoxicity of GLYCOCLIP/DOPE and CLIP/PC liposomes were estimated in experiments with a cultured glyal cell line [44], which is very sensitive to any influence, as described earlier [4]. The influence of the former liposomal preparation on the growth (24hrs) of RGNN cells and the DNA synthesis in these cells was studied. The preparations have almost no effect on cell growth at both concentrations used: 6μg/ml (number of cells survived after 24 hrs incubation was 110.5 +/−3.1% (M+/−s) comparing to the control one) and 60μg/ml (98.6+/−9.3%). The influence on DNA synthesis was evaluated as the extent of incorporation of ^{14}C-thymidine into RGNN cell genomic DNA. It had equally essential effect on the DNA synthesis at both concentrations (6 or 60 μg/ml): the values of the DNA synthesis were 58.5+/−7.8% and 66.3+/−9.2% comparing to the control ones, correspondingly. The influence of CLIP liposomes on DNA synthesis in RGGN cells was not so pronounced (in the range of experimental error), as found for the GLYCOCLIP ones. The CLIP/PC liposomal preparation has no effect on either the cell survival, or the DNA synthesis in RGGN cells at 6μg/ml level. The number of cells survived after 24 hrs incubation was 100.0 +/−5.0% comparing to the control, and the value of the DNA synthesis in the cells was 101.8 +/−7.0% comparing to the control one. Only 10-fold dose of CLIP/PC liposomes (60μg/ml) had an effect on the DNA synthesis: 55.5 +/−11.7% ($p < 0.05$) comparing to the control one. CHOLENIM preparation itself appeared to be completely non-toxic at the range of concentrations used [18].

3.2.2. Gene encapsulation and delivery in vitro

Gene transfer activity of the liposomes based on GLYCOCLIP was studied with the commonly used reporter gene transfer system: transfection of pCMV-Luc plasmid into CHO cells followed by gene transfer efficiency testing using luminometer assay [45]. Figure 3 represents data on reporter gene (pLuc) transfer efficiency with liposomal preparations of compounds I and II into CHO cells in comparison with

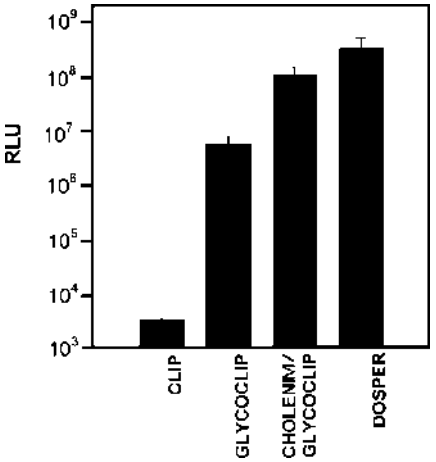

Figure 3. Transfection efficiency of lipoplex and glycolipoplex preparations formed of various cationic lipids, GLYCOCLIP and pCMV-Luc reporter plasmid against CHO cells: 1-CHOLENIM I/GLYCOCLIP V; 2-CLIP IV; 3-GLYCOCLIP VI; 4-Lipofectin; 5-Dosper

corresponding data for commercial gene transfer agent DOSPER. As follows from the results the introduction of a triacetyl-glucose moiety into the structure of a cationic lipid enhances remarkably the transfection: RLU value of GLYCOCLIP/DOPE liposomes equals to 7.10^6 (compare GLYCOCLIP/DOPE and CLIP/PC values).

