Research Article

Inhibition of the Friend Retrovirus by Antisense Oligonucleotides Encapsulated in Liposomes: Mechanism of Action

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Proliferation of the Friend retrovirus was specifically inhibited by the *env* mRNA complementary oligonucleotide encapsulated in pH-sensitive liposomes. This observation was made using the focus immunoassay (FIA) and the reverse transcriptase test. The key finding of the present study was the dramatic impact on liposome penetration. For chronic or de novo infection, the point at which the penetration of liposomes began corresponded to the time needed for the virus to leave the cell. In the absence of the virus, liposomes remained adsorbed onto the cell surface without any internalization. Regardless of the mechanism involved, the fact that a retroviral infection stimulates the cellular uptake of oligonucleotide liposomes widens the spectrum of strategies for specific antiviral action.

KEY WORDS: Friend retrovirus; pH-sensitive liposomes; oligonucleotides; fibroblasts; focus immunoassay.

INTRODUCTION

Oligonucleotides with base sequences complementary (antisense) to a specific cellular RNA can modulate the expression of any individual gene (1,2). However, crucial problems, such as the stability of oligonucleotides in relation to nuclease activity *in vitro* and *in vivo* and the low penetration into cells, have to be solved before oligonucleotides can be used as drugs (3). Resistance of oligonucleotides to nucleases can be achieved by substitution of one of the nonbridging oxygen atoms for either a methyl group (4) or a sulfur atom (5) or functionalization in 5′ or 3′ with polylysine (6) or acridine (7). However, at a high concentration these derivatives may be toxic to cells. The possibility of using colloidal carriers (liposomes, nanoparticles), to increase cellular uptake of oligonucleotides, thereby reducing their degradation by nucleases, has been considered. Association of oligonucleotides with nanoparticles is feasible as shown with an oligothymidylate (8). The *in vitro* protection against degradation of oligothymidylate adsorbed onto nanoparticles was also demonstrated. Further, oligonucleotides against the vesicular stomatitis virus were encapsulated in liposomes (9). However, cellular uptake of nanoparticles or liposomes occurs via the endocytosis pathway, and therefore, the ultimate fate of both carriers and their content is lysosomal degradation (10). There are at least two drawbacks to the intracellular distribution with nanoparticles or liposomes. (i) Oligonucleotides will be released in a subcellular environment with risk of enzymatic degradation. (ii) Delivery may occur in a nonrelevant cellular compartment (the oligonucleotides must diffuse through the lysosomal membrane to reach the mRNA target).

To avoid lysosomal degradation and achieve transport of entrapped oligonucleotides into the cell cytoplasm, we have used pH-sensitive liposomes (11). These phospholipidic bilayered vesicles are destabilized at acidic pH (12). During endocytosis, the pH is reduced in the endosomal compartment which precedes the lysosomes. Consequently, after destabilization and fusion with the endosomal membrane, the liposome should be able to deliver its content into cytoplasm. Encapsulated oligonucleotides in pH-sensitive liposomes have been shown to inhibit specifically the proliferation of the Friend retrovirus in mouse fibroblasts (13).

These results have led us to investigate the mechanism of delivery into cells of oligonucleotides entrapped in liposomes. The present paper shows that cellular uptake of liposomes in fibroblasts is stimulated by the presence of virions.

MATERIALS AND METHODS

Cell Line and Virus

Mouse fibroblasts (DUNNI cells) were used throughout this work. The virus (F-Mulv) was propagated on NIH 3T3 (chronically infected cells) grown in DMEM medium with 5% fetal bovine serum (heated at 56°C for 30 min) and antibiotics. After filtration released viruses were stocked at

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$-70°C$ and titrated by Focus Immuno Assay. Virus stocks contained $2 \cdot 10^4$ focus forming unit (ffu)/mL.

Synthesis and Radiolabeling of Oligonucleotides

Oligonucleotides with the sequence complementary (antisense) to the 5′ region of the initiation codon AUG of the *env* gene were synthesized on an automated DNA synthesizer (Model 380B, Applied Biosystem Foster City, CA). The sequence was 5′ TGAACACGCCATGTC 3′. The control oligomer, which should not affect viral mRNA, had the reverse antisense composition: 5′ CTGTACCGCACAAGT 3′. This oligomer did not match any other sequence in the retrovirus genome.

