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Synthesis and anti-HIV activity of thiocholesteryl-coupled phosphodiester antisense oligonucleotides incorporated into immunoliposomes

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Abstract

Encapsulation of oligonucleotides in antibody-targeted liposomes (immunoliposomes) which bind to target cells permits intracellular delivery of the oligonucleotides. This approach circumvents problems of extracellular degradation by nucleases and poor membrane permeability which free phosphodiester oligonucleotides are subject to, but leaves unresolved the inefficiency of encapsulation of oligonucleotides in liposomes. We have coupled oligonucleotides to cholesterol via a reversible disulfide bond. This modification of oligonucleotides improved their association with immunoliposomes by a factor of about 10 in comparison to unmodified oligonucleotides. The presence of cholesteryl-modified oligonucleotides incorporated in the bilayer of liposomes did not interfere with the coupling of the targeting protein to the liposome surface. Free or cholesterol coupled oligonucleotides associated with liposomes and directed against the *tat* gene of HIV-1 were tested for inhibition of HIV-1 proliferation in acutely infected cells. We demonstrate that the cholesteryl-modified as well as unmodified oligonucleotides acquire the target specificity of the antibody on the liposome. Their antiviral activity when delivered into cells is sequence-specific. The activity of these modified or unmodified oligonucleotides to inhibit the replication of HIV was the same on an equimolar basis (EC_{50} around 0.1 μM). Cholesterol coupled oligonucleotides thus offer increased liposome association without loss of antiviral activity.

Key words: HIV-1; Oligonucleotide; Liposome

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1. Introduction

Use of antisense oligonucleotides constitutes a promising therapeutic approach in viral, malignant and inflamatory diseases since they have been shown to be powerful inhibitors of gene expression (Crooke et al., 1993). However, native oligonucleotides are susceptible to nuclease degradation (Akhtar et al., 1991; Shaw et al., 1991) and their uptake by cells is inefficient (Loke et al., 1989; Yakubov et al., 1989).

An approach to circumvent these problems has been to covalently bind native (phosphodiester) or modified (phosphorothioate) oligonucleotides to phospholipid (Shea et al., 1990) or other hydrophobic residues (Kabanov et al., 1990; Saison-Behmoaras et al., 1991), including cholesterol (Boutorine et al., 1989; Letsinger et al., 1989; Krieg et al., 1993). Cholesterol modification has been shown to increase both the cell association and activity of phosphodiester and phosphorothioate oligonucleotides (Boutorine et al., 1989; Letsinger et al., 1989; Boutorine et al., 1993; Krieg et al., 1993). These compounds have been shown to be taken up, at least in part, by the LDL receptor (de Smidt et al., 1991; Krieg et al., 1993). The mechanism of action of cholesteryl-phosphodiester oligonucleotides is unclear since sequence non-specific effects were found in an HIV model (Letsinger et al., 1989), in which the non-coupled oligonucleotide was reported to act in a sequence-specific manner (Zamecnik et al., 1986; Goodchild et al., 1988). Lack of specificity is presumably caused by the increased hydrophobicity associated with coupling to cholesterol or other hydrophobic residues in several viral and cellular models (Shea et al., 1990; Boutorine et al., 1993). Other authors, however, have reported that cholesteryl-modified oligonucleotides act in a sequence specific manner (Krieg et al., 1993; Svinarchuk et al., 1993).

Another means of bypassing problems of cellular membrane permeability, stability to nucleases and also to confer a cell target specificity on oligonucleotides is the use of antibody-targeted liposomes (immunoliposomes) as a transport system. Immunoliposomes have been shown to enter into lymphoid cells by an endocytic pathway and release the encapsulated product intracellularly (Leserman et al., 1990; Machy et al., 1990). The efficiency of this process depends on the physiology of the targeted surface molecule, the type of cell, and the liposome size (Machy et al., 1983; Matthay et al., 1989; Suzuki et al., 1991). Phosphodiester oligonucleotides encapsulated in liposomes have been shown to be protected from extracellular degradation, to have enhanced delivery into cells and to specifically inhibit the expression of the targeted viral genes (Leonetti et al., 1990; Thierry et al., 1992; Zelphati et al., 1993).