The GLYCOCLIP-based liposomes' RLU values are only slightly less than those of DOSPER mediated gene transfer. Our data on the inhibition of DNA synthesis in RGGN cells after 24hrs incubation with GLYCOCLIP/DOPE liposomes corresponds to the data testifying to the toxicity of many cationic liposomes during *in vitro* experiments [14]. However, it was demonstrated that the efficiency of gene transfer with cationic liposomes is not directly connected with the degree of their toxicity [46], so one may get high transfection efficiency with the use of gene transfer agents demonstrating a certain toxicity *in vitro*. It is possible that lowering the concentration of GLYCOCLIP/DOPE liposomes used for transfection will help to avoid their influence on DNA synthesis. It cannot be excluded that this effect will not appear during *in vivo* transfection. The fact that GLYCOCLIP/DOPE liposomes don't influence the cell growth at least during the first 24hrs is also promising. Thus, partial glyconylation of polylysine has been shown to increase the efficiency of transfection with its participation, and the conjugation of modified polylysine with a few lactose moieties causes appearance of genosome's specificity to cell surface lectin [47, 48]. A series of amphiphilic dendritic galactosides were synthesized to be used for selective targeting of liposomes to the hepatic asialoglycoprotein receptor [49]. Introduction of carbohydrate moieties into the structures involved in lipoplex formation increases the efficacy and the specificity (hepatocytes) of transfection. Lipoplexes composed of galactosylated peptides demonstrate tropicity to hepatocytes [50].

DOPE and PC represent helper lipids, which enhance transfection efficiency being included into liposomes and lipoplex composition [24,51]. The presence of a helper lipid and the difference between the helper lipids (DOPE or PC) in GLYCOCLIP and CLIP liposomal formulations used can give no strong influence on the gene transfer efficiency in the case of CHO cells, because of the endocytotic way of the lipoplex internalization into this cell line [51]. Therefore the enhanced transfection efficiency of GLYCOCLIP liposomes compare to CLIP liposomes can be explained by the presence of carbohydrate (glucose) moiety in the first one. Introduction of CHOLENIM preparation into glycolipoplex composition facilitates the elaboration of DNA from a lipoplex in perinuclear space. That is the main reason for increasing transfection efficiency of GLYCOCLIP/ CHOLENIM/DOPE liposomes comparing to GLYCOCLIP/DOPE ones. Another reason is the higher value (3.2) of +/− ratio. It appeares that mechanism of gene transfer with the glycolipoplex includes both adsorbic endocytosis usual for lipoplex formulations, and receptor-mediated gene transfer characteristic for carbohydrate ligand-mediated gene transfer. Our results represent one of the first examples of the use of a cationic glycolipid, its liposomal formulations, and genosomes/lipoplexes composed of GLYCOCLIP as gene transfer agents. We believe that glycocationic lipids of this type will be effective especially for *in vivo* studies due to the affinity of carbohydrate structures to the cell surface and the vessell's endothelium as well.

3.3. Glycolipid

3.3.1. Gene delivery in vivo

The first stage in the study of the effectiveness of gene transfer using liposomes containing phosphatidylcholine and GLYCOLIPID VI included the determination of the pattern of distribution of ^{14}C-adenosine-labeled eukaryotic DNA in mouse organs. The maximal DNA level (recalculated per gram tissue) was detected in the kidneys (6000–8000 cpm per gram) and liver (4000 cpm per gram). Note that the content of ^{14}C–DNA in the liver was three times greater than in the lungs (Figure 4). It is known that intravenous injections of the complexes of cationic liposomes with DNA are usually characterized by "the effect of the first passage," i.e., the majority of injected liposome with bloodstream get from the heart to the lungs [52]. When using liposomes containing GLYCOLIPID VI, this effect was not observed. In our experiments, we observed certain affinity of the complex of these ^{14}C-DNA-containing liposomes for the liver and kidneys.

The maximal level of ^{14}C-DNA in the kidneys is probably due to the fact that it might have been eliminated as early as 24 h after injection, because kidneys are excretory organs. Then, we studied the expression of the β–galactosidase gene in mouse organs in the case of delivery of the pCMV-SPORT-β-Gal plasmid (100 μg) in the complex with mixed liposomes consisting of phosphatidylcholine, GLYCOLIPID, and dicholenim (160 μg).

