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# Antibody-targeted liposomes containing oligodeoxyribonucleotides complementary to viral RNA selectively inhibit viral replication

(antisense oligonucleotides/vesicular stomatitis virus/antiviral therapy/intracellular delivery)

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**ABSTRACT** **Mouse L929 cells were incubated with antibody-targeted liposomes containing oligodeoxyribonucleotides (oligomers). When the oligomer was a 15-mer complementary to the 5′-end region of the mRNA encoding the N protein of vesicular stomatitis virus, the cells became less permissive for multiplication of that virus; >95% reduction of viral multiplication was achieved. Protection was not seen for "empty" liposomes, liposomes containing a random oligomer sequence, or liposomes containing a sequence complementary to the 5′ end of c-*myc* protooncogene mRNA targeted by the same antibody, nor was it seen when the liposomes containing the N-protein antisense oligomer were targeted by an antibody that does not bind to L929 cells. Antibody-bearing liposomes containing antisense oligomers thus have a double specificity: a particular cell selected by the targeting antibody on the liposome and a particular mRNA in the cell selected by sequence complementarity with the liposome-encapsulated oligomer. Nonencapsulated oligomers are sensitive to nucleases and usually must be administered to cells at high concentrations. Oligomers encapsulated in liposomes resist DNase and are active in amounts 1–2 orders of magnitude lower than for those reported for unencapsulated oligomer sequences.**

Selective inhibition of gene expression can be achieved by the introduction into cells of interfering oligomer sequences complementary to cellular mRNA or to viral RNA or DNA ("antisense" sequences). This technology offers the potential for studying the role of individual proteins in living cells and may have therapeutic potential. Several factors limit use of antisense reagents, the most important of which are their susceptibility to degradation by nucleases and their poor penetration into cells, so that high concentrations (5–500 μM, depending on the system) of oligomer in the medium have been reported to be required for detectable inhibition (for review see ref. 1).

Among different methods investigated to overcome these problems, the use of nonionic methylphosphate oligomers has proved efficient in the inhibition of vesicular stomatitis virus (VSV) (2), herpes simplex virus (3), and human immunodeficiency virus (4) multiplication, but these oligomers usually also need to be used at high concentrations (1). One of our laboratories has shown that conjugation to poly(L-lysine) of oligomers complementary to the 5′ region of the RNA encoding the N protein of VSV was capable of increasing the effect of the antisense reagent to an activity in the nanomolar range against the multiplication of VSV (5). However, these reagents do not associate specifically with cells and can be toxic to some cell lines. Further, the polylysine-coupled oligomer sequence remains sensitive to degradation by nucleases. We examined an alternative strategy for augmenting the cell association and enhancing both the resistance to degradation of oligomers and their entry into cells.

Macromolecules may be stably encapsulated in liposomes, which, by virtue of their limited permeability, restrict access by the milieu and protect their contents against enzymatic degradation. Liposomes may, in addition, be coupled to various ligands, including monoclonal antibodies or protein A, that permit their targeting to specific cell populations. Depending on the target molecule and cell, these liposomes may be taken up and release the encapsulated product intracellularly. We have previously shown that liposomes can be used for intracellular delivery of low molecular weight drugs and of nucleotide-based material such as (2′–5′)$A_n$ (6) and the RNA duplex poly(rI)·poly(rC) (7). In the present study we demonstrate the use of antibody-targeted liposomes for intracellular delivery of oligomers complementary to the 5′ region of the N-protein mRNA of VSV. These liposome antisense reagents are active at low concentrations, resist nuclease degradation, retain their target sequence specificity, and can be directed to selected cell populations *in vitro*.

## MATERIALS AND METHODS

**Cell Line and Virus.** Mouse L929 cells (CCL1; American Type Culture Collection) were used throughout this work. They were grown in RPMI 1640 medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (Boehringer Mannheim) and antibiotics. The Indiana strain of VSV was grown in L929 cells and titrated by a dilution method. This method is easier to implement than plaque titration assays and gives similar results (8).