The 5′ end of all oligonucleotides was labeled with ^{32}P-ATP using T4 polynucleotide kinase (Biolabs) at 37°C for 30 min.

Preparation of Liposomes

Liposomes were prepared by reverse-phase evaporation (14). The organic phase was constituted by ethanol/chloroform at a ratio of 1:1 in which phospholipids were dissolved. In order to compare the efficiency of oligonucleotides delivery by liposomes, two types of composition were used: a pH-sensitive composition which includes dioleylphosphatidylethanolamin (DOPE)/oleic acid (OA)/cholesterol (chol) at a ratio of 10:5:2 and non-pH-sensitive liposomes composed of dioleylphosphatidylcholine (DOPC)/oleic acid (OA)/cholesterol (chol) at a ratio of 10:5:2.

Oligonucleotides, which were encapsulated at a concentration of 600 μg/mL, were suspended in a buffer (Tris–EDTA, 50 m*M*, pH 7.8). The diameter of liposomes was determined with a laser light scattering apparatus (WS-4; Coulter Electronics, Inc., Hialeah, FL).

Nonencapsulated oligonucleotides were separated from liposomes by Sepharose 4B gel filtration. The rate of encapsulation of the oligonucleotides was evaluated using ^{32}P-oligomer. The amount of phospholipids in the final liposomal preparation was determined using ^{14}C-DOPE (Amersham).

Electron Microscopy

An aliquot of the liposomal preparation was negatively stained on carbon-coated grids with 2% aqueous solutions of sodium phosphotungstate (pH 7.0 with sodium hydroxide). Electron microscopy was performed using a transmission electron microscope.

Acidic-Induced Leakage

To evaluate pH sensitivity of DOPE liposomes in different acidic media at 37°C, 1 mL of liposomes (5 μmol of lipids) was placed in buffer (10 mL) at pH 7.6, 5.5, and 4.5. At different time intervals, a sample (1 mL) was removed from the incubation medium and oligonucleotide leakage was determined after passage through Sepharose 4B.

Stability in Culture Medium

The same experimental conditions (volume and concentration of lipids) as described above were used to evaluate the stability of oligonucleotide-liposomes in culture medium.

The release of oligonucleotides labeled with ^{32}P was evaluated after passage through the Sepharose 4B column and radioactivity was then counted.

Focus Immunoassay (FIA)

Dunni cells (24,000/well) were infected by viruses (diluted to obtain 100 foci/well). At the same time either oligonucleotide-liposomes, empty liposomes, or free oligonucleotides were added at different concentrations. After 96 hr of incubation a focus immunoassay was used to determine the presence of gp70 (an envelope protein of the virus) at the surface of the cell membrane. All the infected cells expressed this protein in their membrane. At 96 hr, the cell supernatant was also removed and used for the reverse transcriptase test. These cells were first incubated with a mouse antibody against gp70 (15). After rinsing, a goat anti-mouse antibody conjugated to peroxidase was added. Thirty minutes later cells were rewashed and the final step of the test was performed with a peroxidase substrate (3-amino-9-ethylcarbazole) (Sigma), dissolved in a dimethylformamide solution containing acetoacetate and H_2O_2 (16). The cells were then rinsed and the brown foci counted.

It was different to score foci of chronically infected cells as they were numerous. The supernatant was therefore removed 24 hr after the addition of oligonucleotides in culture medium and used to reinfect Dunni cells. The FIA was performed 96 hr after reinfection.

Reverse Transcriptase Assay

To evaluate the production of viral particles 96 hr after the incubation of the infected cells with oligonucleotides which were either encapsulated or not, a reverse transcriptase test was performed.