When this lipoplex was injected into the portal vein, the *Lac Z* gene was expressed predominantly in hepatocytes. However, despite the presence on the surface of

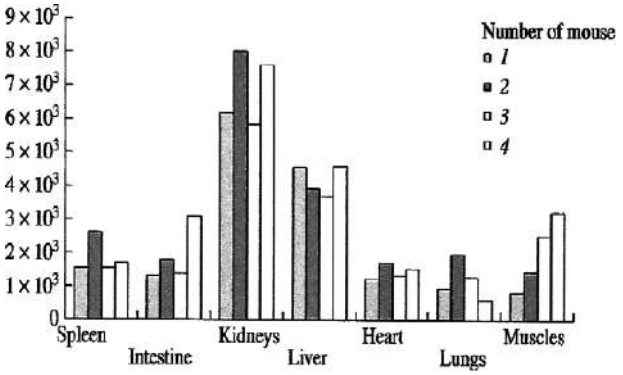

Figure 4. Distribution of the lipoplex formed by ^{14}C-adenosine-labeled DNA and liposomes comprised of phosphatidylcholine, lactosolipid, and dicholenim in mouse organs after injection into the portal vein of the liver (cpm/min per gram organ; $n = 4$)

liposomes of a lactose residue, which exhibits affinity for the lectin located on the surface of hepatocytes, the degree of expression was low, and expression was observed mostly in epithelium of blood vessels and in the immediate vicinity of them. This fact is indicative of a low permeability of tissues for such complexes. A more long-term incubation with the substrate led to appearance of the dye indigo in the form of small (less than 1 μm) bright blue granules both in the control and experimental liver section. It can be assumed that this phenomenon may be accounted for by location of the endogenous enzyme in lysosomes or other compartments of the cytoplasm of hepatocytes. In the lungs and spleen, the level of expression of the *Lac Z* gene (reaction with X-Gal) was high (Figure 5).

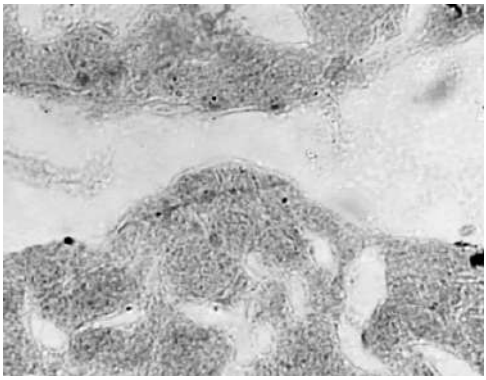

Figure 5. Histochemical assessment of expression of the *LacZ* gene (the pCMV-SPORT-β-Gal plasmid) in the ICR mouse spleen after injection of the lipoplex based on the pCMV-SPORT-β-Gal plasmid and liposomes composed of phosphatidylcholine/GLYCOLIPID/dicholenim (ratio 1:1.6, w/w) into the portal vein of the liver. Dark areas indicate the sites of the highest expression (magnification ×200)

A high endogenous activity of β-galactosidase was detected in the kidneys, which hampered the assessment of the effectiveness of the exogenous enzyme. For quantitative estimation of expression of the β-galactosidase gene in mouse organs after injecting the complex of the plasmid with the liposomes consisting of phosphatidylcholine, lactosolipid, and dicholenim, the activity of this enzyme in tissues was determined spectrophotometrically.