**Oligodeoxyribonucleotide Synthesis.** Reagents for oligomer synthesis were supplied by Milligen (Bedford, MA) and Applied Biosystems. Oligomers were synthesized on a riboadenosine-derivatized support (9) with a Biosearch Cyclone or an Applied Biosystems automatic DNA synthesizer, at 15- and 10-μmol synthesis scales, respectively. Oligomers were then purified by serial precipitation in ethanol saturated with ammonium acetate, and purity was checked by reversed-phase chromatography. The oligomer CATTTTGATTACTGTrA is complementary to the 5′-end sequence of VSV N-protein mRNA. The VSV N protein is implicated in the control of transcription of viral mRNAs (10). The oligomer CCTAGCACCACArA has a random nucleotide composition (nonsense sequence). AACGTTGAGGGGCATrA is

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Abbreviations: VSV, vesicular stomatitis virus; MHC, major histocompatibility complex; VSV liposomes, liposomes containing 15-mer oligodeoxyribonucleotide (oligomer) complementary to the 5′ end region of the mRNA encoding the N protein of VSV; nonsense liposomes, liposomes containing a random oligomer sequence; myc liposomes, liposomes containing oligomer complementary to the 5′ end of human c-*myc* protooncogene mRNA; moi, multiplicity of infection.

complementary to the initiation codon and four downstream codons of the c-*myc* protooncogene mRNA (11).

**Preparation of Liposomes.** Liposomes composed of 80 μmol of total lipid [65% (molar basis) dipalmitoyl phosphatidylcholine (Nattermann Phospholipid, Cologne, F.R.G.), 34% cholesterol (Sigma), and 1% *N*-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia)-modified (12) phosphatidylethanolamine (Sigma)] were prepared with the aqueous phase composed of a solution of oligomer at 15 mg/ml (≈ 2.5 mM) in 145 mM NaCl/10 mM Hepes, pH 7.45 (or with buffer alone, to produce "empty" liposomes). The solution was alternately frozen and thawed by heating to 60°C five times with intermittent gentle mixing on a Vortex mixer. The multilamellar liposomes thus produced were passed through an "Extruder" (Lipex Biomembranes, Vancouver) mounted with 0.2-μm polycarbonate filters (Nuclepore) at 60°C. According to the manufacturer's instructions and published references, 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 Nuclepore filters used (13). The encapsulation efficiency was about 3% of the aqueous phase for all preparations. Liposomes were covalently coupled to *Staphylococcus aureus* protein A (Pharmacia), as described (12). Uncoupled protein A and unencapsulated oligomers were separated from liposomes on a Sepharose 4B (Pharmacia) column. The solution was diluted to a final concentration of 5 μM oligomer for incubation with cells, as determined by the use of oligomer that was trace-labeled with $^{32}P$ at the 5′ end by T4 polynucleotide kinase (Amersham) according to the supplier's instructions. In these experiments reduction of the amount of oligomer in culture was achieved by dilution of liposomes, all of which contained the initial concentration (2.5 mM) of oligomer encapsulated in their aqueous space.

**Antibodies.** Mouse IgG2a,κ monoclonal antibodies were purified from supernatant fluids of cultured hybridoma cells on protein A-Sepharose (Pharmacia) affinity columns. The target specificity of these antibodies is the mouse major histocompatibility complex (MHC)-encoded H-2K molecule, for H100.5.28, and the human MHC-encoded HLA-B and C molecules, for B1.23.2; the latter antibody is immunologically non-cross-reactive with the former antigen. The use of these protein A-binding monoclonal antibodies in conjunction with protein A-bearing liposomes has been reported (14).

**Incubation of L929 Cells and Virus.** Cells ($2 \times 10^5$ per well in 24-well tissue culture plates) were incubated for various periods of time with liposome-encapsulated oligomers at the required concentration and with relevant (H-2K-specific) or control (HLA-specific) monoclonal antibodies (5 μg/ml). The cells were then infected with VSV at a multiplicity of infection (moi) of 1 and were frozen at −20°C 18 hr later. Virus was then titrated as described (8).

**VSV Protein Synthesis in Cultured Cells.** VSV protein synthesis was evaluated in monolayer cultures of L929 cells ($2 \times 10^5$ cells per well). The cells were incubated with liposome-encapsulated oligomers (2 μM) and H-2K-specific mAb (5 μg/ml) for 12 hr before infection with VSV at a moi of 5. Ten hours after infection, the medium was removed and the cells were incubated for 30 min in methionine-free medium supplemented with 10% (vol/vol) dialyzed fetal bovine serum and [$^{35}S$]methionine (20 μCi/ml; 1 μCi = 37 kBq). The cells were then lysed in 1% (wt/vol) sodium dodecyl sulfate (SDS) and proteins were precipitated in 8% (wt/vol) trichloroacetic acid. After centrifugation the pellets were washed with diethyl ether, solubilized in sample loading buffer (4 M urea/80 mM Tris·HCl, pH 6.8/2% SDS/50 mM 2-mercaptoethanol), and electrophoresed in a 10% (wt/vol) polyacrylamide gel (15). Fluorography was performed as described (16). The gel was exposed to Kodak X-Omat film at −80°C with an intensifier screen.