The liposome technology presents a major inconvenient feature, however, which is the poor efficiency of encapsulation of agents in liposomes sufficiently small to be taken up by target cells. We have consequently investigated the biological activity of oligonucleotides covalently coupled to cholesterol via a potentially bioreversible disulfide linkage which have improved incorporation into liposomes (Oberhauser and Wagner, 1992). The advantage of this reagent is that it offers the potential both of increasing inclusion of the reagent in the lipid bilayer and permitting release of the associated oligonucleotide in the reducing environment of endocytic vesicles (Feener et al., 1990; Oberhauser and Wagner, 1992). 2′-*O*-methyl-oligoribonucleotide thiocholesterol conjugates have been shown to be effectively incorporated into liposomes and to have

increased uptake by cells (Oberhauser and Wagner, 1992), but their capacity to exert antisense effects has not been reported.

In the present study, we have synthesized oligodeoxyribonucleotide thiocholesterol conjugates complementary to the *tat* gene and evaluated their incorporation into antibody-targeted liposomes and their anti-HIV-1 activity. These experiments were done in comparison with unmodified oligonucleotides encapsulated in immunoliposomes. We demonstrate that the cholesteryl-modified oligonucleotides associate efficiently with liposomes, acquire the target specificity of the antibody on the liposome and are specific in their antiviral activity when delivered into cells.

2. Materials and methods

2.1. Synthesis and purification of unmodified and 3'-amino-modified oligodeoxynucleotides

All syntheses were performed with an automated DNA synthesizer (Applied Biosystems, model 380-B). Unmodified oligonucleotides (*n*-anti-*tat* and *n*-scrambled-*tat*) and 3'-amino-modified oligonucleotides were synthesized by Genset (Paris, France) by the standard phosphoramidite procedure (Uhlmann et al., 1990). All purifications were performed by reverse-phase high-performance liquid chromatography. The target region chosen in the HIV-1 genome was the translation initiation region of the *tat* gene. Antisense oligonucleotide (*n*-anti-*tat*) was a 16-mer with the following sequence: 5'-CTAGGATCTACTGGCT-3'. To verify the sequence specificity of the antisense oligonucleotides we have used a scrambled sequence of the same base composition for unmodified normal oligonucleotide 16-mer *n*-scrambled-*tat*: 5'-TGCCGTCGAAG-TATTC-3'.

2.2. Synthesis of thiocholesterol-modified oligodeoxynucleotides

The synthesis was performed by a modification of the technique described in (Oberhauser and Wagner, 1992). Minor changes in the isolation procedure had to be made due to the reduced solubility of the thiocholesterol-modified 16-mers in water.

A solution of 970 μg 3'-amino-modified anti-*tat* oligodeoxynucleotide in 1 ml 50 mM HEPES buffer (pH 7.9) was treated with a solution of 5.7 mg (100 equivalents) of *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, Pharmacia) in 400 μl ethanol for 4 h at room temperature. The mixture was then subjected to gel filtration (Sephadex G25 PD10 column, Pharmacia) with water as eluent. The dithiopyridine-modified oligonucleotide (950 μg) was dissolved in 800 μl of a solution containing 30 mg 3,3,6,9,9-pentamethyl-2,10-diazabicyclo [4.4.0] dec-1-ene (PMDBD "Heinzer base", Fluka) acetate salt (pH 6.5), in methanol/water (15:85). The solution was subjected to gel filtration on a Sephadex G25 PD10 column (15% aqueous methanol). The oligonucleotide-containing fractions (790 μg) were concentrated in a Speedvac to remove methanol, lyophilized and then dissolved in 2 ml of an anhydrous methanolic 60 mM PMDBD acetate buffer (pH 8.5). To this solution was added 3 mg thiocholesterol (Sigma) in 3 ml

