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Purpose. To investigate the potential use of polymeric nanoparticles for the delivery of antisense oligonucleotides in HIV-1-infected cell cultures.

Methods. Phosphorothioate oligonucleotides were encapsulated into poly (D,L-lactic acid) nanoparticles. Two models of infected cells were used to test the ability of nanoparticles to deliver them. HeLa $P_{4\text{-}2}$ CD_4^+ cells, stably transfected with the β-galactosidase reporter gene, were first used to evaluate the activity of the oligonucleotides on a single-round infection cycle. The acutely infected lymphoid CEM cells were then used to evaluate the inhibition of the viral production of HIV-1 by the oligonucleotides.

Results. The addition to infected CEM cells of nanoparticles containing gag antisense oligonucleotides in the nanomolar range led to strong inhibition of the viral production in a concentration-dependent manner. Similar results were previously observed in HeLa $P_{4\text{-}2}$ CD_4^+ cells. Nanoparticle-entrapped random-order gag oligonucleotides had similar effects on reverse transcription. However, the reverse transcriptase activity of infected cells treated with nanomolar concentrations of free antisense and random oligonucleotides was not affected.

Conclusions. These results suggest that poly (D,L-lactic acid) nanoparticles may have great potential as an efficient delivery system for oligonucleotides in HIV natural target cells, i.e., lymphocytic cells.

KEY WORDS: phosphorothioate oligonucleotide; nanoparticle; drug delivery; HIV-1; reverse transcription.

INTRODUCTION

Antisense oligonucleotides are designed to modulate the information transfer from the gene to protein during the RNA processing. In this way, these antisense compounds have the potential to be used as therapeutic agents to control the synthesis of a deleterious protein associated with viral, neoplastic, or other diseases (1,2). The development of antisense oligonucleotides is in constant progress, particularly with the synthesis of novel modified classes of oligonucleotides such as phosphorothioates, which are less sensitive toward nucleases than phosphodiesters. Some phosphorothioates are currently being evaluated in clinical trials for treatment of cancer, targeting PKCα (3), C-raf kinase (4), or Bcl-2 (5). Vitravene, an antisense phosphorothioate oligonucleotide recently approved by the FDA, is currently in clinical use for the intraocular treatment of retinitis caused by cytomegalovirus (6). Because of the emergence of drug-resistant HIV variants, antiviral therapies for HIV infection include anti-HIV drug combinations such as reverse transcriptase (RT) and protease inhibitors (7).

Among several approaches used to inhibit retroviral replication *in vitro,* the antisense strategy has also been proposed to block various viral replication steps, in particular reverse transcription. Indeed, RT is essential for HIV replication, performing the conversion of the single-stranded RNA genome of the virus into a linear double-stranded DNA, a step indispensable for subsequent integration into the host cell chromosomes and expression of the provirus (8). Thus, targeting HIV-1 RT is an attractive approach for the treatment of AIDS by the use of an oligonucleotide-based therapy. Phosphorothioate oligonucleotides have been used as inhibitors of HIV replication in HIV-1-infected cells, but the apparent mechanism of HIV-1 inhibition has been shown to be mediated by antisense as well as nonsequence-specific mechanisms and has differed, depending on the experimental cell culture model used (for a review see Ref. 9). Indeed, because of their polyanionic nature, phosphorothioate oligonucleotides have poor subcellular availability due to nonspecific interactions with cellular or extracellular components. Moreover, the immune stimulation induced by some specific CpG motifs with the appropriate flanking sequences may adversely affect the potential of these oligonucleotides as therapeutic agents (10). All of these problems preclude potential applications of antisense-based specific inhibition of viral reverse transcription. Significant progress has been made to resolve these problems and to render antisense oligonucleotides useful as a therapeutic alternative via the development of transport systems into cells (for a review see Ref. 11). Delivery agents should protect oligonucleotides from the environment. Protection from enzymatic hydrolysis and prevention of protein binding can also be expected, permitting targeting of the drug to specific cells or tissues, ensuring an improved therapeutic index (12). However, the rate of oligonucleotide release from the nanoparticle as well as the location (extra- or intracellular compartment) where the oligonucleotide is released should also have an influence on undesirable interactions.