## RESULTS

L929 cells were incubated with H-2K-specific antibody and dilutions of protein A-bearing liposomes containing oligomers complementary to the 5′ end of N-protein mRNA (VSV liposomes), of random sequence (nonsense liposomes), or complementary to the 5′ end of human c-*myc* mRNA (myc liposomes). After 4 hr at 37°C, VSV was added, and after 18 hr of additional incubation, virus was titrated. Results of a representative experiment are presented in Fig. 1. Dose-dependent inhibition of virus multiplication was observed for VSV liposomes, >95% at a final concentration of 800 nM (1:3125 dilution of liposomes containing 2.5 mM oligomer); nonsense and myc liposomes had no effect. The unencapsulated VSV N-protein antisense oligomer inhibited viral replication 90% at 40 μM when incubated in serum-free medium; lower concentrations were inactive, and the presence of serum eliminated the protective effect. The unencapsulated nonsense and myc antisense oligomers were inactive (data not shown).

The kinetics of the antiviral effect were studied in a separate experiment. Cells were incubated with H-2K-specific antibody and VSV, empty, or myc liposomes; VSV was added immediately, or after various times, and infectious virus was assayed 18 hr after virus addition. No protective effect was seen when infection immediately followed liposome binding, or at any time for myc or empty liposomes. Some protection was seen for VSV liposomes after 2 hr; optimal protection was seen after 5 hr and protection persisted even when virus was added after 10 hr (Fig. 2).

The protective effect was strictly dependent on the use of an antibody specific for the H-2K molecules expressed by the target cell. When cells were incubated with VSV liposomes in the absence of antibody or in the presence of an HLA-specific antibody, no inhibition of viral multiplication was observed (Fig. 3).

The effect of VSV liposomes on protein synthesis in infected cells was also evaluated. L929 cells were incubated with H-2K-specific antibody and sufficient VSV liposomes so that the amount of VSV antisense oligomer in culture was 2 μM. Twelve hours later cultures were infected with VSV, and after 10 hr the effect on incorporation of [$^{35}S$]methionine into viral proteins was determined. We observed (Fig. 4) a marked reduction of synthesis of all viral proteins, compared to cells incubated with empty or myc liposomes. The synthesis of cellular proteins unaffected by viral infection was

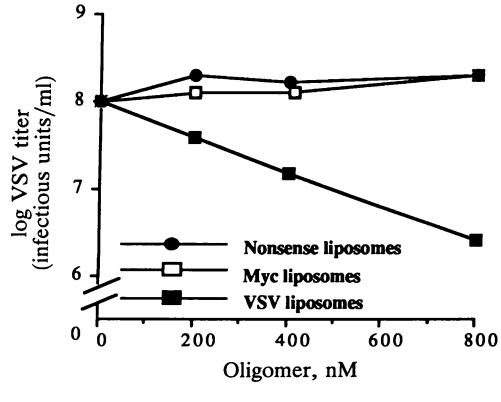


FIG. 1. Action of antibody-targeted liposomes. Cells were incubated with H-2K-specific antibody (5 μg/ml) and various concentrations of VSV liposomes (■), nonsense liposomes (●), or myc liposomes (□). After 4 hr at 37°C, VSV was added at a moi of 1, and after 18 hr of additional incubation at 37°C, the virus was titrated.

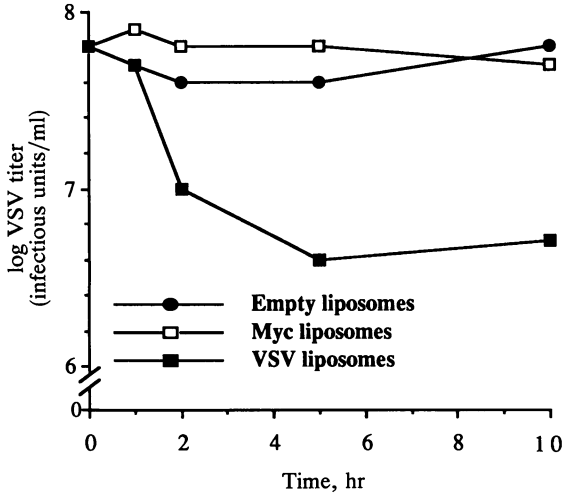


FIG. 2. Kinetics of antiviral activity. Cells were incubated with VSV (■) or myc (□) liposomes (final concentration; 250 nM oligomer) or empty liposomes (●) and H-2K-specific antibody (5 μg/ml) at 37°C. VSV (moi, 1) was added at intervals thereafter (abscissa). Virus was titrated for each sample 18 hr after virus addition.