dichloromethane. The reaction mixture was kept under argon for 18 h at room temperature. The solution was concentrated to about 1 ml in the Speedvac and extracted 10 times with 400 μl of 80 mM triethylammonium acetate in methanol/water (20:80). The aqueous extracts were combined and fractionated in five portions by reverse-phase HPLC (Nucleosil RP-18 column; buffer A: 40 mM triethylammonium acetate (pH 7); buffer B; acetonitrile; flow; 0.9 ml/min; gradient: 0–100% B) yielding 237 μg of the thiocholesterol-modified oligodeoxynucleotide in 11 ml buffer, eluting at a concentration of 63–65% acetonitrile. A thiocholesterol-modified oligonucleotide with the scrambled *tat* sequence was prepared in the same manner.

2.3. End-labelling of unmodified and modified oligodeoxynucleotides

Unmodified oligonucleotides were trace-labeled with [γ-^{32}P]ATP (Amersham) at the 5′ end by T4 polynucleotide kinase (Gibco, BRL) according to the supplier's instructions. The labeled oligonucleotide was then purified on a Elutip-D column (Schleicher and Schuell). Thiocholesterol-modified oligodeoxynucleotides were 5′-^{32}P-labeled using an incubation buffer with a minimum amount of reducing agents. Five pmol of oligonucleotide was incubated in 55 μl buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol) with 20 units T4 polynucleotide kinase and 10 pmoles of [γ-^{32}P]ATP for 40 min at 37°C. The labeled modified-oligonucleotide was then purified by gel filtration on Sephadex G-25 (50 mM Hepes (pH 7.3)–20% Ethanol) (Pharmacia) after addition of 50 pmol unlabeled oligonucleotide to minimize unspecific absorption.

2.4. Preparation of liposomes with unmodified oligodeoxynucleotides

Liposomes composed of 80 μmol total lipid (64 mol% dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL, USA), 35 mol% cholesterol (Sigma), and 1 mol% dipalmitoylphosphatidylethanolamine (Sigma) modified with SPDP (Pharmacia) (Leserman et al., 1980), were prepared with the aqueous phase (total volume: 1 ml) composed of a solution of oligonucleotides at 8 mg/ml in 145 mM NaCl/10 mM Hepes (pH 7.45), or with this buffer alone to produce "empty" liposomes as control. The solution was alternately frozen in liquid nitrogen and thawed by heating to 55°C five times with intermittent vortex mixing. The liposomes thus produced were passed through an "Extruder" (Lipex Biomembranes, Vancouver, Canada), mounted with 0.1 μm polycarbonate filters (Nucleopore, Pleasanton, CA, USA) at 55°C (Hope et al., 1985). Size determinations performed for liposomes of similar composition show liposomes formed by this technique to be primarily unilamellar, and their diameter corresponds closely to the pore size of the Nucleopore filters used. Liposomes were covalently coupled to *Staphylococcus aureus* Protein A (Pharmacia), as described (Leserman et al., 1980). Uncoupled Protein A and unencapsulated products were separated from liposomes on a Sepharose 4B (Pharmacia) column. Liposomes were sterilized by filtration through 0.45 μm Gelman filters. Liposomes always contained in their aqueous space oligonucleotides at their original concentrations. The final concentration of oligonucleotides in the preparation was determined by the use of ^{32}P

end-labeled oligonucleotide. Liposomes containing buffer were diluted to equivalent phospholipid concentrations, as determined by the method of Stewart (1980).