To enhance their pharmacologic efficiency, oligonucleotides have been associated or conjugated with liposomes (13), cationic lipids (14), cationic polymers (15) fusogenic peptides (16), dendrimers (17), and surfactants (18). To date, efforts have been made in liposomal composition to show tolerable toxicity (19) and improved stability in plasma and serum (20). Among delivery systems, polymeric particles encapsulating the active compound into a solid core have also been developed. This approach has been validated with different oligonucleotides and various polymers such as methylmethacrylate (21), polylactic acid (22,23), and poly(lactide-co-glycolide) copolymer (24). The 25-mer phosphorothioate oligonucleotide GEM91, directed toward gag mRNA was selected as a model in this study (25). GEM91 has been shown to inhibit viral replication but is also known for its *in vitro-in vivo* toxic effects (26). The purpose of this work was then to investigate the potential of poly (D,L-lactic acid) nanoparticles as oligonucleotide carriers in reducing the side effects of the GEM91

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oligonucleotide. The efficiency of encapsulated oligonucleotides for their ability to enhance the inhibition of HIV-1 reverse transcription in lymphocytic cells was compared with free oligonucleotides.

MATERIALS AND METHODS

Oligonucleotides, Polymer, and Reagents

An antisense phosphorothioate oligodeoxynucleotide, designed to bind to the initiation site of the gag region of HIV-1 (GEM91), and a random sequence phosphorothioate of equivalent length (5′ N_{25} 3′), made by a random mixture of the equivalent molar quantities of the four nucleotides used for the antisense sequence, were synthesized by Genset (Paris, France). A hydrophobic complex of oligodeoxynucleotide was prepared by association with a cationic surfactant, cethyltrimethylammonium bromide (CTAB; Fluka, Buchs, Switzerland) as previously described (23). Poly (D,L-lactic acid) (Medisorb 100DL) was obtained from Alkermes (Alkermes, Inc., Cincinnati, OH). As a stabilizing hydrocolloid, poly(vinyl alcohol) (Mowiol 4-88, Hoechst, Germany) was chosen. Benzyl alcohol was purchased from Merck (Darmstadt, Germany).

Preparation of Oligonucleotide-Loaded Nanoparticles

Nanoparticles were produced by the emulsification-diffusion method as described previously (22,23). Briefly, a benzyl alcohol solution of poly(D,L-lactic acid) and oligonucleotide-CTAB complex was emulsified in an aqueous solution of poly(vinyl alcohol). Subsequent dilution of the emulsion with distilled water induced the diffusion of benzyl alcohol in the external phase of the emulsion and then the formation of nanoparticles. The nanoparticle dispersion was then purified by centrifugation and freeze-dried.

Cell Lines and Virus

Human 293T and HeLa $P_{4\text{-}2}$ CD_4^+ adherent cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin (250 U/mL), streptomycin (250 μg/mL) and 2 mM L-glutamine. The CEM human lymphoid T cell line was maintained in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Basel, Switzerland), penicillin (250 U/mL), streptomycin (250 μg/mL), and 2 mM L-glutamine at 37°C in 5% CO_2. The HIV-1 R9 clone used in these experiments was described previously (27). Briefly, plasmid R9 is a chimera made between HIV-1_{HXB2} and HIV-$1_{NL4\text{-}3}$ isolates, and it expresses functional forms of all HIV-1 genes.

Transfections and Infections

HIV-1 R9 infectious virus stock was produced by transfecting 293T cells with 40 μg of R9 DNA (28). Briefly, the subconfluent 293T cells were transfected by calcium phosphate precipitation, washed the day after, and supernatant was collected 48 h after transfection. The conditioned medium containing the virus was filtered on a 0.45-μm membrane. CEM cells or HeLa $P_{4\text{-}2}$ CD_4^+ cells were infected for 2 h at 37°C with HIV-1 R9 at different multiplicities of infection (MOI) ranging from 0.001 to 0.1. Then the infected cells were washed to remove unadsorbed virus. Fresh complete growth medium was added and cells were plated into 12-well plates (1 mL per well).

Antiviral Assays

To determine infectious titers of viral stocks, the $P_{4\text{-}2}$ cell line was used (8). The $P_{4\text{-}2}$ indicators cells are HIV-1-susceptible HeLa-CD_4^+ cells carrying the bacterial lacZ gene driven by the HIV-1 long terminal repeat (LTR). The β-galactosidase expression is induced by the LTR transactivation by the viral Tat protein produced during infection. The cells were then fixed with 1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) for up to 4 h. The infection level of the viral suspension on $P_{4\text{-}2}$ cells, cultivated with the different preparations, is expressed by counting the number of blue foci. To determine the effect of oligonucleotides on a single round of infection, $P_{4\text{-}2}$ CD_4^+ cells were infected with HIV-1 (MOI of 0.1) and cultured for 24 h in the presence of free oligonucleotide or oligonucleotide loaded-nanoparticles.