Cell culture supernatants were centrifuged for 2 hr at 12,000*g* and 4°C. Virus pellets were resuspended in a detergent (NP40, 5%) in order to lyse the viral envelope. The polymerase activity of F-Mulv reverse transcriptase was determined on the oligo pdT_{12-18}:poly A template by measuring the incorporation of ^3H-dTTP into the acid-precipitable fraction. The reaction mixture contained 20 m*M* Tris HCl (pH 7.5), 10 m*M* $MgCl_2$, 50 m*M* dithiothreitol, 20 m*M* NaCl, 150 μ*M* poly(A) (Pharmacia), 75 μ*M* dT_{12-18} (Pharmacia), and 1.5 μCi (^3H)dTTP (NEN). After 1 hr of incubation at 37°C, the reaction was stopped using 1 μl of EDTA (0.1 *M*) and 5 μL of calf thymus DNA (1 mg/mL). The reaction mixture was spotted onto 0.45-μm filters (Type HA, Millipore) and washed with a solution of trichloroacetic acid (TCA; 10%), Na_2HPO_4 (1%), followed by another wash in TCA (5%) and two further washes in ethanol. Scintillation counting was performed after drying.

Uptake of Oligonucleotide-Liposomes by Cells

Labeled oligonucleotide-liposomes were added to infected and noninfected fibroblasts (2×10^5 cells). Cells were pelleted, washed three times in PBS, and lysed by adding water (cell fraction). Supernatants were pooled (supernatant fraction). Uptake was determined by counting the cell and supernatant fractions.

We then determined whether pH-sensitive liposomes

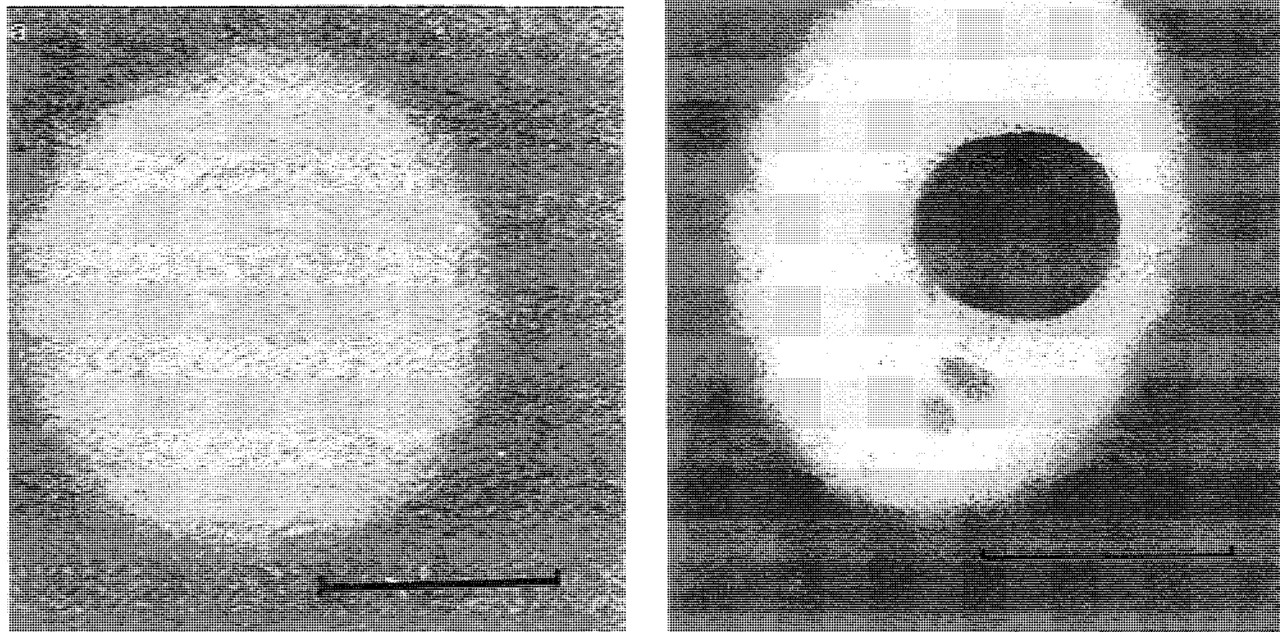

Fig. 1. Morphologic structure of liposomes observed by electron microscopy. Liposomes were stained with a 2% aqueous solution of sodium phosphotungstate. (a) Empty liposomes; (b) oligonucleotide liposomes. Bar scale = 100 nm.

were localized in the cell cytoplasm. Liposomes were labeled with ^{14}C-DOPE (360 μmol of phospholipids, 10^4 cpm for $2 \cdot 10^5$ fibroblasts). Once oligonucleotide liposomes were incubated with cells, the latter were pelleted and washed three times with PBS, and the supernatants were pooled. Cells were then lysed by adding water. The suspension was centrifuged (12,000*g* for 5 min), and the membrane pellet was washed once again with water. The cytoplasmic supernatant and the subsequent wash were pooled. The three fractions (extracellular medium, cytoplasm, and membranes) were counted to evaluate the uptake and the association with membrane.