2.5. Incorporation of thiocholesterol-modified oligodeoxynucleotides into liposomes

20 μmol total lipid (dissolved in chloroform/methanol) described above were mixed with the aqueous phase (water/methanol, total volume: 0.5 ml) composed of a solution of thiocholesterol-modified oligodeoxynucleotides at 0.4–0.6 mg/ml, or with water/methanol alone to produce "empty" liposomes as control. This preparation was evaporated under nitrogen gas and lyophilized overnight. The dry lipidic film was then mixed with the aqueous phase (145 mM NaCl/10 mM Hepes (pH 7.45), total volume: 0.8 ml), to produce liposomes. The production of unilamellar vesicles and the covalent coupling to Protein A were the same as described above. The final concentration of oligonucleotides in the preparation was determined by the use of ^{32}P end-labeled oligonucleotide. Liposomes containing buffer were diluted to equivalent phospholipid concentrations, as determined above.

2.6. Treatment of liposomes by DNase I

A part of each liposome preparation (10 000 cpm/0.2 ml) were incubated alone or with DNAse I (50 μg/ml, 10 mM $MgCl_2$) 40 min at 25°C. After the treatment, liposomes and released ^{32}P were separated by gel filtration on Sepharose 4B columns (Pharmacia) and analyzed by Cerenkov counting.

2.7. Antibodies

The target specificity of antibodies used in this study are: the human major histocompatibility complex (MHC)-encoded HLA (Human Leukocyte Antigen)-B and C molecules for antibody B1.23.2 (Rebaï et al., 1983) and the mouse (MHC)-encoded H2-K molecule for antibody H100.5/28 (Lemke et al., 1979). This antibody does not bind to human cells and was used as a control. Both are mouse $IgG2_{a,\kappa}$ monoclonal antibodies purified from supernatant fluids of cultured hybridoma cells on Protein A-Sepharose CL-4B (Pharmacia) affinity columns. The use of these protein A-binding monoclonal antibodies in conjunction with protein A-bearing liposomes has been reported (Machy et al., 1982).

2.8. Cells and virus

CCRF-CEM cells (CEM), a T lymphoblastoid cell line, were obtained from American Type Culture Collection, Rockville, MD, USA (Ref. CCL 119). HIV-1 (BRU) (Barré-Sinoussi et al., 1983) provided by L. Montagnier (Institut Pasteur, Paris, France), was maintained and amplified on CEM cells. Uninfected and infected cells were cultivated in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 2 mM sodium pyruvate, 2 mM non essential amino acids and antibiotics at 37°C in a 5% CO_2 atmosphere.

2.9. Antiviral assay in acutely infected CEM cells

CEM cells, at a concentration of 2×10^6 cells/ml, were inoculated with 1000 $TCID_{50}$ (50% tissue culture infectious dose) of virus and incubated for 30 min at 4°C in Eppendorf tubes, in a total volume of 1 ml. Cells were washed, diluted to 2×10^5 cells/ml and incubated at 37°C in the presence or absence of 20 μg/ml of antibody and various concentrations of oligonucleotides encapsulated in liposomes, in a total volume of 500 μl, in 24 well tissue culture plates. At day four, cells were split and incubated in fresh medium without readdition of oligonucleotides until day seven, which represents the peak of the reverse transcriptase (RT) activity. At this time samples were removed to determine RT activity as described previously (Rey et al., 1984) and p24 expression as described below.

2.10. Determination of p24 expression

The assay is a twin-site sandwich ELISA in which p24 antigen is captured from a detergent lysate of virions onto a polyclonal anti-p24 antibody (Ref. D7320, Aalto Bioreagents, Dublin, Eire) adsorbed on a solid phase support (MaxiSorp, Nunc, Roskilde, Denmark). Bound p24 was detected with an alkaline phosphatase-conjugated anti-p24 monoclonal antibody (Ref. EH12E1, MRC Reagent Programme) and the AMPAK ELISA amplification system (DAKO, France).

3. Results

3.1. Encapsulation efficiency and conformational study of liposome-associated unmodified and thiocholesterol-modified oligonucleotides.