Separate experiments were also performed to test the antiviral activity of the oligonucleotides on acutely infected lymphocytes. CEM cells (2×10^5 cells/mL) were infected with the HIV-1 virus (MOI of 0.1) as previously described. Cells were treated every 3 or 4 days for up to 20 days with oligonucleotides added either free to the cultures at indicated concentrations or loaded in nanoparticles. Cell-free supernatants were collected every 3 to 4 days and tested for the presence of reverse transcriptase (RT) activity as described below. In the same time, the cytotoxicity of the formulations tested was measured as described below.

Cytotoxic Assays

In a preliminary set of experiments to determine whether the free antisense or random oligonucleotides exerted a toxic effect on the noninfected CEM cells in culture, the number of viable cells remaining in the culture medium after oligonucleotide treatment was determined by counting live cells every 3 or 4 days by trypan blue dye exclusion. Cell viability and cytotoxicity were also assayed by using the CellTiter96 Aqueous Nonradioactive Cell Proliferation Assay as recommended by the manufacturer (Promega. Madison, WI). Aliquots of infected CEM cells treated with free oligonucleotides or loaded nanoparticles were taken each 3 or 4 days during the experiment and dispensed in quadruplicate into a 96-well microculture plate (5000 cells/well) to control the degree of cell proliferation, which was determined according to the manufacturer's protocol.

Reverse Transcriptase Assay

The reverse transcriptase activity found in the cellular supernatant is directly proportional to the amount of virus produced by the infected cells. Because of this, after treatment with drug, a decrease in reverse transcriptase activity in the culture supernatant, should reflect inhibition of viral replication. Aliquots of culture supernatants from infected CEM cells were then treated by triton X-100 1% for 30 min to inactivate the virus. The reverse transcription assay (protocol adapted from Ref. 29) was performed on 10 μL of this lysed

virus added to 20 μL of reaction mix containing 50 mM Tris-HCl (pH 7.8), 75 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 0.1% NP40, 5 μg/mL polyrA-oligodT, ^{3}H-TTP (40 μCi/mL). The reaction was stopped after a 2 h incubation at 37°C by freezing at –20°C. The entire reaction was spotted on 2.3-cm Whatman DE81 circles. The filters were washed three times for 5 min in a 2×SSC buffer to eliminate nonincorporated ^{3}H-TTP and then once for 5 min in 95% EtOH, air dried, and counted by scintillation. The amount of radioactivity products bound to the filter is proportional to the quantity of the viral reverse transcriptase present in the sample.

RESULTS

Cytotoxicity Assays

In the first set of experiments, the cellular toxicity of free phosphorothioate oligonucleotides in noninfected cells was evaluated by cell counting via trypan blue dye exclusion test. Generally, cell growth was slightly affected at oligonucleotide concentrations below 2 μM. At this concentration, a decrease in cell viability (40%) showed the toxicity of the free oligonucleotide (Fig. 1A).

The cytotoxicity of olignucleotide-loaded nanoparticles was then determined by the CellTiter96 assay (Fig. 1B). Limited cytotoxic effects in infected cell cultures were observed with oligonucleotide-loaded nanoparticles at the various concentrations tested (Fig. 1B). For concentrations of oligonucleotide-loaded nanoparticles > 40 μg/mL (100 mM oligonucleotide), cytotoxic effects were observed, whereas no cytotoxicity was noticed with blank NP (without oligonucleotide) whatever the concentration used (data not shown).

Antiviral Activity in $P_{4\text{-}2}$ Cells

The Hela-CD_4^+-β-galactosidase cells were used to evaluate the effects of antisense oligonucleotides (free or loaded into nanoparticles) on a single-round infection cycle. Cells were then successively infected with R9 stock and treated with the different compounds. If the viral infection in these infected cells is inhibited, this should involve a decrease in the *tat* gene product, which in turn would cause a decrease in the number of blue cells. The results show that indeed oligonucleotides inhibit β-galactosidase production with an IC_{50} of 50 nM when they are loaded into nanoparticles. Moreover, free oligonucleotides tested at the higher concentration of 100 nM inhibit β-galactosidase production of only 30% (Fig. 2).