RESULTS

Physical Properties of Liposomes

Characterization

Empty liposomes were oligolamellar as observed by electron microscopy (Fig. 1a). However, dramatic morphological changes were observed for liposomes containing oligonucleotides (Fig. 1b); their multilamellar appearance had completely disappeared. The reason for such a morphological change has yet to be investigated. The unusual appearance of oligonucleotide liposomes might be due to a strong ionic interaction between DOPE and the oligomers, as suggested previously with DNA (17). Both liposomes, with and without oligonucleotides, were approximately 170 nm in diameter. The entrapment yield was 10% of the total oligonucleotide and its efficiency was 2.5 μg oligonucleotide/μmol lipid. The oligonucleotide concentration in the final suspension was approximately 12 μ*M*, and that of phospholipids 3.5 m*M*.

Stability in Buffers

pH-sensitive liposomes with carboxyfluorescein were destabilized at a pH below 6, unlike their non-pH-sensitive counterparts (results not shown). It is noteworthy that DOPE liposomes with entrapped oligonucleotides remained pH sensitive despite their nonlamellar morphology. Indeed, 90% of ^{32}P-labeled oligonucleotides were released from DOPE liposomes after 90 min of incubation at pH 5.5. In contrast, only 15% of ^{32}P-oligonucleotides incubated under the same experimental conditions were released with the non-pH-sensitive composition (Table I). This result confirmed that DOPE/OA/chol liposomes delivered their content faster than those with a non-pH-sensitive composition in an acidic environment.

Stability in Culture Medium

Approximately 60% of oligonucleotides remained entrapped in liposomes after 24 hr of incubation at 37°C in a culture medium, regardless of the nature of the liposomes (pH sensitive or not) (Fig. 2). This percentage was significantly higher than with other previously described pH-sensitive formulations (12).

Inhibition of Virus Spreading

Significant inhibition of virus spreading, quantified by

Table I. Release of Oligonucleotides from pH-Sensitive and Non-pH-Sensitive Liposomes at Different Acidic Levels and at 37°C[a]

	pH-sensitive liposomes			Non-pH-sensitive liposomes		
hr	pH 4.5	pH 5.5	pH 7.5	pH 4.5	pH 5.5	pH 7.5
0	1	1	1	1	1	1
0.5	50	50	1	1	1	1
1	100	70	1	12	13	12
1.5	100	90	18	14	13	12

[a] The values are expressed as percentage of released oligonucleotides. Each point is the mean of two experiments.

the focus immunoassay (FIA), was obtained after the addition of oligonucleotide liposomes either in the de novo or in the chronic infection model. Indeed, after 96 hr, the FIA—used for the immunologic detection of the *env* protein—revealed an IC_{50} of about 0.04 μM with pH-sensitive liposomes, whereas this value was 0.12 μM for non-pH-sensitive liposomes (Fig. 3a). pH-sensitive liposomes inhibited 80% of virus spreading, whereas free oligonucleotides were unable to inhibit virus proliferation until the concentration attained 15 μM (data not shown). Empty liposomes did not affect the FIA. In the chronic infection model, known to be more resistant to drugs, a 60% inhibition was achieved for the pH-sensitive formulation and only 30% for non-pH-sensitive liposomes (oligonucleotide concentration of 0.16 μM) (Fig. 3b). It could be argued, however, that the FIA is capable of measuring *env* protein inhibition at the surface of cells but not the inhibition of viral proliferation. It was therefore necessary to quantify the gp70 measurement of the retroviral infection independently, by measuring reverse transcriptase (RT) activity of the virions.