Phosphodiester and 3′-thiocholesteryl modified phosphodiester oligonucleotides synthesized as described in Section 2 were 5′-end labeled with ^{32}P and encapsulated or incorporated into small unilamellar antibody-targeted liposomes (100 nm diameter). Their incorporation or encapsulation efficiency were determined by comparing the radioactivity added to the lipid film at the start of the liposome preparation and the radioactivity recovered after the antibody-targeted liposomes were formed and sterilized. For free phosphodiester oligonucleotides we typically obtained 2.5–3.5% encapsulation efficiency. Encapsulation varies with the quantity of lipid used, since this determine the number of liposomes formed. The number of molecules encapsulated per liposomes depends on their solubility in the aqueous phase. In this case, we obtained between 85–160 oligonucleotide molecules per liposome based on an estimate of 7.2×10^{12} liposomes of 0.1 μm diameter per μmol of phospholipid. On the other hand, 20–30% of cholesterol-coupled oligonucleotides were incorporated in the liposomes, representing an increase of efficiency by a factor of about 10. With these liposomes we obtained 42–70 oligonucleotide molecules per liposome when 1% of the cholesterol used for the DPPC/cholesterol mixture from which the liposomes were formed consisted of cholesterol coupled to oligonucleotides. The number of liposome-associated molecules was

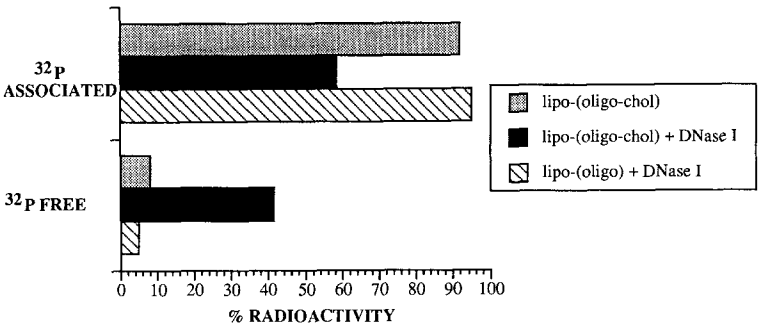

Fig. 1. Treatment of liposomes by DNase I. ^{32}P-labeled unmodified and cholesterol-modified oligonucleotides incorporated or encapsulated with immunoliposomes were treated or not with DNase and subjected to gel filtration as described in Section 2. ^{32}P associated with liposomes or liberated by treatment were analyzed by Cerenkov counting and results were expressed as % of cpm recovered after filtration.

two times lower with modified oligonucleotides than for free oligonucleotides, but these were prepared at a 4-fold lower lipid concentration and using 15–22 fold fewer oligonucleotides. Thus, the number of cholesterol bound oligonucleotides per liposome may be augmented by increasing the cholesterol-oligonucleotide content of the preparation.

The disulfide bond binding the cholesterol and oligonucleotide did not interfere with the efficiency of coupling Protein A to the liposomes (which requires formation of a disulfide bond between SPDP-modified phosphatidylethanolamine in the liposomes and Protein A), since coupling efficiency was the same in the presence and absence of the thiocholesterol-modified oligonucleotides.

Given that cholesteryl-modified oligonucleotides were expected to be incorporated in the bilayer of liposomes, if the liposomes are unilamellar about 50% of the oligonucleotides should be directed toward the inside and 50% toward the outside of the liposomes, whereas unmodified oligonucleotides should be 100% encapsulated in the aqueous phase of liposomes as previously suggested (Leonetti et al., 1990; Akhtar et al., 1991; Oberhauser and Wagner, 1992).

To verify this hypothesis, we incubated liposomes with DNase I and after separation by gel filtration measured the radioactivity that was still liposome-associated (excluded from Sepharose 4B) or liberated by treatment. Results presented in Fig. 1 show that after DNase I action, 58% of radioactivity remained associated with liposomes and 42% was free. In contrast, with lipo-(oligo) 94.5% of the radioactivity was recovered associated with liposomes. This confirms the result observed for alkaline phosphatase cleavage of 2′-*O*-methyl-oligoribonucleotide thiocholesterol conjugates (Oberhauser and Wagner, 1992).