Inhibition of Viral Replication in CEM Cells

Because CD_4^+ T lymphocytes are one of the main natural targets of HIV-1 infection *in vivo,* the antiviral activity of the antisense oligonucleotides, either free or loaded into nanoparticles, were evaluated on the human CEM lymphoid cells infected *de novo* by HIV-1 R9, for up to 20 days. The nanoparticle concentrations used were lower than the cytotoxicity threshold determined in previous experiments. The cells were monitored every 3 or 4 days for reverse transcriptase activity in the cell-free culture medium. The inactivity of blank nanoparticles (without oligonucleotide) on the inhibition of the reverse transcriptase activity has first been checked (data not shown). Figure 3 shows the dose-response curve of the oligonucleotide-loaded nanoparticles vs. free oligonucleotides. The inhibition of HIV-1 reverse transcriptase by the antisense oligonucleotide-loaded nanoparticles (Fig. 3A) is concentration dependent and was observed from a concentration of 10 μg/mL of nanoparticles, which corresponds to 25 nM of oligonucleotide. Moreover, suppression of HIV-1 replication was already observed at the beginning of infection (day 6) for the highest concentrations of nanoparticles tested (40 μg/mL of nanoparticles or 100 nM of oligonucleotides). Free antisense oligonucleotides used at equivalent concentrations did not prevent the reverse transcription of HIV-1 (Fig. 3B). Reverse transcriptase activity in the supernatants of cultures treated with antisense oligonucleotide-loaded nanoparticles was strongly reduced, whereas with free antisense oligonucleotides, little effect on viral production was seen. Therefore, as measured by the reverse transcriptase assay, the nanoparticles improved oligonucleotide antiviral activity in this cell culture model compared with addition of the free oligonucleotides directly in the culture medium. The contribution of the

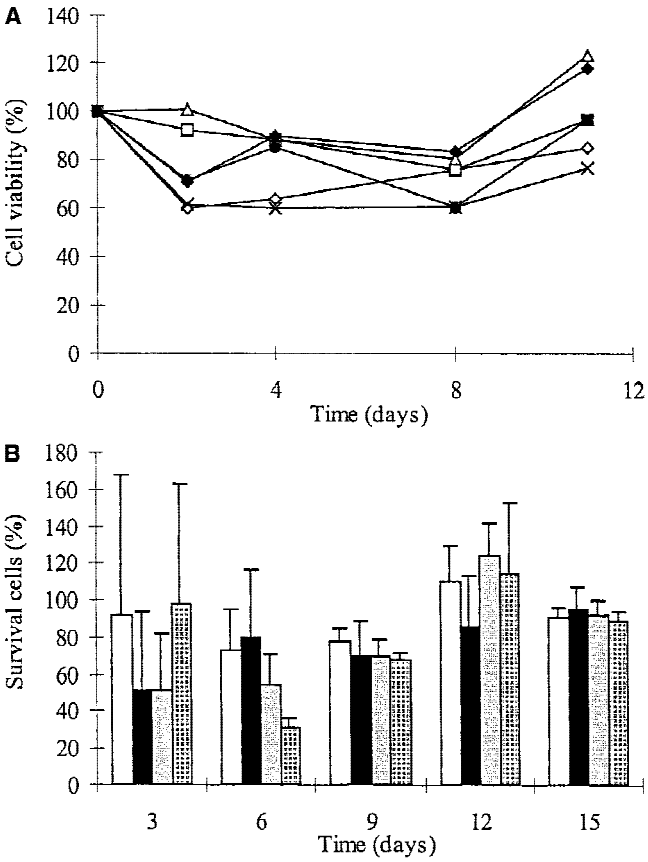

Fig. 1. (A) Cell toxicity of free antisense (AS) or random (R) oligonucleotides added to infected CEM cells at various concentrations: (◇) AS 2 μM, (□) AS 200 nM, (△) AS 20 nM, (×) R 2 μM, (*) R 200 nM, (•) R 20 nM. Cell counts were determined by the trypan blue dye exclusion technique. (B) Cell toxicity of oligonucleotide-loaded nanoparticles (NP) added to infected CEM cells at various concentrations (white bars: 10 μg/mL of NP containing 25 nM of oligonucleotide; black bars: 20 μg/mL of NP containing 50 nM of oligonucleotide; gray bars: 30 μg/mL of NP containing 75 nM of oligonucleotide; square bars: 40 μg/mL of NP containing 100 nM of oligonucleotide. Survival cells were determined by the CellTiter96 Aqueous Assay and reported as percent cell viability compared with infected cells, unexposed cell cultures (means ± SD, n = 4).