The oligonucleotide effect measured using the reverse transcriptase assay (Table II) was found to be similar to that obtained with the FIA assay in both chronic or de novo infection (96 hr postincubation). Whereas several days were required for the formation of *foci* with the FIA, reverse transcriptase activity could be measured at intermediate time intervals with the RT assay.

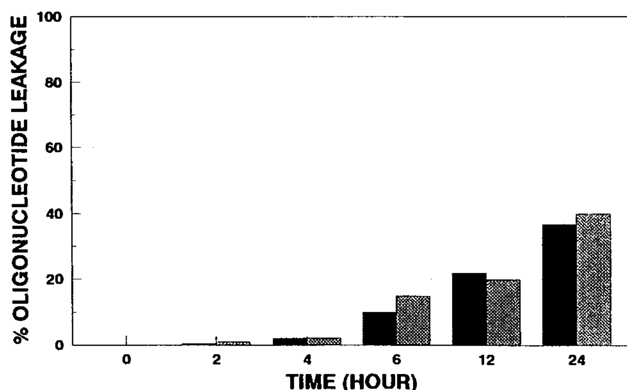

Fig. 2. Stability of oligonucleotide non-pH-sensitive (▩) or pH-sensitive (■) liposomes in culture medium at 37°C. Released oligonucleotides were separated from oligonucleotide liposomes after passage through a Sepharose 4B column. Results are the mean of two experiments.

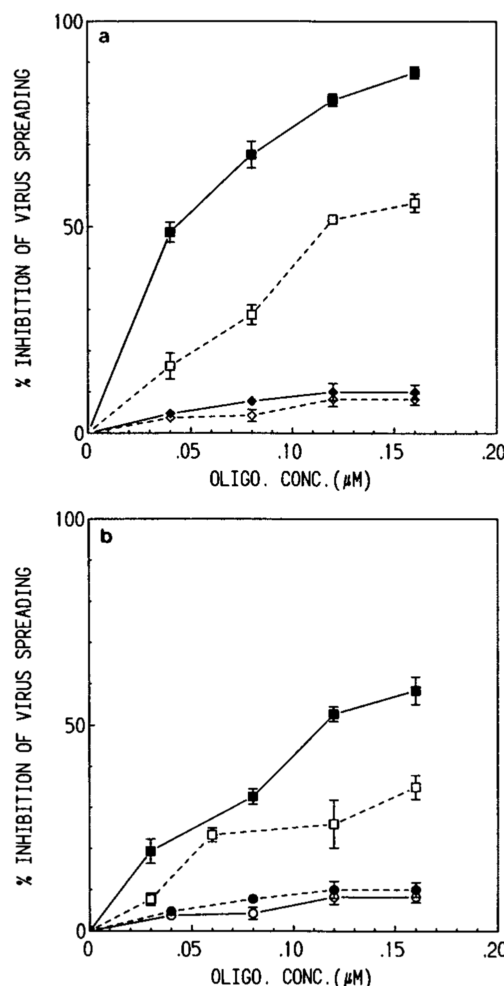

Fig. 3. (a) FIA-measured antiviral activity of encapsulated oligonucleotides in de novo infection (■) Antisense oligonucleotide in pH-sensitive liposomes. (□) Antisense oligonucleotide in non-pH-sensitive liposomes. (♦) Control oligonucleotide in pH-sensitive liposomes. (◇) Control oligonucleotide in non-pH-sensitive liposomes. Results are the mean ± SD of three experiments. (b) FIA-measured antiviral activity of encapsulated oligonucleotides in chronic infection. (■) Antisense oligonucleotide in pH-sensitive liposomes. (□) Antisense oligonucleotide in non-pH-sensitive liposomes. (●) Control oligonucleotide in pH-sensitive liposomes. (○) Control oligonucleotide in non-pH-sensitive liposomes. Results are the mean ± SD of three experiments.

We have tested higher doses of oligonucleotide liposomes (0.20 and 0.30 μM). However, at those concentrations, control oligonucleotide liposomes, which, in principle had no sequence complementary with the retrovirus genome, exhibited strong toxicity. Therefore, cells were incubated with a 0.16 μM dose of oligonucleotides twice, with an interval of 24 hr between additions (for details, see Materials and Methods). Under such conditions, pH-sensitive liposomes with antisense oligonucleotides were able to induce the inhibition of 100% of virus spreading (Fig. 4).