The maximal activity of the enzyme was observed in the spleen (data are not shown), and equal activity was detected in the lungs and liver. A high level of endogenous activity of β-galactosidase in some organs hampers quantitative assessment of expression. Thus, the results of this study showed that GLYCOLIPID VI containing a lactose residue, which was used in the form of liposomes to transfer ^{14}C-adenosine-labeled or plasmid DNA, determined the affinity of lipoplexes for kidney, liver, and spleen tissues. The effect of the first passage, characteristic of cationic complexes, was not observed when ^{14}C-DNA was injected in the complex with liposomes comprised of phosphatidylcholine, lactosolipid, and dicholenim, was considerably decreased when the plasmid was injected in the complex with liposomes comprised of phosphatidylcholine, lactosolipid, and dicholenim. In the last case, the expression of β-galactosidase was maximum in the spleen. GLYCOLIPID VI, which determines the affinity of lipoplexes for tissues, as well as glycolipids on the whole, is a prospective tool for designing on its basis of nonviral vectors of a new generation for targeted gene delivery to tissues.

3.4. Modified Chitosan (mCHIT)

We used natural polycationic polysaccharide, chitosan, which can usually be prepared by deacetylation of chitin – linear poly– (N-acetyl-glucosamine) to gene transfer against cultured cell lines. Chitosan macromolecule represents linear polymer of glucosamine, part of whose primary amino-groups (normally 5–20%) are still acetylated. It is well–known that chitosan being one of the most widespead biomass represents non-toxic, biocompatible biopolymer [53, 54], which is suitable for gene delivery purpose [14–16],[55–57]. However, in our preliminary study we also worked to get reporter gene transfer of transformed cells using non-modified chitosan preparations. After the data on efficient transfection of 3T3 and HepG2 cells with complexes of plasmid DNA and polyethylenimine (PEI) were published [41], Dr. G.G. Krivtsov decided to introduce the secondary and tertiary aminogroups into chitosan structure to use modified chitosan preparations (mCHIT) for gene transfer. The matter is that PEI contains the secondary amino groups along with the primary and tertiary ones. He synthesized the chitosan preparation, containing N-ethyl- (the secondary one) and N,N-dimethyl amino (the tertiary one) groups to facilitate ionic interaction of chitosan with the DNA and to increase transfection efficiency against different transformed cell lines, especially the suspension blood cell ones. The latter topic is an acquit area of research now, and is also very important for development of non-viral delivery systems for *ex vivo* gene therapy of variety of genetic diseases and cancer pathologies [58–60].

A number of papers on the usage of different chitosan preparations and nanospheres as transfection agents have been published [14–16],[55–57]. It was reported that unmodified chitosan has ability to condense DNA and form small discrete particles [57]. They can transfect HeLa cells (β-gal [14] or Luc [57] genes) independently of the presence of 10% fetal serum. Gene expression gradually increased with time, being at 96 hours 10 times more efficient, than polyethylenimine [57]. It was suggested that non-ionic interactions between chitosan macromolecule and cell surface might play an important role in chitosan-mediated transfection [56]. pH-sensitive endosomolytic peptide enhanced gene expression in COS-1 cells by factor 4, but during in vivo experiments on rabbits (intestine and colon) gene expression appeared to be still low [15]. Hydophobically modified chitosan (containing five deoxycholic acyl moieties per 100 anhydroglucose units) was prepared, its aggregates being 162 +/−18 nm in diameter [14]. Transfection of COS-1 cells using self-aggregates/plasmid DNA complexes at +/− charge ratio 4 was reported. Nanospheres composed of cDNA and gelatin or chitosan (200–750 nm) were used for in vitro transfection, efficiency being lower than in the case of lipofectamine-mediated and Ca–phosphate ones [55]. Method for oral DNA delivery with N-acetylated chitosan was reported [16].