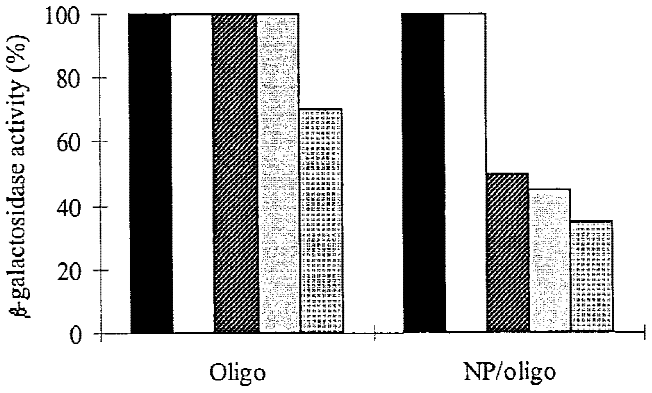

Fig. 2. Hela-CD_4-β-galactosidase cell assays. Hela-CD_4-β-galactosidase cells were incubated in medium containing virus at MOI 0.1 and treated with antisense oligonucleotides free (Oligo) or loaded into nanoparticles (NP/oligo) at different concentrations (black bars: control cells; white bars: 10 μg/mL of NP containing 25 nM of oligonucleotide; hatched bars: 20 μg/mL of NP containing 50 nM of oligonucleotide; gray bars: 30 μg/mL of NP containing 75 nM of oligonucleotide; square bars: 40 μg/mL of NP containing 100 nM of oligonucleotide). At 48 h postinfection, the cells were fixed and stained with X-gal. Blue cells were then counted under an inverted microscope.

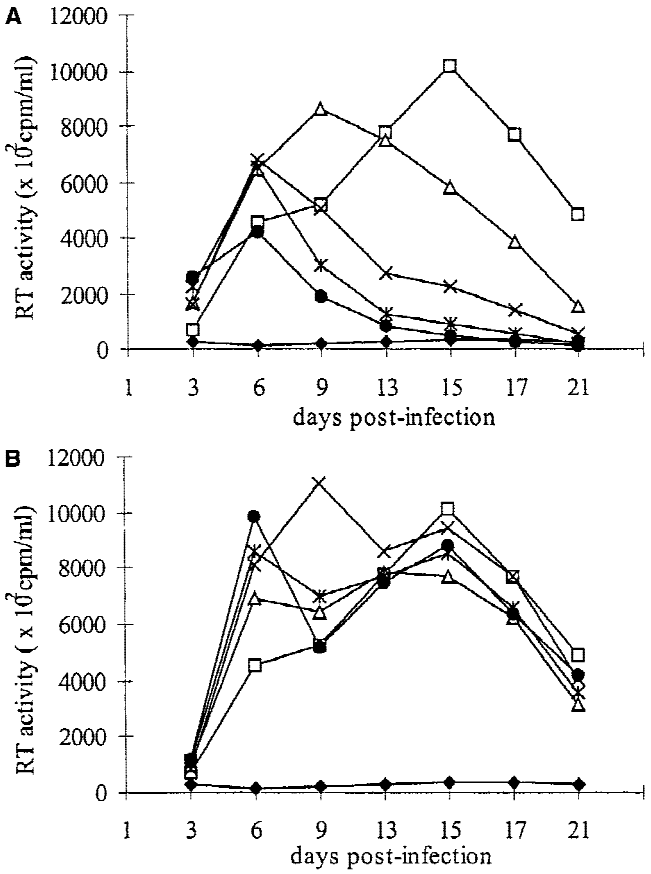

Fig. 3. Inhibition of HIV replication in infected CEM cells. CEM cells were *in vitro* infected with HIV-1 at an MOI of 0.1 and treated with (A) antisense oligonucleotide-loaded nanoparticles or (B) free antisense oligonucleotide: (△) 25 nM of oligonucleotide free or in 10 μg/mL of NP (×) 50 nM of oligonucleotide free or in 20 μg/mL of NP (*) 75 nM of oligonucleotide free or in 30 μg/mL of NP (•) 100 nM of oligonucleotide free or in 40 μg/mL of NP. Cell-free supernatants were tested every 3 or 4 days for the presence of reverse transcriptase activity (RT). HIV replication was compared with that in control infected CEM cells (□). Noninfected CEM cells (♦) were used as the negative control. (Duplicate samples were analyzed for all-time points).

free oligonucleotide to the biological activity, even at the higher concentration tested of 100 nM, is negligible. Regarding the experiments performed with the random oligonucleotide, results similar to those obtained with antisense oligonucleotides were achieved (data not shown). Moreover, because the inhibition seems to be nonspecific, by adding antisense or random oligonucleotides in presence of supernatant of infected CEM cells during the reverse transcritpion assay, we checked that the RT activity is not inhibited by the oligonucleotides (data not shown). The inhibition of viral replication, even when the random oligonucleotides are loaded into nanoparticles, showed that although nanoparticles are able to enhance the activity of the loaded oligonucleotide vs. free oligonucleotide, they are not able to increase the specificity of the oligonucleotide even when its concentration was reduced.