not reduced by any liposome treatment. Together, these data indicate that antiviral oligomer sequences may be introduced into specific cell populations *in vitro* and exert a specific effect.

The resistance of the encapsulated antisense sequence to degradation by DNase was evaluated. Cells were incubated with or without DNase I (*Escherichia coli*, Sigma) at 20 units/ml. To some wells was added the 15-mer VSV antisense oligomer coupled to polylysine, which was previously shown to inhibit VSV infection (5). Other wells received the VSV antisense in protein A-bearing liposomes, together with H-2K-specific antibody at 5 μg/ml. After 5 hr, virus was added, and the virus titer was determined after additional incubation for 18 hr. The action of the VSV antisense sequence coupled to polylysine was completely inhibited by the DNase, but there was little or no effect on the antiviral activity of VSV liposomes (Fig. 5).

## DISCUSSION

The VSV and myc antisense oligomer sequences used in these experiments were selected because of their activity in inhibiting synthesis of the complementary proteins in other

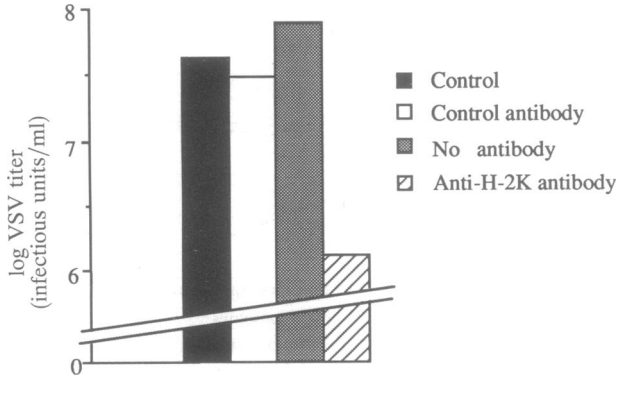


FIG. 3. Antibody dependence of the antiviral effect. Cells were incubated without liposomes (black bar), with VSV liposomes without antibody (gray bar), or with antibody (5 μg/ml) specific for a nonexpressed HLA molecule (white bar) or for the expressed H-2K molecule (hatched bar). The final concentration of antisense oligomer was 215 nM. Virus was added after 5 hr and was titrated 18 hr later.

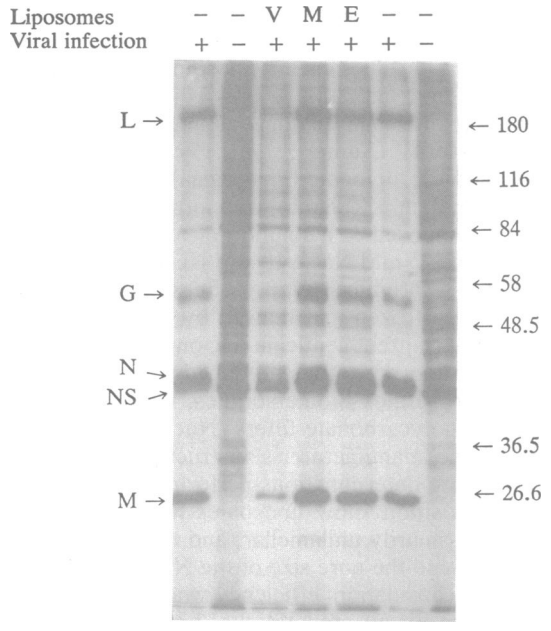


FIG. 4. Effect of VSV liposomes on viral protein synthesis. Cells preincubated for 12 hr with empty (E), VSV (V), or myc (M), liposomes (2 μM) and H-2K-specific antibody (2 μg/ml) were infected with VSV (moi, 1). Ten hours after infection the cells were pulse-labeled with [$^{35}$S]methionine for 30 min. Proteins were analyzed by SDS/polyacrylamide gel electrophoresis followed by fluorography. Positions of viral proteins are indicated at left. Positions and sizes (kDa) of standard proteins run in the same gel are indicated at right.