Studies on Uptake of Oligonucleotides and Liposomes

The cellular association of ^{32}P-labeled oligonucleotides in pH-sensitive liposomes was first investigated under the

Table II. Summary of Inhibition of Virus Proliferation by Antisense Oligonucleotides and by Control Oligonucleotides Encapsulated in pH-Sensitive Liposomes: Assays Were Performed by Either RT or FIA[a]

	Oligonucleotide conc. (μM)			
	0.04	0.08	0.12	0.16
Antisense				
De novo infection				
RT assay	34 ± 5	51 ± 7	72 ± 8	87 ± 6
FIA	32 ± 4	59 ± 2	76 ± 2	80 ± 4
Chronic infection				
RT assay	10 ± 3	24 ± 5	32 ± 5	46 ± 4
FIA	14 ± 4	28 ± 4	32 ± 3	59 ± 4
Control				
De Novo infection				
RT assay	2 ± 0.5	5.5 ± 1.5	6 ± 1.5	6.5 ± 1
FIA	3.7 ± 1.5	4.2 ± 2.5	8.1 ± 3	8.2 ± 3
Chronic infection				
RT assay	2.4 ± 2	4.5 ± 5	5 ± 2	6 ± 1
FIA	3.5 ± 1.5	4 ± 1	4.5 ± 2	5.1 ± 2

[a] The values are expressed as percentage of inhibition of the virus proliferation ($n = 2$).

following conditions: in cells without virus and in cells with chronic or de novo infection. The association of ^{32}P-oligonucleotide-liposomes was very low with noninfected cells compared to that observed with infected cells (Fig. 5). Further, the more virions were added, the greater was the oligonucleotide cell association. It was also striking to note that significant Dunni cell association of ^{32}P occurred only 24 hr after the addition of virions. This time interval roughly corresponded to the period necessary for the first virions to leave the cells after a complete viral cycle. On the contrary, oligonucleotide-liposomes were captured quickly in chronically infected cells.

The cellular association of pH- and non-pH-sensitive liposomes was also compared. ^{32}P radioactivity of oligonucleotides with pH-sensitive liposomes in infected cells was twofold that of oligonucleotides with non-pH-sensitive liposomes (Fig. 6).

The cellular association of the encapsulated reverse antisense oligonucleotide (used as a control in the inhibition experiments) was also measured. The amount of cell-associated ^{32}P at a given postinfection time was two- to threefold higher than for the anti-*env* oligonucleotides (Fig. 6).

In order to determine whether cellular uptake of oligonucleotides was induced by the intracellular capture of lipo-

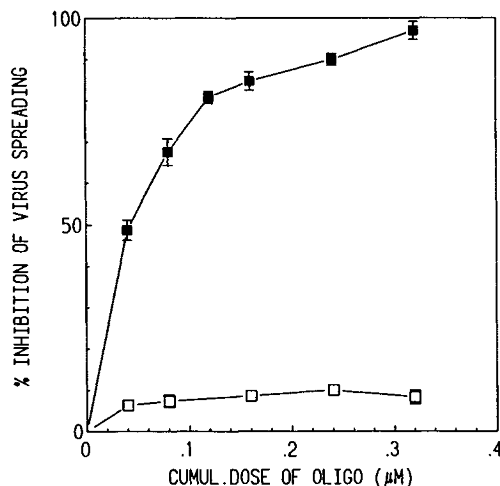

Fig. 4. Two successive additions of oligonucleotides completely inhibit the retrovirus proliferation followed by FIA. Liposomes were added at the time of infection and a second dose 24 hr later. (■) Antisense oligonucleotides in pH-sensitive liposomes. (□) Control oligonucleotides in pH-sensitive liposomes. Oligonucleotide doses result of the cumulative added doses. Each point is the mean ± SD of two experiments.