DISCUSSION

In previous studies, the evaluation of the potential of phosphorothioate antisense oligonucleotides as HIV therapeutic agents has been difficult, because suitable controls were generally not included. In addition, contributions from the nonspecific effects of these compounds, due to their charged nature, were also not always taken into account. Several possible mechanisms of action were postulated to account for the anti-HIV-1 activity of the oligonucleotides, such as the blocking of viral adsorption and/or the inhibition of viral reverse transcriptase (30). However, specific RNase H cleavage of HIV-1 gag mRNA has been shown in both a cell lysate assay and in cells acutely infected with virus (25). Although these results support the potential of antisense oligonucleotides as efficient agents to regulate gene expression, the uptake of these drugs, especially into appropriate cellular compartments, remains a major obstacle to their therapeutic application. Therefore, the aim of this work was not to confirm the mechanism of action of the gag oligonucleotide but, rather, to investigate the efficacy of a delivery system to improve the cellular uptake of the oligonucleotides.

The experimental results presented in this study report that poly (D,L-lactic acid) nanoparticles could be used for improving cellular delivery of the oligonucleotides. Nanoparticles allow oligonucleotides to exert their antiviral activity in a more effective manner than free oligonucleotides. The poly (D,L-lactic acid) nanoparticle formulation enabled delivery of entrapped oligonucleotides to both $P_{4\text{-}2}$ CD_4^+ and CEM cell lines at nanomolar concentrations. The first experimental model, selected to test oligonucleotide activity, relied on the use of cells containing the Tat activation region (TAR) of the HIV-1 LTR, which controls the transcription of the β-galactosidase reporter gene. Subsequent to HIV infection, the Tat protein is synthesized and binds directly to the TAR, leading to the expression of the β-galactosidase gene. Here, a decrease in the β-galactosidase activity was detected 48 h postinfection when the oligonucleotide-loaded nanoparticles were added to the cell cultures at the time of virus infection.

These results suggest that oligonucleotide-loaded nanoparticles act in inhibiting early events in the viral cycle life. Indeed, these results obtained during one cycle of infection are encouraging because *in vivo,* a defect in the replication in the early viral stage may induce a decrease of the viral multiplication. In the second experimental model, the inhibitory activity of oligonucleotides was studied in acutely infected CEM cells. In this type of infection, free phosphorothioate oligonucleotides have been shown to behave like polyanions and to interfere with virus adsorption by binding to CD_4 and to the V3 loop of viral gp120, thus inhibiting viral entry by nonsequence-specific effects (26). In the present study, the level of HIV-1 reverse transcriptase activity, in the presence of oligonucleotide-loaded nanoparticles, was markedly reduced and maintained at a low level during the entire time of the experiment. However, these results do not permit assignment of a definite mechanism of action of the oligonucleotide-loaded nanoparticles. Thus, the inhibition of viral replication also observed with random encapsulated nanoparticles may indicate that the oligonucleotides can leak from nanoparticles in sufficient quantity to inhibit the gp120-CD_4 interaction via this mechanism. Nevertheless, because of the inactivity of the free oligonucleotides and of the blank nanoparticles, we can propose that the loading of oligonucleotide in nanoparticles allows increased intracellular availability. This may occur by the bypassing of vesicular trafficking, resulting in a significant improvement in efficacy.