3.2. Antiviral effects of oligonucleotides encapsulated or incorporated in immunoliposomes on acutely infected cells

In previous studies, we and others have shown that phosphodiester oligonucleotides free in solution were unable to inhibit HIV replication (up to a concentration of 50 μM)

because their degradation by extracellular nucleases is rapid and their uptake relatively poor (Matsukura et al., 1987; Kim et al., 1991; Kinchington et al., 1992; Zelphati et al., 1993). To protect oligonucleotides from nuclease action and to concentrate them at the cell surface we encapsulated oligonucleotides or incorporated oligonucleotides coupled to cholesterol in liposomes and evaluated their antiviral activity.

In these experiments, we used liposomes coupled to protein A which binds with high affinity to the Fc region of several antibody classes including the mouse $IgG2_a$ antibodies used here. The targeted cell surface determinant selected was the HLA class I molecule since we have previously reported the fixation and internalization of fluorescent, methotrexate- or oligonucleotide-containing liposomes by human T cells when liposomes were targeted to this determinant (Suzuki et al., 1991; Zelphati et al., 1993).

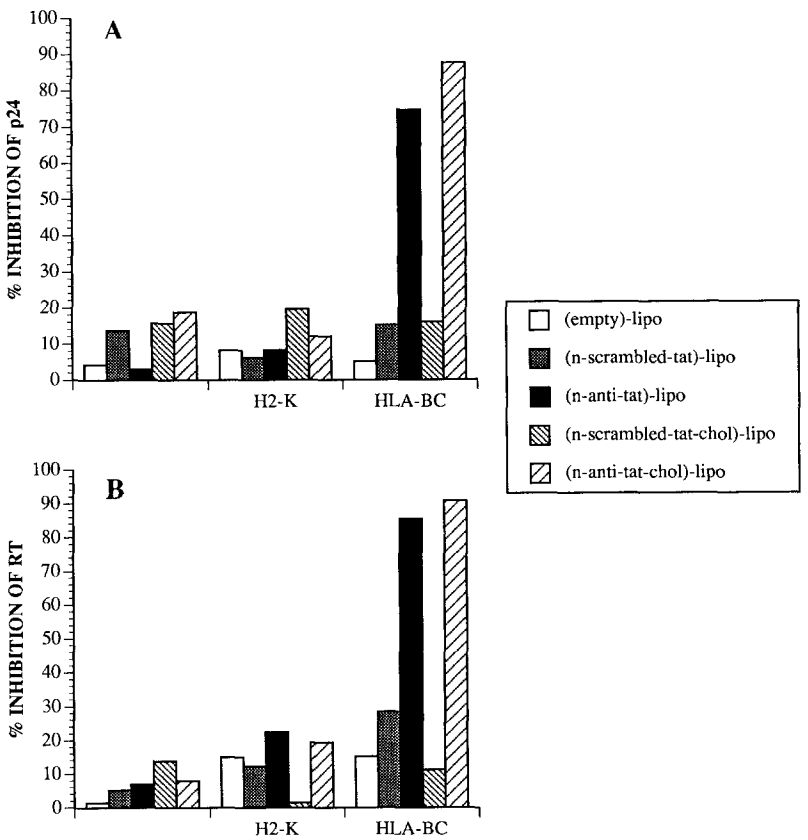

Fig. 2. Antiviral effects ot unmodified and cholesterol-modified oligonucleotides encapsulated in immunoliposomes on acutely HIV-1-infected cells. CEM cells were inoculated with HIV-1. Oligonucleotides encapsulated in liposomes were subsequently added to some wells, at a final concentration of 0.25 μM, in the presence (as specified), or not (-), of antibodies. The total incubation time post infection was 7 days. The RT activity and p24 expression was determined in the supernatants. The data are given as percent of inhibition of p24 expression (**A**) and RT activity (**B**), compared with the infected controls. The means of three separate experiments are given. The S.D. did not exceed 20% of this mean.