All groups that have been working with chitosan preparations as gene delivery systems used non-N-alkylated chitosan samples containing only primary amino-groups along with N-acetyl moiety. These preparations usually represent particles of small size (80 nm) as measured by variety of techniques [14–16],[55–57]. Chitosan preparation hydrophobized with deoxycholeic acyl moieties (5%) forms self-aggregates of medium size (200 nm) itself. Nanosheres formed of chitosan are even bigger: 200–700 nm [14, 15],[55–57]. These chitosan preparations are characterized with ability to form DNA aggergates with supercoiled plasmid like cationic liposomes and other polycations usually do [39]. The size of these aggregates is even bigger. All known chitosan preparations tested for gene delivery in vitro and/or in vivo are far from being as effective as any commercial gene transfer ones, e.g. PEI™. We usually obtained low transfection efficiency values with non-modified chitosan preparations. The reason for these, by our opinion, is unsufficient ability of polysaccharide bearing only primary glucosamine moieties and forming big aggregates to be as stable as to survive in endosome-lyzosomal complex. There are very few reasons to add any hydrophobic moieties (like choleic acid) into glucosamine residue, as chitosan biomacromolecule having well-known hydrophobic properties is able to bind 10-fold amount (w/w) of fat molecules [61].

Transfection was carried out with two various reporter gene plasmids: pEQ176 (β-galactosidase) and pCSEAP (secreted alkaline phosphatase) (under IE CMV promoters) against transformed cell lines with different ethiology: three adherent cell lines (MeWo, HeLa, and HOS-1) and two suspension cell cultures (U937 and Jurkat) as well. Transformed blood cell lines had been cultured by conventional methods. A number of transfection techniques (Ca-phosphate; pH-sensitive amphiphylic liposomes/Ca^{2+}- and PEI-mediated gene transfer) were used for comparing results of mCHIT glycoplex transfection. Glycoplex composition was choosen with mCHIT

nitrogen/DNA phosphorus ratio equalling to 10:1 which corresponds to +/− charge ratio 8. At other ratio values we got a decrease of efficacy by decreasing the ratio, and an increase of toxicity by increasing the ratio in the case of both mCHIT and PEI (data are not shown).

It follows from data on efficiency of transfection of pEQ176 plasmid into selected transformed cell lines, that mCHIT and PEI preparations demonstrated maximum transfection activity (up to 100%) for human melanoma cell line (MeWo). However, gene transfer efficacy appeared to be lower for HeLa and HOS-1 cell lines: from 2 to 5% of bacterial β-gal gene expressed cells, which is in the connection with the results of liposomes/Ca ions-mediated [28, 39] and Ca-phosphate transfection method [38]. Gene transfer activity of mCHIT preparation against immortalized premonocytes (U937) (10% of cells are expressing bacterial β-galactosidase gene) was higher than PEI activity by factor 10. mCHIT demonstrated also the ability to transfect transformed lymphocyte cell line (Jurkat), which is very difficult to be transfected, 10 fold higher then PEI (0.01% and 0.001% β-gal expressing cells, correspondingly).

The similar results were obtained in the case of transfection experiments with another plasmid, pCSEAP, containing secreted alkaline phosphatase gene with one exception. We were not able to detect expression of SEAP gene after transfection with Ca-phosphate precipitates. PEI and mCHIT preparations showed the same level of SEAP gene transfer activity against adherent cell cultures. Lowest level of transfection was found for HeLa cells, twice higher - for HOS-1 cells, and 8 fold higher – for melanoma cells MeWo. Ca-phosphate precipitate transfection demonstrated the same level of gene transfer efficiency for all adherent cell lines, as mCHIT and PEI-mediated showed in the case of HeLa cells. Amphiphilic PC liposomes in the presence of Ca ions (>15 mM) [28, 39] were active only in the case of MeWo, but twice more effective than Ca-phosphate technique. Glycoplex preparation was twice more effective against U937 cells higher than PEI. pH-sensitive PC liposomes/Ca^{2+} also showed sufficient transfection in the case of U937 cells (6 fold lower than mCHIT).