To understand these results, based on previous data, several hypotheses have been proposed. A preliminary work (22) showed that oligonucleotide-loaded nanoparticles are internalized by DU145 prostate cancer cells. However, the intracellular internalization pathway seems to be different if the oligonucleotides are free or loaded into nanoparticles. Apparently, poly (D,L-lactic acid) nanoparticles deliver oligonucleotides into a nonsequestering intracellular compartment. It can be assumed that the increase of cellular association of the oligonucleotides arises from the adsorption of nanoparticles at the cell surface, where carrier bioerosion should occur followed by the delivery of the encapsulated oligonucleotide. Therefore, the oligonucleotide release should occur close to the cellular membrane. Because the oligonucleotide release from the nanoparticles in cell culture medium is quite fast, it can be also supposed that oligonucleotides are principally located close to the nanoparticle surface, which are then able to release oligonucleotide by desorption, independent of the degradation of the polymer. Moreover, because of the inhibitory results obtained with random oligonucleotide-loaded nanoparticles, the antisense activity reported in all cell assays may have been due to the nonsequence-specific effect of GEM91. GEM91 alone (100 nM) did not produce a significant inhibition of HIV, but its incorporation into nanoparticles resulted in approximately 90% inhibition.

As a conclusion, this work showed that the main advantage of the nanoparticulate oligonucleotide carrier used is increased cellular uptake. A concentration effect with an increase in the entrapped oligonucleotide efficiency has been observed. Therefore, nanoparticles seem to be a suitable tool for oligonucleotide delivery. Nevertheless, further investigation is required to adjust the rate of the oligonucleotide release from the polymeric system, which may, in turn, facilitate long-term treatment. Moreover, the use of compounds other than amphiphilic molecules is needed to develop hydrophobic oligonucleotides able to be incorporated into polymeric matrices.

REFERENCES

1. B. F. Baker and B. P. Monia. Novel mechanisms for antisense-mediated regulation of gene expression. *Biochim. Biophys. Acta* **1489**:3–18 (1999).
2. S. T. Crooke. Molecular mechanisms of action of antisense drugs. *Biochim. Biophys. Acta* **1489**:31–44 (1999).
3. J. Nemunaitis, J. T. Holmlund, M. Kraynak, D. Richards, J. Bruce, N. Ognoskie, T. J. Kwoh, R. Geary, A. Dorr, D. Von Hoff, and S. G. Eckhardt. Phase I evaluation of ISIS 3521, an antisense oligodeoxynucleotide to protein kinase C-alpha, in patients with advanced cancer. *J. Clin. Oncol.* **17**:3586–3595 (1999).
4. C. C. Cunningham, J. T. Holmlund, J. H. Schiller, R. S. Geary, T. J. Kwoh, A. Dorr, and J. Nemunaitis. A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. *Clin. Cancer Res.* **6**:1626–1631 (2000).
5. J. S. Waters, A. Webb, D. Cunningham, P. A. Clarke, F. Raynaud, F. di Stefano, and F. E. Cotter. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J. Clin. Oncol.* **18**: 1812–1823 (2000).
6. K. P. Anderson, M. C. Fox, V. Brown-Driver, M. J. Martin, and R. F. Azad. Inhibition of human cytomegalovirus immediate-early gene expression by an antisense oligonucleotide complementary to immediate-early RNA. *Antimicrob. Agents Chemother.* **40**:2004–2011 (1996).
7. C. A. Deminie, C. M. Bechtold, D. Stock, M. Alam, F. Djang, A. H. Balch, T.-C. Chou, M. Prichard, R. J. Colonno, and P.-F. Lin. Evaluation of reverse transcriptase and protease inhibitors in two-drug combinations against human immunodeficiency virus replication. *Antimicrob. Agents Chemother.* **40**:1346–1351 (1996).
8. P. Charneau, G. Mirambeau, P. Roux, S. Paulous, H. Buc, and F. Clavel. HIV-1 reverse transcription: A termination step at the center of the genome. *J. Mol. Biol.* **241**:651–662 (1994).
9. S. Agrawal. Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides. *Biochim. Biophys. Acta* **1489**:53–68 (1999).
10. A. M. Krieg. Mechanisms and applications of immune stimulatory CpG oligodeoxynucleotides. *Biochim. Biophys. Acta* **1489**: 107–116 (1999).
11. I. Lebedeva, L. Benimetskaya, C. A. Stein, and M. Vilenchik. Cellular delivery of antisense oligonucleotides. *Eur. J. Pharm. Biopharm.* **50**:101–119 (2000).
12. R. L. Juliano, S. Alahari, H. Yoo, R. Kole, and M. Cho. Antisense pharmacodynamics: Critical issues in the transport and delivery of antisense oligonucleotides. *Pharm. Res.* **16**:494–502 (1999).
13. O. Zelphati, J. L. Imbach, N. Signoret, G. Zon, B. Rayner, and L. Leserman. Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms. *Nucleic Acids Res.* **22**:4307–4314 (1994).
14. C. F. Bennett, D. Mirejovsky, R. M. Crooke, Y. J. Tsai, J. Felgner, C. N. Sridhar, C. J. Wheeler, and P. L. Felgner. Structural requirements for cationic lipid mediated phosphorothioate oligonucleotides delivery to cells in culture. *J. Drug Target.* **5**:149–162 (1998).
15. C. Pichon, M. B. Roufaï, M. Monsigny, and P. Midoux. Histidylated oligolysines increase the transmembrane passage and the biological activity of antisense oligonucleotides. *Nucleic Acids Res.* **28**:504–512 (2000).
16. J.-P. Bongartz, A.-M. Aubertin, P. G. Milhaud, and B. Lebleu. Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide. *Nucleic Acids Res.* 22:4681–4688 (1994).
17. H. Yoo, P. Sazani, and R. L. Juliano. PAMAM dendrimers as delivery agents for antisense oligonucleotides. *Pharm. Res.* **16**: 1799–1804 (1999).
18. J. A. Hughes, A. I. Aronsohn, A. V. Avrutskaya, and R. L. Juliano. Evaluation of adjuvants that enhance the effectiveness of antisense oligodeoxynucleotides. *Pharm. Res.* **13**:404–410 (1996).
19. G. J. Nabel, E. G. Nabel, Z. Y. Yang, B. A. Fox, G. E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, and A. E. Chang. Direct gene