Oligonucleotides encapsulated or incorporated in immunoliposomes targeted to HLA class I molecules by specific antibody strongly blocked (at an oligonucleotide concentration of 0.25 μM) the expression of the viral protein p24 and the activity of the RT (Fig. 2). This inhibition was sequence specific since antisense *tat* oligonucleotides were active but both free and cholesterol-coupled scrambled sequence of the same base composition were without effect. Both preparations were very similar in their efficiency since (*n*-anti-*tat*)-lipo and (*n*-anti-*tat*-chol)-lipo targeted to acutely infected CEM cells

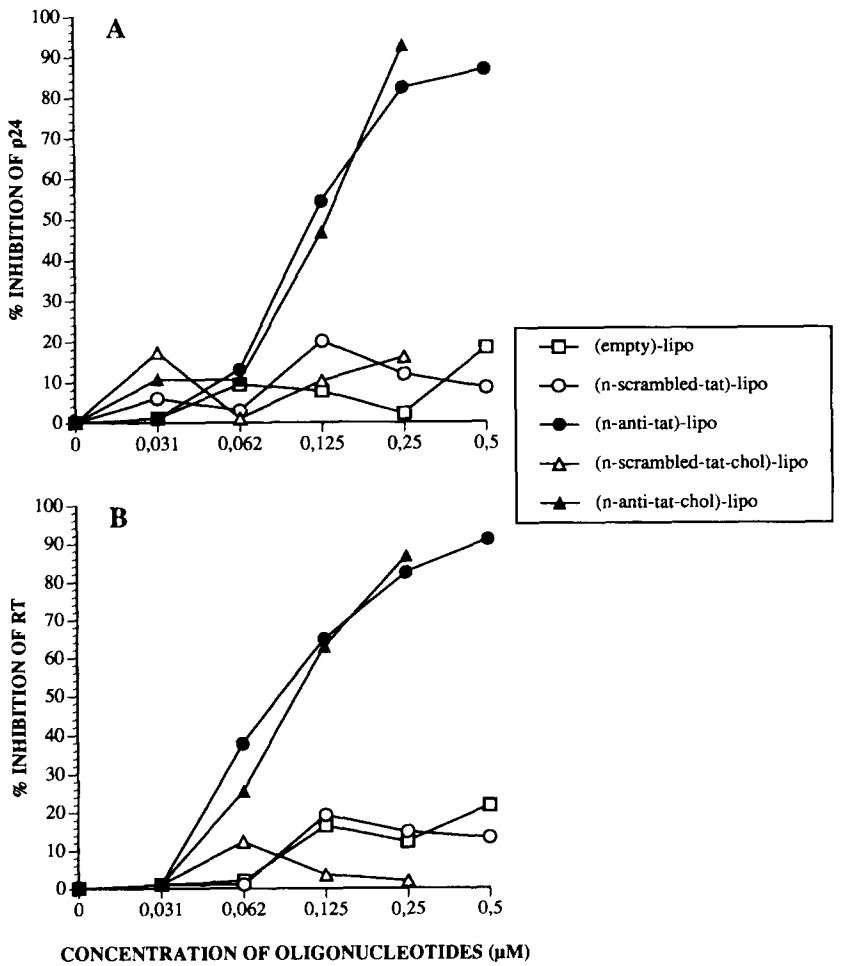

Fig. 3. Dose-response curve of antiviral effects of unmodified and cholesterol-modified oligonucleotides encapsulated in immunoliposomes on acutely infected cells. Acutely infected CEM cells were incubated alone or with anti-HLA antibody (B.1.23.2) and with various concentrations of oligonucleotides encapsulated in liposomes. At day seven, p24 expression and RT activity were determined in the supernatants. The data are given as percent of inhibition of p24 expression (**A**) and RT activity (**B**), compared with the infected controls. The means of two experiments are given. The S.D. did not exceed 10% of this mean.

by HLA specific antibodies inhibited HIV-1 p24 expression (75% and 87% inhibition, respectively) and RT activity (85% and 91% inhibition, respectively) (Fig. 2).