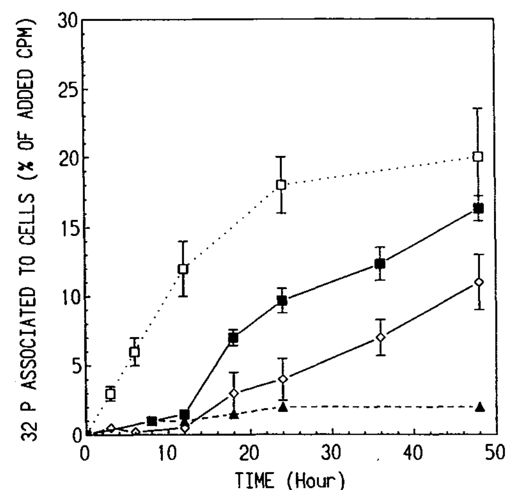

Fig. 5. Association of ^{32}P-antisense oligonucleotide in pH-sensitive liposomes with chronically infected cells (□), infected cells by virus ($2 \cdot 10^3$ pfu/mL) (■), infected cells by virus ($2 \cdot 10^2$ pfu/mL) (◇), or noninfected cells (▲). Oligonucleotides were added to fibroblasts; cells were pelleted, washed three times in PBS, and lysed by adding water. Radioactivity associated and nonassociated to cells was counted (supernatants and cells). Results are the mean of four experiments; bars show the standard deviation.

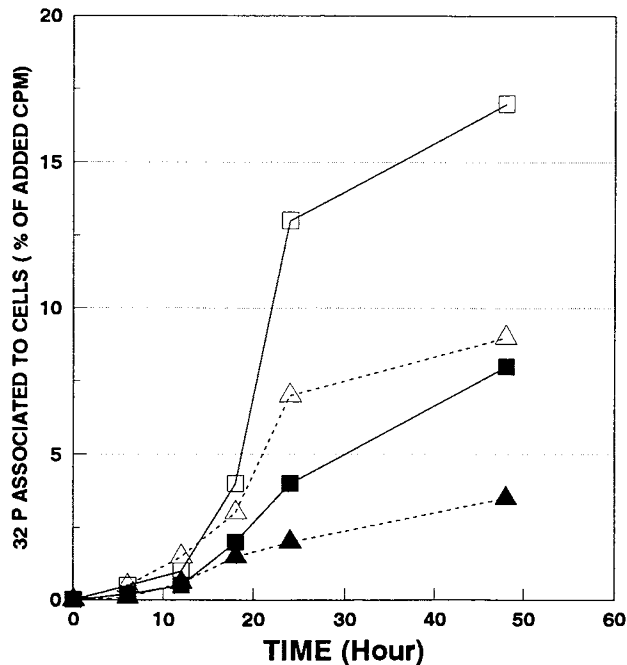

Fig. 6. Comparison between the association of ^{32}P-antisense oligonucleotide in pH-sensitive (—■—) and non-pH-sensitive (▲) liposomes and control oligonucleotides in pH-sensitive (—□—) and non-pH-sensitive (-△-) liposomes with infected cells (2 · 10^2 pfu/mL). Radioactivity associated and nonassociated to cells was counted (supernatants and cells). Results are the mean of three experiments.

somes, ^{14}C-DOPE was measured in cytoplasmic and noncytoplasmic cellular compartments. Let us assume that radioactivity found in the noncytoplasmic fraction is likely localized in the membranes. As shown in Table III, A, the bulk of the radioactivity remained in the membranes of noninfected cells; only a small amount was detected in the cytoplasm. The ratio membrane-versus-cytoplasm was inverted for the infected cells. Finally, the amount of ^{14}C found in the membranes of infected cells was significantly higher with antisense than with control oligonucleotides (Table III, C and B). This observation suggests that liposomal uptake is likely linked with retrovirus budding.

Table III. Distribution of Oligonucleotide ^{14}C-DOPE Liposomes in Dunni Cells According to Time: (A) Distribution of Antisense Oligonucleotides in Noninfected Dunni Cells; (B) Distribution of Control Oligonucleotides in Infected Dunni Cells (2 · 10^2 pfu/mL); (C) Distribution of Antisense Oligonucleotides in Infected Dunni Cells (2 · 10^2 pfu/ml)[a]

hr	Cytoplasm	Membrane	Non-cell-associated radioactivity
		A	
6	1	1	98
18	2	2	96
24	2.5	3.5	94
36	3.5	20	76.5
48	5	35	60
		B	
6	2	1	97
18	2.5	2.5	94
24	3.5	4.6	91.8
36	20	5	75
48	30	18	52
		C	
6	1	1	98
18	3	2	95
24	5	5	90
36	8	12	80
48	15	25	60

[a] After addition of oligo ^{14}C-DOPE liposomes, the three fractions (medium, cytoplasm, and membranes) were counted to evaluate cell uptake and membrane association (n = 3).