transfer with DNA-liposome complexes in melanoma: Expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA* **90**:11307–11311 (1993).
20. J. G. Lewis, K. Y. Lin, A. Kothavale, W. M. Flanagan, M. D. Matteucci, R. B. Deprince, R. A. Mook, R. W. Hendren, and R. W. Wagner. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc. Natl. Acad. Sci. USA* **93**:3176–3181 (1996).
21. H.-P. Zobel, M. Junghans, V. Maienschein, D. Werner, M. Gilbert, H. Zimmermann, C. Noe, J. Kreuter, and A. Timmer. Enhanced antisense efficacy of oligonucleotide adsorbed to monomethylaminoethylmethacrylate methylmethacrylate copolymer nanoparticles. *Eur. J. Pharm. Biopharm.* **49**:203–210 (2000).
22. M. Berton, L. Benimetskaya, E. Allémann, C. A. Stein, and R. Gurny. Uptake of oligonucleotide-loaded nanoparticles in prostatic cancer cells and their intracellular localization. *Eur. J. Pharm. Biopharm.* **47**:119–123 (1999).
23. M. Berton, E. Allémann, C. A. Stein, and R. Gurny. Highly loaded nanoparticulate carrier using an hydrophobic antisense oligonucleotide complex. *Eur. J. Pharm. Sci.* **9**:163–170 (1999).
24. J. K. Lewis, W. J. Irwin, and S. Akhtar. Development of a sustained-release biodegradable polymer delivery system for site-specific delivery of oligonucleotides: Characterization of P(LA-GA) copolymer microspheres in vitro. *J. Drug Target.* **5**:291–302 (1998).
25. G. J. Veal, S. Agrawal, and R. A. Byrn. Sequence-specific RNaseH cleavage of gag mRNA from HIV-1 infected cells by an antisense oligonucleotide in vitro. *Nucleic Acids Res.* **26**:5670–5675 (1998).
26. K. Yamaguchi, B. Papp, D. Zhang, A. N. Ali, S. Agrawal, and R. A. Byrn. The multiple inhibitory mechanisms of GEM91, a gag antisense phosphorothioate oligonucleotide, for human immunodeficiency virus type 1. *Aids Res. Hum. Retrov.* **13**:545–554 (1997).
27. T. Gallay, T. Hope, D. Chin, and D. Trono. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci. USA* 94:9825–9830 (1997).
28. A. Mangasarian, V. Piguet, J.-K. Wang, Y.-L. Chen, and D. Trono. Nef-induced CD4 and major histocompatibility complex calss I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. *J. Virol.* **73**:1964–1973 (1999).
29. S. Goff, P. Traktman, and D. Baltimore. Isolation and properties of moloney murine leukemia virus mutants: Use of a rapid assay for release of virion reverse transcriptase. *J. Virol.* **38**:239–248 (1981).
30. J. Ojwang, A. Elbaggari, H. B. Marshall, K. Jarayaman, M. S. McGrath, and R. F. Rando. Inhibition of human immunodeficiency virus type 1 activity in vitro by oligonucleotides composed entirely of guanosine and thymidine. *J. AIDS* **7**:560–570 (1994).