We determined that all liposome preparations were devoid of cytotoxicity for uninfected CEM cells as measured by trypan blue exclusion and radiolabelled base incorporation (data not shown). The antiviral effects depended on specific cellular delivery of the liposome-containing antisense reagents, as none were seen for targeted empty liposomes or for (*n*-anti-*tat*)-lipo and (*n*-anti-*tat*-chol)-lipo in the absence of antibody, or targeted to H2-K molecules which are not expressed on CEM cells (Fig. 2).

From the dose response curve (Fig. 3), the 50% antivirally effective concentration dose (EC_{50}) of (*n*-anti-*tat*)-lipo and (*n*-anti-*tat*-chol)-lipo targeted to HLA class I molecules were evaluated for acutely infected CEM cells. They were both about 0.125 μM in the cases of inhibition of p24 expression and around 0.1 μM for inhibition of RT activity. Thus, no difference were seen between liposomes containing equimolar concentrations of free or cholesterol-coupled oligonucleotides in their capacity to inhibit the replication of HIV in acutely infected CEM cells.

4. Discussion

An approach to bypass problems of extracellular nuclease degradation and poor cell permeability of oligonucleotides composed of unmodified (phosphodiester) nucleotides is to use a transport system such as immunoliposomes. These have been shown to protect oligonucleotides against nucleases and to increase their cellular delivery (Leonetti et al., 1990; Thierry et al., 1992; Zelphati et al., 1993). However, the efficiency of encapsulation of oligonucleotides in small immunoliposomes at the lipid concentration used was very low (2–3%) and constitutes the major inconvenient feature of this transport system. Increasing the size of the liposomes reduces the ability of target cells to take up the liposomes by receptor-mediated endocytosis and also reduces their ability to remain in the circulation (Machy et al., 1983; Senior, 1987).

In the present study we have evaluated the efficiency of liposome association and biological activity of oligonucleotides coupled to cholesterol. The synthesis of such compounds with a reversible disulfide linkage was previously described (Boutorine et al., 1990; Oberhauser and Wagner, 1992). The method of Oberhauser and Wagner for the coupling of 2′-*O*-methyl-oligoribonucleotides was modified for coupling to oligodeoxyribonucleotides. This conjugate had increased association with liposomes by a factor of about 10 for the cholesterol concentration used here and did not influence the efficiency of subsequent coupling of Protein A to the liposome surface. Cholesterol-coupled oligonucleotides have been shown to be incorporated into the bilayer of liposomes with about half of the oligonucleotides directed toward the interior and half toward the exterior surface of the liposomes. The phosphodiester oligonucleotides facing the medium were sensitive to degradation by DNase I at high concentrations (Fig. 1), but as they are coupled to cholesterol at their 3′ extremity they should be protected to some extent against nucleases present in serum (Boutorine et al., 1992; Gamper et al., 1993) which are principally 3′-exonucleases (Shaw et al., 1991).

Liposomes containing these oligonucleotides were shown to strongly inhibit the replication of HIV. This activity depended on the association of targeting antibodies with the liposomes which demonstrated that the cholesteryl-coupled oligonucleotides were not released from the liposomes in the medium, but rather entered into the cells in their liposome-associated form. An antiviral effect was seen for the antisense but not the scrambled anti-*tat* sequence. This indicates that the antiviral effect was due to an interaction with viral RNA, rather than with RT and/or on the HIV entry process (binding gp120-CD4, or fusion, or entry of the viral genome into cell) as reported for phosphorotioate oligonucleotides coupled or not to cholesterol (Stein et al., 1991). The interaction could be with genomic RNA, prior to reverse transcription and integration, or with mRNA newly produced by the integrated virus, or both. Experiments to evaluate the mechanism of action of liposome-associated oligonucleotides and their extension to other target sequences are in progress.

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