DISCUSSION

Proliferation of the Friend retrovirus was specifically inhibited by the *env* mRNA complementary oligonucleotide encapsulated in liposomes. This oligonucleotide has already demonstrated its efficacy in inhibiting the synthesis of *env* protein in cell lysates at a concentration of 10 μM (7). However, no further activity was displayed with the oligonucleotide alone when retrovirus proliferation took place in cells. Virus inhibition by encapsulated anti-*env* oligonucleotide was ascertained by measurements performed either with the focus immunoassay (FIA) or with the reverse transcriptase assay. The specificity was highlighted by the lack of activity of encapsulated random control oligonucleotides and unloaded liposomes. DOPE pH-sensitive liposomes seemed to be roughly twice as effective as their DOPC non-pH-sensitive counterparts.

We succeeded in completely inhibiting virus spreading by successive additions of oligonucleotides encapsulated in pH-sensitive liposomes (with a 0.16 μM oligonucleotide concentration added twice).

The key finding in the present study was the dramatic impact of the virus on liposome penetration into the cells. For both chronic and de novo infection the point at which liposome penetration began corresponded well to the time needed for the virus to leave the cell: approximately 6 hr for chronically infected cells and 18 hr for de novo infected cells. In the absence of the virus, liposomes remained adsorbed onto the cell surface without any significant internalization. Since fibroblasts are nonprofessional phagocytes, it is unlikely that liposomes could massively enter the infected cells as a consequence of the stimulation of the phagocytosis process. It is more likely that the exit of the virus facilitates cellular uptake of liposomes, although the exact mechanism involved remains unclear. This hypothesis is also consistent with the difference observed in the capture of antisense and control encapsulated oligonucleotides. Antisense oligonucleotides inhibited *env* synthesis and, thereby, the amount of budding virus. This may account for the relatively poor liposomal uptake which ensued when such oligonucleotides were encapsulated. On the contrary, with control oligomers, no perturbation of the viral cycle was expected to occur, and a higher quantity of the virus was released in the extracellular medium, thus allowing more significant liposomal uptake. The exit of the retrovirus induces budding and desta-

bilization of the cell membrane which is part of the viral envelope (18). Some previous studies have shown a modification of the lipid composition of virus infected cells (19,20). The facilitated uptake of oligonucleotide liposomes in Friend retrovirus-infected cells could be related to this modification.

A key question was whether pH-sensitive liposomes reacted as was originally intended. Two reasons led us to believe that the improved efficiency of these liposomes was not induced by the destabilization of DOPE liposome bilayers. First, it was reported that the efficiency of the viral inhibition obtained with encapsulated oligonucleotides in pH-sensitive liposomes was only twice that of entrapped oligonucleotides in non-pH-sensitive liposomes. Second, a two-fold increase in cell association was also observed when pH-sensitive liposomes were compared to non-pH-sensitive liposomes. Szoka *et al.* observed the same results with the pH-sensitive composition containing cholesterylhemisuccinate (CHEM)/DOPE, and non-pH-sensitive CHEM/DOPC (12). Thus, the higher association of DOPE-containing liposomes with cells probably triggers a better delivery into the cytoplasm. This is essential for the activity of antisense oligonucleotides because their targets are located in this intracellular compartment. The situation is different from other techniques such as microinjection or electropermeabilization for which oligomers are localized in the nucleus (21,22).

Further experiments are needed to determine the pathway that liposomes and oligonucleotides pass through after incubation with retroviral infected cells. Regardless of the precise mechanism involved, the fact that a retroviral infection stimulates the cellular uptake of liposome-encapsulated antisense oligonucleotides widens the spectrum of strategies for the treatment of retroviral infections and possibly that of HIV infection, in which cells are reported to be modified (20).

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