studies (5, 17). The suitability of a sequence for antisense intervention may depend on many factors, including the function of the target protein and the concentration, stability, and accessibility of the RNA encoding it, though the mechanism(s) of antisense action are not entirely understood. The 3′ ribonucleotide group on the oligomers (9) was included for the purpose of improving the efficiency of the covalent coupling in earlier work from one of our groups showing action of polylysine-coupled oligomers (5). The use of the same reagent for liposome and polylysine delivery permits us to have a control for the activity of the preparation. Since the

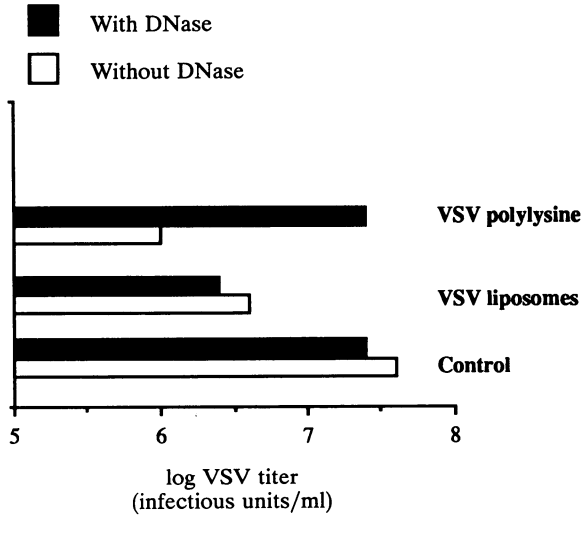


FIG. 5. Effect of DNase on antisense effect. Cells were incubated with (solid bars) or without (open bars) DNase alone (Control) or with the VSV liposomes (together with H-2K-specific antibody at 5 μg/ml) or the VSV antisense oligomer coupled to polylysine. Virus was added after 5 hr and was titrated 18 hr later.

free 3′-ribonucleotide oligomer has about the same activity as that reported for conventional oligomers (inhibition at 40 μM), the addition of the ribonucleotide seems to have little effect on its stability.

In this study a liposome-encapsulated antisense oligomer specific for viral mRNA, but not a nonsense oligomer or an oligomer specific for c-*myc* mRNA (17), specifically reduced virus production *in vitro*. The amount of liposome-encapsulated antisense oligomer required for activity was 100 times lower than for the nonencapsulated oligomer. Class I molecules, encoded by genes of the MHC and expressed at variable levels on practically all nucleated cells, were used to mediate cell association of liposomes, with the specificity of action depending on the encapsulated oligomer sequence. In the presence of antibodies to the class I molecules expressed on L cells, protein A-bearing liposomes will bind to these molecules and be internalized by endocytosis. Nonspecific association between cells and protein A-bearing liposomes is very low in the absence of a ligand that becomes cell-bound (18), and inhibitory effects were not seen in these conditions. Although liposomes are diluted in the medium and have only limited encapsulated volume, they contain concentrated oligomers and, in the presence of the appropriate ligand, accumulate at the cell surface. In the present experiments, excess liposomes and antibodies remained in the culture throughout the incubation, and multiple rounds of internalization were possible. High ratios of antisense to coding (sense) sequences may be necessary for efficient antisense action, but at the present time we cannot estimate the efficiency of the process of transfer of functional oligomers to their intracellular site of action. The liposomes used in these studies are not known to fuse with cell membranes (19), and a large fraction of the endocytosed oligomers may be degraded in lysosomes.

The fate of liposomes taken up by receptor-mediated endocytosis is determined by the fate of the cell-surface molecules to which they bind. This may depend on both the cell type and the target molecule. Liposomes bound to MHC-encoded class I target molecules on L cells are internalized via noncoated pits (20), whereas the same molecules on T lymphoid cells are internalized via coated pits (21). The role of the compartment in which antisense oligomers enter into cells in relation to their capacity to regulate the translation of their complementary mRNAs is an area for further study.

The protection against degradation, the cell specificity, and the enhanced intracellular delivery made possible by antibody targeting provide a convenient means of introducing oligomer sequences into cells and will help us to understand the relationship between expression and function of the targeted RNAs. The ability to inhibit viral proliferation needs to be tested in a number of models to determine whether this technology may offer potential for therapeutic applications.

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