Remarkable gene transfer properties of mCHIT glycoplex preparation, which contains secondary and tertiary amino groups, compare to PEI (one of the most powerful gene transfer agent now) appear to be connected, first, with enhanced endocytosis of glycoplex particles through mono- and lymphocyte cytoplasmatic membrane (probably, receptor-mediated transfer). N-acetylglucosamine residues (N-AGA), which are normally present in any commercial chitosan preparation, can be considered as the most probable candidate for a ligand in receptor-mediated endocytosis. Corresponding fraction of immunoglobulins was found in patient's blood. Those proteins are also exposed on cytoplasmatic membrane, their nature being different for various cell types. Second, mCHIT bearing secondary and tertiary amino groups and being higher positively charged can form more tough and stable complexes permitting plasmid DNA to survive through endosome-lyzosome complexation [43]. Third, mCHIT preparations, being not so highly positively charged as quarternary cationic lipids, provide the type of DNA complexation with

mCHIT which resembles the interaction of DNA with PEI and facilitate an easy escape of DNA from the complex at nuclear membrane or/and perinuclear space to be transcribed in the nuclei [62].

The mCHIT preparation demonstrated the highest gene transfer activity for all types of cells used and for both of β-Gal and CSEAP plasmids. It appears that the data obtained reflect a difference in value and structural homogenity of negative potential/charge of cytoplasmatic membrane of transformed cells of different tissue genesis. This issue can be supported by transfection efficiency data for two suspension cultures of white blood cells. The most important result we got is the comparatively high efficiency of transfection of suspension cell lines, especially for Jurkat transformed lymphocyte cell line, which is usually very difficult to be transfected with any delivery sytem.

Gene transfer with amphiphilic liposomes containing pH-sensitive agent α-tocopherol ester of succinic acid and complexed with plasmid DNA in the presence of high concentration of Me (II) ions (20 mM Ca ions and higher concentrations) [28,39,40] appeared to be even more active than Ca-phosphate precipitate technique. The former one is promising for targeted delivery in combination with the use of addressing groups. Reporter genes can be easily substituted in GLYCOPLEX by therapeutic genes, e.g. suicide genes, ADA gene, because of still big size (up to 8–10 kb) for the purpose of *ex vivo* gene therapy.

4. CONCLUSIONS

Introducing the cholesterol moiety into the structure of oligoethylene imines improves the characteristics of the corresponding complexes: increases the hydrophobicity/hydrophilicity ratio, stabilizes the lipoplex, and ensures optimal CMC values. The existence of stable DNA/CHOLENIM complexes and electrostatic interaction of positively charged groups with negatively charged phosphate groups of DNA are confirmed, the deoxyribose phosphate backbone being apparently involved in the stabilization of genosomes. CHOLENIMS interact with DNA to form a hydrophobic coat around its double helix. CHOLENIM-based lipoplex provides reporter DNA retard circulation in blood. Mono-, di-, and tri CHOLENIMS-based lipoplexes are characterized by various tissue distributions in animal experiments.

The enhanced transfection efficiency of GLYCOCLIP V liposomes compare to CLIP liposomes can be explained by the presence of carbohydrate (glucose) moiety in the first one. Introduction of CHOLENIM preparation (as helper lipid) into glycolipoplex composition facilitates the elaboration of DNA from a lipoplex in perinuclear space. It appeares that mechanism of gene transfer with the glycolipoplex includes both adsorbic endocytosis usual for lipoplex formulations, and receptor-mediated gene transfer characteristic for carbohydrate ligand-mediated gene transfer. We believe that glycocationic lipids of this type will be effective especially for *in vivo* studies due to the affinity of carbohydrate structures to the cell surface and the vessell's endothelium as well.

It is shown that GLYCOLIPID VI containing a lactose residue, which was used to form liposomes for gene delivery into tissues of ^{14}C-adenosine-labeled or plasmid DNA, expessed the affinity of corresponding lipoplexes for kidney, liver, and spleen tissues. GLYCOLIPID VI is a prospective tool for designing on its basis of nonviral vectors of a new generation for targeted gene delivery to tissues. The mCHIT preparation demonstrated high gene transfer activity (β-Gal and CSEAP plasmids) against both monolayer and suspension cell lines.

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